Carles Blasi Brugué

Advances
in feline
transfusion
medicine

Avances en medicina
de transfusión felina

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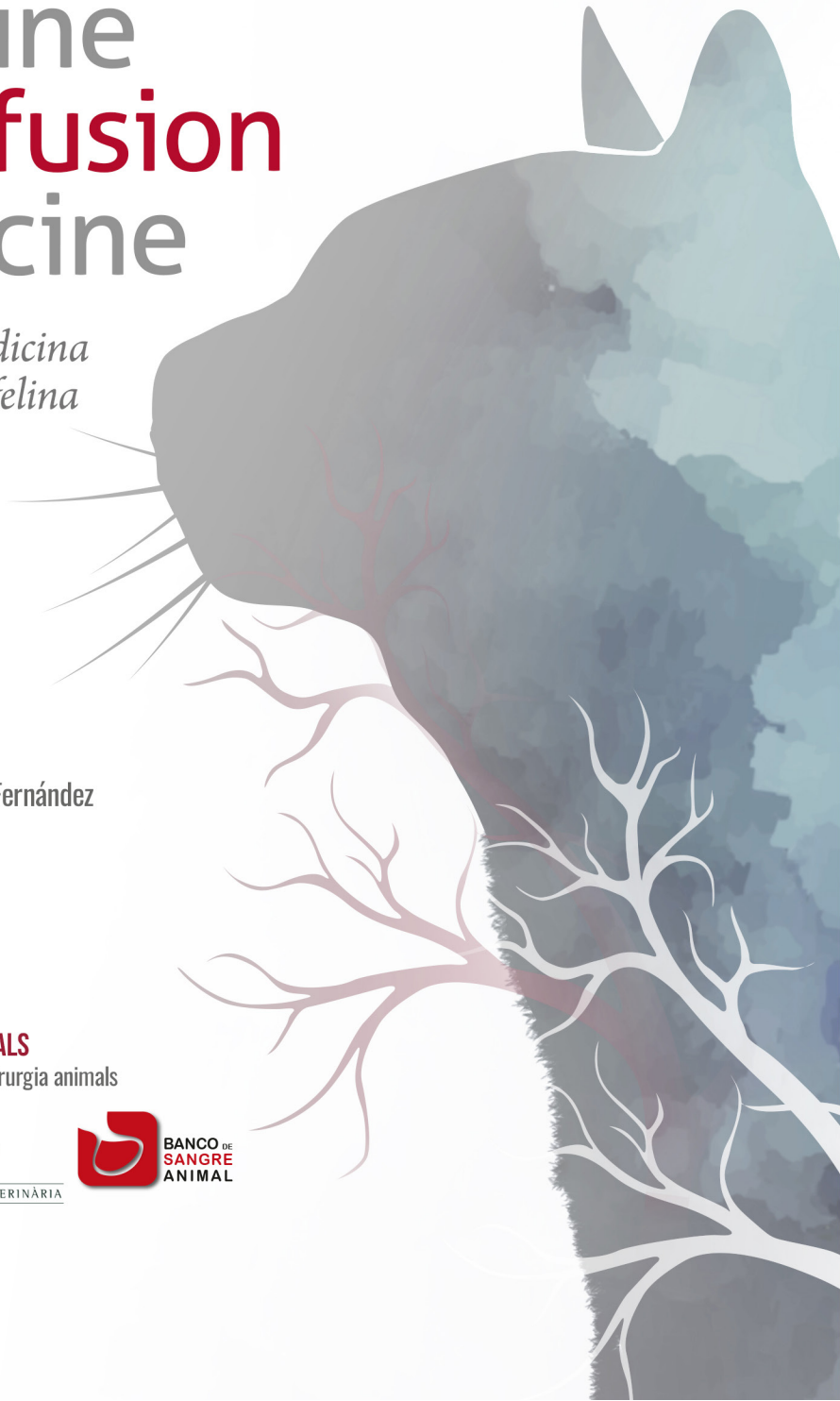**MEDICINA I SANITAT ANIMALS**
Departament de medicina i cirurgia animals

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The Doctor

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REPORT THAT:

The thesis titled

"Advances in feline transfusion medicine"

presented by the veterinary graduate

CARLES BLASI BRUGUÉ

to obtain the degree of Doctor in Veterinary Medicine,
has been carried out under my direction and,
considering it satisfactorily completed,
I authorize its submission to be evaluated
by the corresponding commission.

And for the record for the appropriate purposes,
I sign this report in Bellaterra, 8 June 2021.

Rafael Ruiz de Gopegui Fernández

Nothing in life is to be feared,
it is only to be understood.
Now is the time to understand more,
so that we may fear less.

Marie Curie

Contents

Studies

List of Abbreviations

AKI: acute lung injury
APTT: activated partial thromboplastin time
ARDS: acute respiratory distress syndrome
ATP: adenosine triphosphate
CPD: tri-sodium citrate, sodium phosphate and dextrose
CVs: coefficients of variation
DE: *desviación estándar* (*spanish*)
fCE: *concentrado de eritrocitos felino* (*spanish*)
CE: *concentrado de eritrocitos* (*spanish*)
fFFP: feline fresh frozen
FFP: fresh frozen plasma
FP: frozen plasma
fPFC: *plasma fresco congelado felino* (*spanish*)
FWB: fresh whole blood
Hb: haemoglobin
HGB: haemoglobin
H2O2: hydrogen peroxide
IU: international unit
MPs: micro-particles
OH•: hydroxide
O-2: oxide
PCV: packed cell volume
PFC: plasma fresco congelado
PFK: phosphofructokinase
PIP: peristaltic infusion pumps
PlGF: placenta growth factor
pRBC: packed red blood cells
PT: prothrombin time
RBC: red blood cells
ROS: reactive oxygen species
SAGM: adenine, dextrose, mannitol and sodium chloride
SD: standard deviation
SNO: S-nitrosylation
US FDA: United States Food and Drug Administration
WB: whole blood

Resumen

La medicina transfusional es una terapia de reemplazo con componentes sanguíneos en una situación de déficit. Con frecuencia, es un procedimiento urgente y necesario para mantener al paciente con vida, aún así no está exenta de riesgos, que dependen en gran medida de los métodos de producción, almacenamiento y administración.

Los hemoderivados son productos que se generan a partir del procesado de la sangre entera. El uso de hemoderivados frente al de la sangre completa minimiza el riesgo de reacciones transfusionales, maximiza la eficacia de la transfusión, aumenta el tiempo de almacenaje y por lo tanto la disponibilidad del producto, y optimiza las donaciones de sangre para poder tratar múltiples pacientes a partir de una única donación de sangre. Esto es esencial en medicina felina por la dificultad adicional en encontrar donantes adecuados. Los principales hemoderivados son el concentrado de eritrocitos (CE) y el plasma fresco congelado (PFC).

La medicina transfusional felina esta asociada a muchas dificultades técnicas, por lo que históricamente se ha visto restringida al uso de sangre entera. Aún así, en las últimas décadas, se han producido grandes avances y cada vez más bancos de sangre han empezado a producir y ofrecer hemoderivados felinos. Estos se producen mediante la adaptación de los métodos de procesado y almacenaje de hemoderivados usados en medicina humana y canina. Sin embargo, falta evidencia científica que apoye la seguridad y eficacia de estos métodos en medicina felina. Debido al menor tamaño de los donantes y receptores de sangre, así como sus diferencias fisiológicas y hemorreológicas respecto a los perros y humanos, extrapolar la información entre especies puede suponer un riesgo para la seguridad y la eficacia de las transfusiones felinas.

Durante el almacenaje o la administración, las unidades de hemoderivados sufren cambios que pueden tener consecuencias en la eficacia de la transfusión y aumentar el riesgo de efectos adversos sobre el receptor.

Los concentrados de eritrocitos sufren una serie de cambios metabólicos derivados de la conservación, incluyendo liberación de subproductos al medio, daño oxidativo, destrucción de eritrocitos y su pérdida de propiedades, así como la acumulación de hemoglobina libre. Todas estas alteraciones pueden suponer consecuencias negativas para el receptor. La forma mas común y aceptada de cuantificar el daño eritrocítico es determinar el grado de hemólisis. Las unidades adquieren un grado de hemólisis durante el procesado y almacenaje, y este puede aumentar durante la transfusión según el método de administración utilizado.

Para el plasma fresco congelado el mayor indicador de calidad es la actividad de los factores de coagulación y su estabilidad durante el almacenaje. La administración de factores de coagulación es la principal causa de transfusión de

derivados del plasma, y estos se pueden degradar durante el proceso de producción y conservación. Si pierden su funcionalidad, la transfusión pierde su efecto terapéutico. En la actualidad se desconoce tanto la actividad de los factores de coagulación en el plasma fresco congelado felino como su estabilidad, así como qué factores son lábiles y, en consecuencia, críticos para el control de calidad. Los controles de calidad son esenciales para poder determinar si un hemoderivado es apto para la transfusión. No se puede autorizar un sistema de producción y conservación de plasma felino sin poder realizar estos controles de forma adecuada.

1

Este proyecto se compone principalmente de tres estudios, el primero se basa en las hipótesis de que las unidades de concentrado de eritrocito obtenidas mediante un sistema semicerrado y con SAGM (adenina, dextrosa, manitol y cloruro de sodio) como solución de preservación, presentarán niveles de hemólisis inferiores al 1% dentro de las primeras 6 horas después de la separación de los eritrocitos de la sangre entera mediante la centrifugación a 2000 g durante 15 minutos a 20° C con 80 segundos de aceleración y 110 de deceleración. También que el tiempo de almacenaje a 4° C en unidades de concentrado de eritrocitos felino procesadas mediante el mismo método induce al incremento del porcentaje de hemólisis. *El objetivo principal de este estudio era establecer las bases para el almacenaje de concentrado de eritrocitos felino* mediante cuatro **objetivos secundarios y específicos: 1) evaluar la calidad de los concentrados de eritrocitos felinos** almacenados con SAGM durante 29 a 35 días y durante 36 a 42 días a 4 C mediante el análisis del porcentaje de hemólisis y la hemoglobina libre, **2) contribuir a establecer el tiempo de almacenaje máximo** para cumplir con los criterios establecidos por las guías de United States Food and Drug Administration (US-FDA) en medicina humana, **3) analizar la idoneidad de los sistemas semicerrados de extracción de sangre felina** para el procesado y conservación de CE felino (fCE) según el grado de hemólisis, concentración de hemoglobina libre y riesgo de contaminación bacteriana, y **4) determinar si es necesario realizar controles de calidad antes de administrar unidades de fCE almacenadas durante más de 4 semanas** para cumplir con las directrices de seguridad establecidas por la United States Food and Drug Administration (US-FDA) en medicina humana.

En el estudio se analizaron 489 unidades de concentrado de eritrocitos felino. Se realizó un cultivo bacteriano, y se determinó el hematocrito (PCV), hemoglobina libre, hemoglobina total y el porcentaje de hemólisis durante las 6 horas posteriores al procesado. De estas, 180 unidades se

analizaron otra vez entre los 29 y 35 días de almacenaje (t1) y 118 entre los días 36 y 42 de almacenaje (t2), analizando el PCV, la hemoglobina libre, la hemoglobina total y el porcentaje de hemólisis.

En los resultados no se detectó contaminación bacteriana en ninguna unidad de CE. El PCV medio en t0 fue del 52.25% (SD: ±5.27) y disminuyó significativamente (p < 0.001) durante el almacenaje hasta el 48.15% (SD: ±3.79) en t1 y hasta 49.34% (SD: ±4.45) en t2. El porcentaje medio de hemolisis en t0 fue de 0.07% (SD: ±0.06) y aumentó significativamente (p < 0.001) hasta 0.69% (SD: ±0.40) en t1 y 0.81% (SD: ±0.47) en t2. Además, el 13.88% y el 19.49% de unidades superaron el 1% de hemólisis en t1 y t2 respectivamente.

En conclusión, los resultados de este estudio demuestran un incremento del grado de hemólisis durante el almacenaje del fCE a 4° C. Así mismo, teniendo en cuenta las recomendaciones de la US-FDA en transfusión humana que recomiendan un máximo del 1% de hemólisis, los resultados de este estudio muestran que las unidades con menos de 24h de vida media tienen niveles bajos de hemólisis, pero las unidades almacenadas 28 días solo se deberían administrar si previamente se han revisado los niveles de hemólisis, ya que el 13.88% superan el límite del 1%. Además, el sistema semicerrado es seguro para la para la obtención, el procesado y la conservación de fCE ya que no se detectó contaminación bacteriana en ninguna unidad.

2

El segundo estudio adopta la hipótesis siguiente: el uso de dos tipos de bombas de infusión peristálticas para administrar fCE a una velocidad de 25ml/h no producirá niveles significativos de hemólisis. Por lo tanto, *el objetivo general de este estudio es evaluar el riesgo de daño en los eritrocitos provocado por las bombas de infusión peristálticas en la transfusión de concentrado de eritrocitos felino.* **El objetivo específico consiste en evaluar el efecto de dos tipos de bomba de infusión peristáltica sobre la integridad de los eritrocitos en unidades de fCE almacenados durante 35 - 42 días a 4° C mediante la determinación de la hemólisis y la hemoglobina libre.**

Para ello se analizó el efecto de dos bombas de infusión peristálticas (NIKI V4 y Infusomat FmS) a una velocidad de 25 ml/h sobre 15 unidades de CE felino almacenadas entre 35 a 42 días a 4° ºC mediante la medición de la hemoglobina libre y el porcentaje de hemólisis, y se compararon los resultados con el efecto de la administración por gravedad (sin bomba de infusión). El valor medio de hemolisis para las unidades administradas por gravedad fue del 1.12%, que comparado con el grado de hemólisis en las unidades transfundidas con la bomba NIKI V4

(1.13%) o la Infusomant FmS (1.14%) no mostró diferencias estadísticamente significativas, con un aumento en los resultados de solo el 0.01% y el 0.02% respectivamente. **Como conclusión** de este estudio, el uso de dos bombas de infusión peristálticas durante la administración de fCE no produce hemólisis significativamente distinta que la que causa la administración por gravedad a una velocidad de 25 ml/h.

A diferencia de los primeros dos estudios el tercero trata de otro hemocomponente, el plasma fresco congelado felino (fPFC), y se basa en 3 hipótesis. La primera es que preservar fPFC un año a temperatura de entre -18° C y -25° C reducirá de manera significativa la actividad de los factores de coagulación en comparación con las unidades de fPFC almacenadas menos de dos semanas. La segunda es que congelar unidades de plasma felino dentro de las primeras 24h de la extracción de la sangre entera y almacenarla menos de dos semanas no disminuye la actividad de los factores de coagulación si se compara con un banco o mezcla de plasma fresco de gatos sanos. Y la tercera, es que la recuperación de los factores VIII y V será significativamente menor que la recuperación de los factores II, VII, IX, X, XI y XII después de un año de congelación entre -18 y -25° C, y por lo tanto serán los factores mas lábiles durante el almacenaje. *El objetivo general del estudio era investigar la actividad de factores de coagulación específicos después de congelar y durante el almacenaje del fPFC.*

Los objetivos específicos eran: 1) determinar la actividad (concentración y estabilidad) durante el tiempo de almacenaje de los factores de coagulación I, II, VII, IX, X, XI y XII en el plasma fresco congelado felino, **2) identificar qué factores de coagulación disminuyen más su actividad** como consecuencia del tiempo de conservación del fPFC y deben considerarse factores críticos para la producción y control de calidad del fPFC, **3) establecer el volumen de fPFC necesario para administrar 10 UI/Kg de factores de coagulación funcionales** cuando se administran unidades de plasma fresco congelado felino almacenadas hasta un año a una temperatura de -18° C a -25° C.

Para ello, se recogieron 55 unidades de fPFC y se almacenaron a temperatura de entre -18° C y -25° C. De éstas, 21 se almacenaron durante menos de dos semanas y 34 durante un año. Al terminar el período de almacenaje en ambos grupos, se analizó la actividad de los factores de coagulación II, V, VII, VIII, IX, X, XI y XII mediante ensayos de tiempo parcial de tromboplastina y protrombina activados modificados con plasma humano deficiente en cada factor específico. El fibrinógeno se determinó según el método de von Clauss. Los resultados demostraron

una actividad media (± DE) para los factores II, V, VII, VIII, IX, X, XI y XII de 101.94 19.06%, 71.94 ± 24.14%, 102.78 ± 24.69%, 77.52 ± 30.39%, 84.86 ± 29.35%, 96.24 ± 25.10%, 88.76 ± 22.73% y 89.50 ± 21.85% respectivamente en T0, y 73.23 ± 39.06%, 97.87 ± 62.33%, 60.08 ± 38.17%, 50.32 ± 23.8%, 71.37 ± 22.23%, 83.91 ± 49.54%, 66.28 ± 22.20% y 55.46 ± 23.18% respectivamente en T1. La concentración media de fibrinógeno funcional en T1 fue de 2.76 g/l (± 1.09). Se identificó una disminución significativa entre T0 y T1 para los factores II, P=.0008; VII, P=.0003; VIII, P=.0015; XI P=.0011; and XII P=.000003, y un aumento significativo para el factor V, P=.0458.

En conclusión, aunque se detectó una disminución significativa de la mayoría de los factores de coagulación tras un año de almacenaje, el fPFC sigue siendo hemostáticamente activo *in vitro*. Los factores que mostraron más labilidad y deberían ser usados para la determinación del control de calidad, fueron el factor VII y VIII. Según los resultados de este estudio, se calculó que hace falta transfundir de 13 a 20 ml/kg, dependiendo del tiempo de almacenaje, para alcanzar el mínimo de 10 IU/kg de todos los factores de coagulación.

Summary

Transfusion medicine consists in a blood component replacement therapy for patients in a situation of deficiency, being in many cases a lifesaving procedure. Even so, it is not exempt of risks, which are highly influenced by the methods of production, storage and administration.

Blood components are products obtained from the processing and fractionation of whole blood. The use of blood components allows to minimize the risk of transfusion reactions, maximize the efficiency of transfusions, increase the storage time of blood and consequently the availability of the product, and to optimize blood donations by allowing to treat multiple patients out of one single blood donation, which can be of utmost importance in feline medicine due to the reduced availability of suitable donors. The most used blood products are packed red blood cells (pRBCs) and fresh frozen plasma (FFP).

It is considered that feline transfusion medicine is especially associated with technical difficulties and thus, it has historically been restricted to the use of whole blood. Nevertheless, in the recent decades there have been great advances in feline blood banking, and blood banks have begun to produce and offer feline blood components manufactured by extrapolating methods of production and storage from human and canine medicine. The scientific evidence to support the safety and efficacy of these methods in feline blood banking is scant. Due to the smaller size of blood donors and recipients, as well as the physiological differences between species and the hemorheological differences of feline erythrocytes compared to canine and human red blood cells, extrapolating the information between species can pose a great risk regarding the safety and efficacy of feline transfusions.

During storage and administration, blood products undergo changes that can have consequences both in the efficiency of the transfusion and over the adverse effects induced to the recipient. Regarding pRBCs, a series of metabolic changes occur during storage that lead to the accumulation of by-products in the medium, induction of oxidative damage, destruction of erythrocytes and their loss of properties, as well as the accumulation of free haemoglobin. All these alterations can have negative consequences on the recipient. The most common and accepted way to quantify red cell damage is to determine the degree of haemolysis. Packed red blood cells units acquire a basal level of haemolysis during processing and storage, and this may increase during transfusion depending to a large extent on the method of administration used.

Regarding FFP, the highest quality indicator which reflects its therapeutic effect is the activity of coagulation factors. This depends on their stability during storage. The administration of coagulation factors is the main reason of plasma

derivatives transfusion. These can be degraded during the production and storage process, losing their functionality. Currently, the activity of clotting factors in feline fresh frozen plasma and their stability is unknown, as well as the factors considered labile and, consequently, critical for quality control. Quality control is essential to be able to determine if a blood product is suitable for transfusion. Thereafter, a feline plasma production and storage system cannot be authorized without an appropriate method to carry out these controls.

1

This project consists of three studies the first is based on the hypothesis that pRBC units obtained through a semi-closed system with SAGM (adenine, dextrose, mannitol and sodium chloride) preservation solution have haemolysis levels of less than 1% within the first 6 hours after being separated from whole blood by centrifugation at 2000 g for 15 minutes at 20° C with 80 seconds of acceleration and 110 of deceleration, and also that storage time at 4° C in pRBC units processed by the same method induces an increase in the percentage of haemolysis. *The general objective of this study was to establish the basis for feline pRBC blood banking,* **and this was carried out through 4 different specific objectives;** one was by **evaluating the quality of concentrated feline erythrocytes** stored with SAGM for 29 to 35 days and for 36 to 42 days at 4° C by analysing the percentage of haemolysis and free haemoglobin, a second objective was **to contribute to establish the maximum storage time** that should be adequate to meet the criteria established by the United States Food and Drug Administration (US-FDA) guidelines in human medicine, the third specific objective was **to analyse the suitability of semi-closed feline blood collection systems** for the processing and subsequent storage of pRBC units by evaluating the degree of haemolysis and free haemoglobin, as well as the risk of bacterial contamination, and finally **to assess the need to perform quality controls prior to administration of feline pRBC units stored for more than 4 weeks** in order to comply with the safety guidelines established by the US-FDA in human medicine.

In the context of this study, 489 feline pRBC units were analysed. Bacterial culture, packed cell volume (PCV), free haemoglobin, total haemoglobin and percentage of haemolysis were determined within 6 h after processing (t0). From this, 180 units were re-tested for haemolysis and PCV after 29-35 days of storage (t1) and 118 units after 36-42 days (t2), repeating the PCV, free haemoglobin, total haemoglobin and the percentage of haemolysis.

In the results, no bacterial contamination was detected in any pRBC unit. Mean PCV at t0 was 52.25% (SD: ± 5.27) and it decreased significantly

(p <0.001) during storage to 48.15% (SD: ± 3.79) at t1 and up to 49.34% (SD: ± 4.45) at t2. Mean percentage of haemolysis at t0 was 0.07% (SD: ± 0.06) and increased significantly (p <0.001) to 0.69% (SD: ± 0.40) at t1 and 0.81% (SD: ± 0.47) at t2. Furthermore, 13.88% and 19.49% of pRBC units exceeded 1% haemolysis in t1 and t2 respectively.

In conclusion, the results of this study demonstrated an increase in the degree of haemolysis during storage of feline pRBC at 4° C. Likewise, considering the recommendations of the US-FDA guidelines on human transfusion that recommend a maximum of 1% haemolysis, the results of this study showed that all feline pRBC units with less than 24 h of shelf life have low levels of haemolysis. However, units stored for up to 28 days should only be administered if haemolysis levels have been previously tested, since 13.88% exceed the 1% limit. Furthermore, the semi-closed system was considered safe for use in collecting feline blood for pRBC processing as no bacterial contamination was detected in any unit.

2

The second study is based on the hypothesis that the use of two different types of peristaltic infusion pumps in the administration of feline pRBCs at a rate of 25 ml/h does not produce significant levels of haemolysis. Therefore, *the general objective of this study is to evaluate the risk of damage to erythrocytes secondary to the use of peristaltic infusion pumps in the transfusion of feline pRBCs,* **through the specific objective of evaluating the effect of two different types of peristaltic infusion pump on the integrity of erythrocytes** by measuring haemolysis and free haemoglobin in feline pRBC units stored for 35-42 days at 4° C.

To meet this objective, the effect of two peristaltic infusion pumps (NIKI V4 and Infusomat FmS) at a rate of 25 ml/h was analysed on 15 units of feline pRBC stored between 35 to 42 days at 4° C by measuring free haemoglobin and percent haemolysis, and the results were compared with the effect of gravity administration (without the use of an infusion pump). Mean value of haemolysis for units administered by gravity was 1.12%, and when comparing this with the degree of haemolysis in transfused units using the NIKI V4 pump (1.13%) or the Infusomant FmS pump (1.14%), no significant difference was identified, with a difference of only 0.01% and 0.02% respectively. **As a conclusion** to this study, results demonstrated that the use of two common PIPs in veterinary hospitals does not produce levels of haemolysis that are significantly different than that caused by gravity alone during transfusion of feline pRBCs at a rate of 25 ml/h.

3 Finally, unlike the first two studies, the third study investigates feline plasma storage, and is based on 3 hypotheses. The first is that that one year of storage at a temperature between -18° C and -25° C in feline fresh frozen plasma units will induce a significant decrease in the activity of clotting factors when compared to feline fresh frozen plasma stored for less than two weeks. The second is that to freeze feline plasma units within 24h of blood donation and to store them for less than two weeks at a temperature between -18° C and -25° C, does not lead to a decrease in the activity of clotting factors when we compare the results with a pool of non-stored plasma from healthy cats. And the third is that the recovery of factors VIII and V in feline fresh frozen plasma is significantly lower than the recovery of factors II, VII, IX, X, XI and XII after one year of storage at a temperature between -18° C and -25° C and therefore, are candidates to be considered the most labile factors during storage.

Likewise, *the general objective of this study was to investigate the activity of specific coagulation factors after freezing and during storage of feline FFP* **through the specific objectives of evaluating the concentration and stability during storage time** of specific coagulation factors I, II, VII, IX, X, XI and XII in feline fresh frozen plasma, **to identify the coagulation factors that are most affected by feline plasma storage** and therefore must be considered critical factors for the production and quality control of feline fresh frozen plasma, and finally **to help understand the needed volume of fresh frozen plasma to administer 10 IU/Kg of functional coagulation factors** when using units of feline FFP stored for up to one year at a temperature between -18° C and -25° C.

To meet these objectives, 55 units of feline plasma were collected and stored at -18° C to -25° C. From these, 21 were stored for less than two weeks and 34 for a year. After completion of storage, specific coagulation times for factors II, V, VII, VIII, IX, X, XI, XII were tested using modified one-stage activated partial thromboplastin or prothrombin time assays using human specific factor deficient plasma. Fibrinogen was determined using the von Clauss Method.

The results showed that mean ± SD specific activity for factor II, V, VII, VIII, IX, X, XI and XII were 101.94 ± 19.06%, 71.94 ± 24.14%, 102.78 ± 24.69%, 77.52 ± 30.39%, 84.86 ± 29.35%, 96.24 ± 25.10%, 88.76 ± 22.73% and 89.50 ± 21.85% respectively at T0, and 73.23 ± 39.06%, 97.87 ± 62.33%, 60.08 ± 38.17%, 50.32 ± 23.8%, 71.37 ± 22.23%, 83.91 ± 49.54%, 66.28 ± 22.20% and 55.46 ± 23.18% respectively at T1. Fibrinogen was 2.76 g/l (± 1.09) at T1.

A significant decrease was identified between T0 and T1 for factors II, P = .0008; VII, P = .0003; VIII, P = .0015; XI P = .0011; and XII P = .000003, and a significant increase was identified for factor V, P = .0458.

In conclusion, although a decrease in most coagulation factors activities was noted, one-year-old feline FFP is haemostatically active *in vitro*. The most suitable factors for quality control of fFFP are factor VII and VIII. And finally, based on the results of this study, it was calculated that approximately 13 to 20ml of fFFP are required, depending on storage time, to administer a minimum of 10 IU/Kg of all specific coagulation factor's activity.

Justification

Transfusion medicine is a replacement therapy for one or more blood components that aids in the clinical management of patients with a deficiency of these. Whole blood can be processed and divided into different blood products. A correct use of them allows the clinician to apply a specific treatment to each patient, avoiding the transfusion of unnecessary products or components, reducing the risk of transfusion reactions, allowing better a conservation of the blood and improving the efficiency of blood donations.[1,2]

The use of blood products in feline medicine has historically been restricted to the use of whole blood due to the technical difficulty in the processing and storage of feline blood components and due to the unavailability of closed systems to collect blood. In the recent decades feline transfusion medicine has evolved significantly, with the use of feline blood products becoming an increasingly common practice. However, there is lack of information published, and thus still much controversy, regarding the techniques for storing and administering feline blood products, as well as those for collecting and processing blood, being of particular importance the lack of information regarding the optimal duration of storage with the objective of minimizing cell and proteins damage due to storage and maximizing the conservation of erythrocytes or functional proteins. The lack of information regarding the damage induced over erythrocytes by the use of peristaltic infusion pumps, one of the most commonly used in feline pRBC transfusion, is also relevant.[3]

This project was designed because there was scarce information published about processing, storage and administration of feline blood components. As a result, the procedures were being extrapolated from human and canine medicine.[1,4] Cats have a different physiology when compared to human and dogs. Feline erythrocytes have different hemorheological properties, therefore to extrapolate the information from other species and assume that similar techniques will produce similar results may not be accurate.[5] Ensuring the safety of blood products is essential and should be the top priority for blood banks.

The objective of this doctoral thesis is to provide information that allows to establish the bases for the production, conservation, and administration of the most common feline blood products: feline pRBC and feline FFP. With this in mind, the thesis is divided into three sections, two of them about feline pRBC and one centred in feline FFP:

Feline pRBC:

In vitro quality control analysis after processing and during storage of feline packed red blood cells units

During processing and storage, blood cells release by-products to the media and erythrocytes suffer morphologic changes, metabolic alterations and oxidative lesions that might affect its function and circulating lifespan.[6,7] Mechanical and environmental factors may also affect the RBCs viability, and thus blood banking techniques are essential to avoid storage damage and to allow safe transfusions.[8,9] This alterations are known as storage lesions and not only reduce pRBC transfusion effectiveness, but also can induce adverse effects to the receptor.[10–15]

Free haemoglobin and percent haemolysis are the main markers of storage damage used in human medicine. These are the standard indicators stablished in human blood banking to determine pRBC´s shelf life and the quality and viability of the pRBC production, storage and administration techniques.[16–18]

On the other hand, closed collection systems are not always available in feline transfusion medicine. Semi closed systems are used in many blood banks to collect feline blood for blood components processing, but the risk of bacterial contamination is unknown when using this type of system, and this could affect the capacity for storage.[19] Until the publication of the present articles, the information available regarding feline blood administration was scarce. Few articles with low number of blood units tested were available about the risk of bacterial contamination, and all of them tested units collected using open collection systems.[20,21] To the authors' knowledge there are no previous reports aiming to validate the semi-closed collection systems for use in blood banking. There is one article reporting a positive bacterial culture in a feline pRBC unit collected using a semi closed system.[22]

Regarding the storage damage, there were no publications reporting the degree of haemolysis in a large number of feline pRBC units after processing and storage. This article was designed with the aim to establish the basis for feline pRBC blood banking by studying the degree of red blood cell damage during processing and storage in pRBC units, and also evaluating the risk of bacterial contamination using production and storage techniques extrapolated and modified from human and canine methods.

Quantitative assessment of infusion pump-mediated haemolysis in feline packed red blood cell transfusions

In feline pRBC transfusion very often it is essential to accurately control the transfusion rate in order to reduce the risk of severe transfusion reactions including volume overload. To do so, peristaltic transfusion pumps are commonly used in veterinary hospitals and general practices. However, the use of this pumps might cause red blood cell damage and increase the percentage of haemolysis, not only decreasing transfusion efficiency but also increasing the risk of adverse effects. [23–28]

Erythrocyte damage produced by peristaltic infusion pumps has been widely studied and reported in human transfusion medicine, but at the time of publishing this study there were few publications regarding red blood cell damage in veterinary medicine, with only one article testing feline blood in which they analysed the effect of a syringe infusion pump when transfusing autologous whole blood and tested red blood cell survival once transfused to the recipient, but not erythrocyte damage or haemolysis.[29]

This study aimed to determine if the commonly used peristaltic infusion pumps could induce an increased risk of haemolysis over pRBC units.

Feline FFP:

Stability of coagulation factors in feline fresh frozen plasma intended for transfusion after one year of storage

The main use of feline FFP is for transfusion of coagulation factors to patients with coagulation factor deficiency.[30] Although it is accepted that every ml of fresh plasma contains 1IU of coagulation factor activity this is not incontrovertible, and during storage coagulation factor activity can be depleted losing its therapeutic effect.[31] On the other hand it is important to be aware of the coagulation factor activity in plasma derivatives to understand if a transfusion will be efficient. It is also essential to measure coagulation factors to validate plasma processing and storage techniques. In human medicine it is widely known that the most labile coagulation factors are factor VIII and factor V, which measurement is used as representative of coagulation factor activity and thus, are critical for quality control.[32,33] In feline medicine, not only there was no information published about coagulation factors stability during storage or which coagulation factors are most labile, and thus considered essential for quality control, but not even the activity of coagulation factors in feline FFP before storage had been reported. This study aimed to resolve all this incognita, and thus stablish the basis for feline FFP blood banking.

Background

Within blood Banks, whole blood is separated into blood components with the objective to minimize the risk of transfusion reactions, to maximise the efficiency of transfusion and to increase the availability of blood products, as they can be stored for longer periods of time. Also, the collection of more than one blood components from a single donor is attractive, as there is high demand of blood products and the number of blood donations cannot always cope with it, being this especially relevant in feline transfusion medicine.

Traditionally, due to the lack of feline blood banks, the lack of knowledge and the difficulty in obtaining, processing, and storing feline blood components, feline transfusion has been limited to the use of whole blood but, in the las decades, feline transfusion medicine has largely evolved. Nowadays feline blood components are widely used in many countries, although production, storage and administration techniques have not been validated. This means that feline blood blood components are being widely used without the warranty that current production, storage, and administration systems produce safe and efficient blood products.

There are multiple blood components that can be processed from whole blood, the two most relevant ones being packed red blood cells and plasma.

Packed red blood cells (pRBC)

For hypothermic storage of red blood cells, whole blood is centrifuged and, after separating erythrocytes from the supernatant plasma, they are resuspended in an acidic solution containing high concentrations of glucose, producing a pRBC unit. Then, if this units are not immediately used, they are stored at 1-6° C.[3,34]

Packed red blood cells units contain erythrocytes, leucocytes, platelets, a small remanent of plasma and plasmatic proteins, and the components of the nutrient acidic solution used, which helps extend the storage time.[3,35,36] As most of the plasma is separated from the unit, the protein load is reduced and, therefore, the risk of transfusion reactions, including volume overload and immune mediated reactions.

In feline medicine, the main cause of pRBC transfusion is severe anaemia.[1] In cats with anaemia, a situation of tissue hypoperfusion occurs secondary to a severe decrease in circulating red blood cells (RBCs). Anaemia can be secondary to haemorrhage, haemolysis or inefficient erythropoiesis.[1,37] The goal of pRBC's transfusion is to improve the patient's capacity of oxygen perfusion to body tissues by increasing blood haemoglobin concentration.

Blood collection systems

Blood collection must be performed using a sterile system to avoid the risk of bacterial overgrowth. In human transfusion medicine, septic transfusion reactions are the second most common cause of transfusion associated death

in Western countries.[38–40] The use of aseptic techniques in blood donation and functionally closed blood-containing systems in human medicine has led to a drastic reduction in the risk of bacterial contamination.[41] There are multiple commercial closed system options to collect human whole blood, and the same can be used in canine blood banking, but feline patients cannot benefit from the same products, as the needle is too thick and the volume of the collected blood is much lower, and when mixed with the amount of CPD that this systems contain it causes an excessive concentration of anticoagulant solution. For this reason, it is usually difficult to find closed collection systems available and thus, typically open or semi-closed systems are used for feline whole blood collection. This could limit the storage capacity of blood products as it could potentially increase the risk of bacterial infection.[19]

There are three studies reporting bacterial contamination in feline pRBC units, two of them reported growth of *Serratia* spp. and *Pseudomona* spp. in two units collected using open systems and the other reported growth of *Pseudomonas fluorescens* in a unit collected using a semi-closed system.[22,42] Before the publication of the studies of this project, there were no reports published aiming to validate the semi-closed collection systems for use in feline blood banking. There were two studies that investigated bacterial growth in feline pRBC collected using open collection systems which resulted in all units testing negative, but both had a really low number of samples tested, one testing 10 units at day 32 of storage and the other testing 6 units at day 42 of storage, and thus were not reliable to evaluate the risk of bacterial overgrowth.[20,21]

pRBC storage and storage damage

Packed red blood cells transfusions are commonly needed in emergency situations and thus, availability can be critical to stabilize a patient on time and avoid a fatal outcome. The most reliable way to have immediate availability in urgent cases is to have access to stored blood products. Therefore, transfusion of stored pRBC can be lifesaving, however, it may not always be beneficial and may even cause harm.[23,43–47]

During storage, RBC's release by-products to the media and suffer morphologic changes, metabolic alterations and oxidative lesions that might affect its function and circulating lifespan.[6,7] Biologic response modifiers, such as cytokines, chemokines, bioactive lipids, and metabolites, accumulate during storage, and most of them act as pro-inflammatory agents for transfusion recipients.[48–50] All this changes are known as storage lesions, and might not only reduce the transfusion effectiveness, but also can be the cause of transfusion reactions to the recipient.[10–15]

Storage lesion occurs because RBCs are extracted from their physiological *in vivo* situation and stored *ex vivo*, exposing cells to a different set of chemical and

mechanical stressors to which they are not physiologically prepared to cope with. These conditions arise from the isolation of RBCs, the use of additive solutions and the prolonged hypothermic storage in a closed environment (storage bag), as well as the new stressors that appear secondary to the exposure to oxygen in a non-physiologic *ex vivo* situation. RBCs are exposed to storage bag's plasticisers, ambient oxygen diffusing through the blood bag and a progressive acidification of the media as metabolic waste gets accumulated.[34] Additionally, during storage, RBCs lose the biochemical countermeasures that were active *in vivo*, resulting in an increased risk of oxidative stress.[34] All these stressors produce physical damage and biochemical impairment of stored RBCs.

Storage lesions have been reported to appear within hours of storage, and to increase progressively through time. There is limited literature in veterinary medicine.[3] There are two main mechanisms of storage damage: metabolic impairment and oxidative damage.

Metabolic impairment

When stored as pRBCs, erythrocytes suffer metabolic changes secondary to being removed from the donor's circulation, isolated from plasma, and stored in an acidic solution at hypothermic temperature. Although blood cells metabolic activity is reduced during hypothermic storage, chemical reactions continue to occur without the full benefit of the protective mechanisms that operate in circulation. This produces energy consumption, accumulating metabolic waste products and progressively depleting critical substances like adenosine triphosphate (ATP).[51–53] ATP deficiency is one of the main influencing factors for the appearance of haemolysis. Approximately at two weeks of hypothermic storage, a metabolic shift occurs due to the depletion of high energy compounds like ATP.[54] ATP depletion is produced not only because of the consumption of the stored reserves, but also because with storage, ATP synthesis gets compromised and is unable to cope with the ATP consumption needs.[55,56]

Theoretically, one of the possible limiting factors for the production of ATP could be the availability of glucose, but this has largely been resolved by using additive solutions that are loaded with high concentrations of glucose. Thus the limiting factor in pRBC units is usually the impairment of limiting enzymes, and not the lack of substrate.[55,56] As a consequence of glycolysis, pH lowers progressively and the activity of critical enzymes that usually serve to supply energy and antioxidant defences gets impaired, including an activity decrease of the rate limiting enzymes for glycolysis, the pentose phosphate pathway, and the Rapoport-Luebering pathway.[57] Low pH (5.5 to 6) additive solutions can also contribute to this phenomenon. At the same time, glycolytic enzymes are progressively oxidised, which also contributes to the depletion of ATP during storage. [58–60] In human pRBC it has been reported that, after 5 weeks of storage, a decrease of 60% of the intracellular ATP reserves occurs.[61]

When ATP availability decreases under the minimum requirements of the RBCs, they suffer a series of metabolic and morphologic changes.[11,62–67] ATP depletion affects multiple enzymatic functions and ion pumps (like Ca^{2+} pumps) and thus, cation homeostasis is deregulated, and membrane asymmetry disrupted. Transport of negatively-charged phospholipids from the external cell-membrane surface to the internal surface depends on an ATP-mediated active transport and thus, when ATP deficit installs, an externalization of negatively-charged phospholipids at the cell membrane occurs, triggering the exposure of phosphatidylserine and phosphatidylethanolamine, which are normally confined to the inner bilayer.[35] Within other consequences over the receptor, this increases the macrophage clearance following transfusion.[35]

On the other hand, ATP is necessary for the maintenance of the erythrocyte membrane, and when a deficit occurs, it irreversibly loses deformability.[34] At the same time, as result of a protective cellular mechanism against early cell death, micro-vesiculation of the membrane occurs. In physiologic conditions, this microparticles would be removed by the reticuloendothelial system, but in storage conditions, this does not happen, which leads to the accumulation of phosphatidyl-serine-expressing negatively-charged micro-particles (MPs) in the extracellular fluid.[68,69] This MPs are pro-inflammatory and are capable of inducing transfusion reactions, they also express an inactive form of tissue factor and provide a negatively-charged surface that can catalyse the activation of factors IX and X, predisposing to coagulation.[70,71]

Depleted ATP also induces reorganisation of the cytoskeleton, causing echino-cytosis, altering the ability of kinases to phosphorylate proteins and reducing the anti-oxidant capacity, also contributing to damage from oxidative stress.[72,73]

When micro-vesiculation occurs, the loss of membrane area is higher in relation to its volume, losing the excess surface needed to allow passage of erythrocytes through narrow capillaries and therefore, reducing its elasticity and increasing RBC osmotic fragility.[74,75] In addition, the cross-linking of the cytoskeleton and membrane proteins, the dysregulation of cytoskeletal protein phosphorylation and cell dehydration due to Ca^{2+} influx and K^+ efflux, also contributes to reducing erythrocytes deformability.[76–78]

Although some of the metabolic changes are permanent, part of them can be reversible after transfusion, including ATP deficiency which in human patients recovers by 7-72h after being administered to the receptor. Nevertheless, this might not be enough to cope with the supra-physiological metabolic demand in a critically ill patient.[79] Additionally, cells that are in a more advanced degenerative state or senescent will not be able to recover and will be removed by the recipient's reticuloendothelial system.[80]

Oxidative damage

Oxidative stress is considered one of the main elements for the development of storage lesions, and it is mostly initiated by the chemical oxidation of iron in haemoglobin. Haemoglobin is a highly specialized carrier of oxygen which occupies up to 95% of erythrocytes mean volume.[81,82] Oxidative stress is secondary to a dysregulation between production of reactive oxygen species (ROS) and antioxidants. ROS are produced as a natural by-product of the normal metabolism of oxygen, and can also be acquired exogenously by ionizing radiation and xenobiotic compounds.[83–85] As red blood cells act as an oxygen transporter, they are continuously exposed to oxidative stress due to ROS from both endogenous and exogenous sources, which can potentially damage the RBC and alter erythrocyte rheology.[86,87]

Erythrocytes are rich in reactive ferrous iron within the heme prosthetic group of haemoglobin and, when the RBC it is fully oxygenated, also contain high concentration of dissolved oxygen. Thus, a small rate of auto-oxidation in erythrocytes can produce substantial ROS that can cause damage to the RBC.[34,88] Ferrous iron and oxygen have a highly reactive nature, and when oxygen concentration increases, iron passes to a ferric state and methaemoglobin is formed. Also, superoxide anion is generated and converted to H_2O_2 by superoxide dismutase. H_2O_2 is a major ROS and serves as a substrate for hydroxyl radical (OH^-) generation.[34,89] This mechanism of auto-oxidation is a process that happens physiologically in about 3% of the total Hb every day, generating methaemoglobin and O^{-2}.[89]

In an in vivo situation, to mitigate oxidative stress and oxygen consumption, RBCs develop a really strong antioxidant system with the objective to maintain haemoglobin iron in a reduced state even in the presence of high oxygen concentrations.[34] Once haemoglobin gets oxidised (methaemoglobin) it can be reduced back from ferric state to ferrous state by enzymes like methaemoglobin reductase, or can be aggregated forming Heinz bodies and removed by vesiculation.[90] In a hypothermic *in vitro* situation, the activity of reductase enzymes is decreased. This, together with higher dissolved oxygen concentrations due to increased solubility at low temperatures, results in an enhanced production of methaemoglobin and superoxide anion. Methaemoglobin has low stability at hypothermic temperature, and instead of being accumulated, it denatures into globin and haemin or free heme.[34] On the other hand, ferric iron in heme is reduced by the superoxide by-product derived from the methaemoglobin formation. H_2O_2 then reacts with ferric iron by the Fenton reaction to produce net OH^- by the Haber-Weiss reaction, and with oxyhaemoglobin to produce ferryl haemoglobin ($HbFe^{4+}$).[91] Both by-products are highly reactive and oxidise nearby enzymes and lipids.

Oxidative damage caused by ROS and the accumulation of methaemoglobin contributes to the morphologic changes of erythrocytes and the formation of microparticles, inducing distortion of membrane integrity, altering blood flow rheology, inducing membrane transport abnormalities, inducing exposure of phosphatidylserine and predisposing to cell death.[92–94] Oxidised proteins like denatured haemoglobin, bind to the cytoskeleton and damages its structure causing morphological changes and reduced deformability.[95,96] It initially induces a conversion to echinocytes, and when microparticles are released, to the irreversible form of spherocytes with reduced deformability. Furthermore, it also contributes to the externalization of inner membrane phospholipids by oxidating membrane lipids and proteins, which, as mentioned previously, needs expenditure of ATP to be reversed via ATP dependant transporters, and it also induces imbalances in K^+ and Ca^{2+} ions.[93,97–99] Oxidised and denatured proteins aggregate and precipitate in/on the RBC membrane. ROS moreover oxidises haemoglobin at the critical amino acid residue, β-92 histidine, destroying its ability to bind oxygen.[100]

Additionally, lipid oxidation also occurs secondary to lipid peroxidation induced by Ferryl-haemoglobin and OH^-, which can initiate an oxidative cycle sustained by the availability of oxygen and contributes to the membrane bilayer disruption and to the production of biologically active, oxidised polyunsaturated fatty acids (oxylipins).[101,102]

On the other hand, as this process continues, reducing equivalents get depleted and thus the antioxidant capacity of the red blood cell gets reduced, exacerbating oxidative stress damage, but also directly affecting the recipients when the blood is transfused. It is hypothesised that a loss of erythrocytes S-nitrosylation (SNO) during storage interferes with vasodilation in transfusion recipients by affecting nitric oxide availability, although this remains controversial.[103–105] Old RBCs show higher oxidative stress and are removed from the bloodstream by the liver and spleen by macrophages within the reticuloendothelial system.[34]

In human pRBC, it has been reported that after two weeks of storage with SAGM (adenine, dextrose, mannitol and sodium chloride), this cascade exponentially increases its intensity reaching ROS accumulation plateau, and causing reversible and irreversible oxidation of structural and metabolic enzymes, worsening the metabolic impairment.[100,106,107]

RBC damage biomarkers

Multiple red blood cell damage biomarkers have been described in the literature, including free haemoglobin, percentage of haemolysis, potassium, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase concentrations, phosphatidylserine, CD47 and band-3 protein exposure, microvesicle release, osmotic fragility and changes in RBC's morphology.[10,16,34]

In the design of our studies we decided to use percentage of haemolysis and free haemoglobin when evaluating the red blood cell storage damage and to evaluate the potential damage over RBC's produced by peristaltic infusion pumps during transfusion. Free haemoglobin is the most present biomarker in human literature related to erythrocyte damage.[16-18] Percent haemolysis is also widely used and nowadays is considered as the standard in human medicine to determine the quality and viability of the pRBC production, storage and administration techniques, and also to determine the pRBC shelf life.[16-18] Haemolysis is the most severe and significant manifestation of red blood cell storage damage, representing the rupture of erythrocytes releasing haemoglobin to the suspending fluid or the loss of membrane-bound Hb in microvesicles.

Potassium concentration is another widely used biomarker for RBC damage. Potassium release is a process dependant on ATP, and thus, old RBCs that have already depleted the APT stores have already lost more potassium than young cells.[108] Potassium has shown to be much less sensitive than free haemoglobin, probably due to the smaller relative difference between intracellular and extracellular space in stored red blood cells, and its use has not been recommended when old storage units are tested regarding administration induced haemolysis.[26-28,108]

Haemoglobin toxicity

One of the main functions of RBCs is to constitute a physical and biochemical separation between Hb and the extra-erythrocytic environment by using a selective barrier that allows gaseous and other ligand transport, and to provide enzymatic mechanisms to maintain Hb in a functional nontoxic state.[109] This is essential to preserve the balance between normal physiology and toxicity of Hb, as haemoglobin is an essential protein in feline physiology but, when it is free, it can become toxic.[109,110]

Thus, as haemolysis occurs, not only the effectivity of the transfusion is reduced due to the decrease of functional RBCs, but also haemoglobin (Hb) is liberated to the media.[111,112] Free Hb concentrations increases with time and can result in Hb toxicity and in an important cause of transfusion reactions classified as non-immune-mediated haemolytic transfusion reactions.[112] Transfused free Hb can lead mainly to redox injury of the endothelium and the kidney proximal tubule, but other tissues can also be affected.[109,113,114]

In a normal physiologic state, there are several distinct and overlapping protective mechanisms that act clearing free Hb to mitigate its cytotoxic effect. Once free haemoglobin is released to circulation it is usually cleared from plasma with a half time of 20 to 30 minutes. One of the main mechanisms is mediated by haptoglobin, a serum glycoprotein that avidly bounds Hb dimers to form Hb-Haptoglobin complex, which protects the tissues from oxidative

damage.[115–119] Hb-haptoglobin complex is then recognized and internalized by the phagocytic cells in the reticuloendothelial system via its receptor CD163.[115–118] When free Hb is not bound by haptoglobin, Hb can be oxidized or denatured, and in this state it is prone to release free heme. Most of this heme is initially bound to plasma lipoproteins, including low density lipoproteins and high-density lipoproteins before being reversibly bound to albumin, forming metalbumin, with hemopexin, which has high affinity, or with α1-microglobulin.[120,121,130–132,122–129] From all of this, hemopexin has the highest affinity for free heme, and results in the hemopexin-heme complex which, similarly to the haptoglobin-Hb complex, is removed by endocytosis via the CD91 receptors in hepatocytes and macorphages.[128,129,133–137] Another identified minor pathway for Hb clearance is the oxidation of Hb to methemoglobin which can be excreted or hydrolysed to release ferriheme which can also be complexed to hemopexin to be transported to the mononuclear phagocyte system, or bounded to albumin (methemalbumin) for transport to the mononuclear phagocyte system.[115]

Once internalized by the macrophages, Hb is hydrolysed to globin and heme moieties. The globin chains are degraded by proteolytic enzymes and the constituent amino acids are released.[115] Heme is degraded by the rate limiting enzyme heme oxygenase-1 into equimolar amount of iron, biliverdin and carbon monoxide.[123,138,139] As mentioned before, not all heme or Hb is bound to proteins and, when it is free, it causes erythrocyte membrane damage and injury, and activates proinflammatory signalling pathways in RBCs, immune and endothelial cells, hepatocytes, macrophages and neutrophils.[120,140] To avoid this, heme induces a program of antioxidant enzymes that try to compensate for the oxidative stress caused, including glutathione S-transferase pi and NAD(P)H dehydrogenase [quinone] 1.[141]

If the amount of free Hb exceeds the clearing capacity of these mechanisms it can become toxic. Hb is then filtered by the kidney through glomerular filtration, and when this happens, part of the Hb is catabolized by the proximal renal tubular cells, and the excess is eliminated in the form of hemoglobinuria.[142]

In two studies performed in 1949 and 1978, the infusion of a stroma-free Hb solution to humans resulted in hypertension, haemoglobinuria, and acute renal failure. After dose studies it was deduced that the Hb molecule outside the RBC's protective mechanism was the cause of the hemodynamic alterations observed.[143,144]

Free haemoglobin and its degradation products heme and iron lead to oxidative stress, loss of nitric oxide, activation of inflammatory pathways, and immunosuppression, leading to microcirculatory dysfunction, significant injury to all major organ systems, and increased susceptibility to infection.[145–149] Hence, extracellular Hb is capable of causing toxicity to a diverse group of organ systems

including vascular, myocardial, respiratory, hepatic, renal, and central nervous system.[144,150,159–163,151–158] Potential toxicity is increased with time of exposure and co-existing pathologies such as renal insufficiency, atherosclerosis or diabetes mellitus.[110,164]

When free in the extracellular matrix, heme functional groups of Hb interact with multiple ligands, leading to oxidative stress by the formation of ferric, ferryl, ferryl heme radical, ferryl protein radical, hemichromes, protein radical-induced globin chain cross-links, free heme=iron and nonheme radical species (e.g., lipid peroxides).[165]

The pathophysiological relevance of Hb-induced oxidative stress is not completely understood, nevertheless its redox reactions are believed to significantly contribute to oxidative damage at the vascular endothelium and within tissue.[110]

Heme induction of inflammation

Free heme causes systemic inflammation by activating RBCs, macrophages, neutrophils and endothelial cells and inducing them to secret proinflammatory cytokines including toll-like receptors, tumour necrosis factor, interleukin-6, placenta growth factor (PlGF), interleukin 1β and release of erythroid damage-associated molecular patterns that potentiates inflammation. [120,140,172–176,145,147,166–171]

One of the most relevant inflammatory factors released is PlGF, which is a driver of diverse pathologies, especially angiogenesis and inflammation. PlGF has mitogen and migratory effects on endothelial cells and also induces macrophage activation and chemoattraction. [177]

Hypertension

Extracellular Hb has been shown to induce hypertension by a vasoactive mechanism. Although its mechanism is not yet fully understood, traditionally the main hypothesis has been largely attributed to the pharmacologic interaction between nitric oxide (NO) and heme iron. Hb has shown to react with NO significantly faster than RBC haemoglobin, inducing vasoconstriction.[6,61,110,178–181] On the other hand, RBCs contain arginase, and when haemolysis occurs, this is also released to the media.[182] Free arginase consumes plasma L-arginine, which is needed as a substrate for NO production, and thus contributes to decrease NO bioavailability.[183–185] Although NO deficiency has been identified as a major sequelae of haemolysis, the current knowledge is growing towards the idea that the induction of hypertension is most probably due to a multifactorial mechanism, as there is known to exist a vast range of potential vasoactive substances (e.g., eicosanoids, angiotensin, endothelin, and serotonin) that have a longer pharmacodynamic activity comparing to NO, being this a short-lived diatomic gas.[110,186]

Other mechanisms that probably contribute to the hypertensive state secondary to Hb exposure could be the physical properties of extracellular Hb, adrenergic-serotonin system interactions, Hb-induced free radicals, prostaglandins, and numerous pathways involving vasoactive peptides.[187] Also, this hypertensive consequences could be pathway specific on every type of vascular tissue.[110] Hypertension resulting from any of the mentioned mechanisms results in a limitation of tissue blood flow and tissue oxygenation.

In addition to hypertension, depletion of NO could contribute to promote vasculopathy endophenotype that predisposes to pre-capillary pulmonary hypertension, leg ulceration, cerebrovascular arteriopathy, chronic kidney disease and priapism.[188]

Endothelial damage

Endothelial dysfunction is a major concern when high levels of Hb are present in circulation. Endothelial cells demonstrate high susceptibility to the cytotoxic effects of heme group in Hb. Heme interacts with autacoids that regulate vascular tone and produces local oxidative processes that modifies the physiology and biochemical behaviour of the vascular endothelium and vascular smooth muscle. Heme can be adhered to vascular endothelium, were it scavenges NO and thereby mediates oxidative stress.[6,189,190] Heme has also been described as inducing disruption of microvessel architecture in the endothelial cells.[191] Exposure of endothelial cells to Hb aggravates existing atherogenesis and can induce the onset and progression of endothelial dysfunction.[109,192]

Cardiovascular damage

Cardiovascular toxicity has been reported in a species specific way, with variable and inconsistent toxicity for some species like rabbits and monkeys, and showing no toxicity in other species like mice, rats and dogs.[159] To the author's knowledge, there are no published studies performed in cats at the moment of writing this thesis.

In a species specific way, Hb has been reported to induce transient myocardial tissue necrosis, and it is thought to be secondary to NO scavenging by Hb.[159,193] Myocardial lesions are usually focal, but in some cases it can induce a diffuse degeneration of cardiac myofibers described histologically as coagulative with eosinophilic staining in the left ventricle or the cardiac septum and papillary muscle.[110]

On the other hand, there has been accumulating evidence that PlGF dysregulation, which has been associated with free heme, is present in multiple heart conditions although it is unclear if it acts only as a biomarker or is causative of pathogenesis.[194–196] This association between increased PlGF and worsening of cardiovascular disease has also been observed in human patients with chronic kidney disease.[197,198]

Central nervous system damage

Central nervous system toxicity should be a concern mainly in those patients with traumatic brain injury so that Hb might be in direct contact with neurons. Oxidative stress secondary to Hb is the main mechanism proposed for CNS damage, but other theories have received attention, including the relation between Hb and vasospasm when subarachnoid haemorrhage occurs.[154,199,200] In an *in vitro* setting, neurons have been shown to be susceptible to Hb toxicity, even at subtoxic concentrations Hb has been reported to increase neuronal susceptibility to exocytotoxic injury.[153,201]

Renal system damage

When free Hb is filtered by the kidney, it initially gets reabsorbed by the proximal tubular cells. Once the reabsorption capacity is exceeded, Hb is excreted through the urine.[110,202] Once excreted, heme proteins can precipitate and form casts, predisposing to tubular obstruction and contributing to renal injury. Other described mechanisms are ischemic injury due to an increase in vascular resistance secondary to vasoconstriction and oxidative damage secondary to the effect of the heme group and iron.[110]

On the other hand, PlGF has been reported as significantly upregulated in patients with chronic kidney disease and decreased renal function, which supports a potential link between PlGF and kidney function.[197,203] As mentioned before, it has been reported that free heme stimulates PlGF secretion and additionally, administration of exogenous heme in healthy and sickle cell mice has been reported to induce upregulation of PlGF in the kidneys in agreement with heme uptake from renal cells.[177,204]

On the other hand, in human patients with sickle cell disease with associated albuminuria, renal biopsies have been shown to contain hemoglobin-laden eMPs adherent to the capillary endothelium, suggesting a contribution to renal injury.[190]

Respiratory system damage

In human medicine, plasma Hb levels have been clinically correlated with pulmonary hypertension in patients with sickle cell disease, in which it has been identified as a serious complication associated with high mortality.[188,205,206] On the other hand, short term pulmonary hypertension has been described with the administration of Hb and during acute haemolytic events, although it is hypothesized that long term situations and repeated exposure to Hb have the highest risk, in which chronic exposure might induce permanent changes in vascular compliance, elasticity, distensibility and stiffness.[188,207,208] The main suspected mechanisms are autacoid dysregulation and oxidative stress.[209–213]

Interestingly, free haemoglobin and heme have been found to have a role in acute lung injury/acute respiratory distress syndrome (ALI/ARDS) associated to trauma-haemorrhage and stored RBC transfusion, but also to halogen toxicity, endotoxemia, bacterial pneumonia and ventilator-induced acute lung injury.[149,214–217] On the other hand, not only free haemoglobin in blood has been associated to lung injury, also free haemoglobin levels have been found elevated in bronchoalveolar lavage fluid from ARDS patients when compared with control patients with hydrostatic pulmonary oedema.[215]

In one study performed in dogs with experimentally induced pneumonia, transfusion of 42 days stored blood worsened pulmonary hypertension, gas exchange and induced ischemic vascular damage in the infected lung, increasing mortality when compared to transfusion of 7 days old blood.[148] In a further study, they additionally analysed identically treated animals with lower or higher bacterial dose, and they concluded that the increase in free Hb reported with older units requires the presence of stablished infection to worsen outcome, and that the transfusion of old RBCs increase the risks form infection in septic dogs.[218]

Quality control of pRBC

To avoid the untoward consequences of haemolysis, human transfusion regulatory agencies have set haemolysis standards as a condition of RBC processing, management or storage system licensure.[111,219,220] In human blood banking, the Council of Europe and the U.S. Food and Drug Administration (US FDA) guidelines state that at the end of storage no more than 0.8% and 1% haemolysis, respectively, is surpassed to meet the recommended quality control and avoid the administration of damaged units to the patients, with a minimum of 90% and 95% respectively of the units meeting this expectations.[219,220] However, similar recommendations lack in veterinary medicine.

This values are calculated to obtain medically safe transfusions, as it is considered that normal haptoglobin content of blood is enough to efficiently remove free hemoglobin contained in this units.[111]

Transfusion of old storage pRBC units

Nowadays in human medicine there is still controversy over if transfusing older stored blood negatively affects outcome despite the strict quality control measurements and requirements. There are studies that suggest that there could be a link between stored blood age and increased morbidity and mortality, but others have not found any relationship.[221,222]

In one study performed in critically ill injured trauma human patients, they observed that tissue oxygen saturation decreased in people receiving blood stored for >21 days compared to people receiving blood stored for <21 days.[223] In another study performed with anaemic but otherwise stable trauma intensive

care unit human patients, they reported that transfusions of older RBC units were associated with the inhibition of regional microvascular perfusion.[224] On the other hand, there are several studies that could not find any significant relationship, like a study performed in humans undergoing cardiac surgery, in which they compared tissue oxygenation in patients transfused with red blood cells stored ≤10 days or ≥21 days and they found no differences in cerebral StO_2 or sublingual microcirculatory blood flow.[225] Or a study performed among children with lactic acidosis due to severe anaemia, in which transfusion of longer-storage compared with shorter-storage RBC units did not result in inferior reduction of elevated blood lactate levels.[226] In a similar way, in a randomized trial performed in humans undergoing cardiac surgery they were not able to find a difference in morbidity and mortality between patients transfused with units stored for ≤14 days or for ≥20 days.[227] And in another article performed with human healthy volunteers they reported that transfusion of 1 unit of 42-day-stored RBCs had no overt detrimental effect on tissue oxygenation or the microcirculation.[228]

Overall, recent literature reviews and other recently published randomised controlled trials concluded that there appears to be no evidence of an effect on mortality related to length of storage of transfused RBCs, and indicate that transfusion of fresh stored blood does not decrease the risk of mortality when compared to the standard of care, including massively transfused critically ill patients.[226,229–236]

This must be understood bearing in mind that only units stored within the maximum permitted time of 42 days and using techniques that comply with the standards of human guidelines were used, which has been stablished as safe regarding low risk of a percentage of haemolysis higher than 0.8% to 1%, all of which is not accomplished nowadays in feline transfusion medicine.

PRBC administration

When performing a transfusion, it is important to consider all aspects of the process to avoid contributing to erythrocyte damage, aiming to perform a safe and efficacious transfusion.

Blood components are administered using specific infusion sets with a filter of usually 170 to 260 μm. This are designed to filter blood clots and big particles that could be damaging to the receptor. In feline transfusion also 18 μm paediatric filters can sometimes be used, this are able to filter degenerated platelets, white blood cells and fibrine strands that appear after 5 days of storage.[29,237–239]

Although different routes are described like the intraosseous, mostly used in small cats and kittens, blood components are usually administered through an intravenous catheter.[239,240] There are different administration techniques or devices available on the market, and they can function by gravity (allowing

the fluid to flow through the infusion system) or by adding external pressure using infusion pumps. To be able to control the administration rate is of utmost importance in feline transfusion medicine to avoid volume overload, especially in patients at risk as cats with cardiovascular disease. It is also important initially to maintain a slow rate (0.25 mL/kg[1] for 15-30 minutes) to evaluate possible transfusion reactions before a large amount of blood has been transfused.

On the other hand, any transfusion technique must be evaluated before being safely used, as red blood cells are fragile and can be damaged during the process, decreasing the efficiency of the transfusion and liberating by-products to the supernatant, which can induce an increase in the risk of adverse effects and transfusion reactions.

Infusion devices

Infusion devices can be classified in regard to their power source as gravity controllers or infusion pumps, and the latter may be powered by pneumatic, mechanical or electrical forces.[241]

Gravity controllers are devices that set the desired flow rate in drops per minute and are controlled by battery or mains powered line occlusion valves, relying solely in gravity to provide the infusion pressure. A drop sensor can be used in some cases to monitor the flow rate and, in some devices, a variable mechanical clamp controls the flow by changing pressures to achieve the designated rate. The accuracy of this type of mechanism relies on the conversion from drops to ml, and this is always approximate. The actual drops/ml may differ considerably when comparing different situations as it depends, on one hand, on the fluid's composition, temperature and surface tension and, on the other hand, on the size, shape and the conditions of the orifice.[241] Another important influencing factor is the infusion rate as the drops/ml value decreases and the drop volume grows when the delivery rate is increased.[241] This methods are efficient, but in feline transfusion medicine, due to the higher risk of transfusion reactions including volume overload, its lack of accuracy is not optimal.[25] In cases were accuracy is critical, volumetric or syringe infusion pumps should be used.

Infusion pumps are medical devices used to deliver fluids in controlled amounts. It allows the user to control the rate of infusion by applying positive pressure to the infusion set, and to administer small volumes of fluids accurately.[16,242,243] Thus, infusion pumps are those that have an active mechanism to overcome resistance to flow.

Although gravity controllers have a lower risk of inducing RBC damage, infusion pumps are commonly used in feline transfusion medicine because they allow the clinician to deliver fluids at precise rates and volumes, and to maintain a constant, low-flow rate.[3,239] This is important to reduce the hemodynamic stress

of the associated volume load and to avoid transfusion reactions.[239] However, the mechanical force exerted by syringe pumps or peristaltic pumps may cause haemolysis, with potentially deleterious effects from the released haemoglobin and potassium.[16]

This chapter of the project will focus on the study of infusion pumps and its effect over red blood cell integrity.

❶ Types of infusion pumps

There are different types of infusion pumps and they can be classified regarding to their flux control mechanism as: elastomeric pumps, volumetric infusion pumps and syringe pumps.[16] There are also other more specific types like patient-controlled analgesia pumps, anaesthesia pumps, and ambulatory pumps, which are not adequate for transfusion and will not be discussed further, and drip rate pumps which are considered outdated and no longer available in most countries.

Elastomeric pumps are non-electronic single use pumps, they are considered not accurate and thus, their use for transfusion is not recommended.

Syringe pumps drive the plunger of a syringe forward at a controlled rate. Although syringe pumps are recommended for lower volume and low flow rate infusions, their use in feline transfusion medicine is not recommended when blood components are used because the blood has to be transferred to a syringe, and by doing so, the usually closed system (blood bag) is disrupted endangering the isolation of the unit and consequently increasing the risk of bacterial growth.

Regarding volumetric pumps, they show a wide range of features allowing widely varied performances, including pRBC transfusion. They usually perform correctly at rates down to 5ml/h and can be programmed to lower rates.[244] There are two types of volumetric infusion pumps: dedicated cassette and peristaltic. Within the latter, also two subtypes are described: linear peristaltic and rotary peristaltic. Both types of peristaltic infusion pumps have mechanisms based on fingers, cams or rollers that pinch off a section of the set. The difference between them is that the first uses a wave movement and the second works based on compression on the linear plaques or gyrating rollers.[242,243] Linear peristaltic pumps are the most common, and its mechanism uses fingers or cams that are located on a camshaft, as the shaft rotates, each "pinched off" section delivers its volume to the patient. In rotary peristaltic mechanisms rollers are located on a hub that rotates delivering the volume in each "pinched off" section.[242–244]

As mentioned previously, when applied to transfusion of pRBCs or whole blood, each of these mechanisms is capable to induce mechanical stress over red blood cells, producing erythrocyte damage and haemolysis.[16,27,245] Peristaltic infusion pumps are believed to be the most commonly used pumps in veterinary transfusion medicine.

❷ Haemolysis in peristaltic infusion pumps

As mentioned previously, the use of infusion pumps gives numerous advantages such as control of the infused volume and rate, and even alarms that give increased capacity of reaction to problems during the infusion, but there is still controversy on their use in transfusion therapy due to the effects of the infusion pump mechanism over the integrity of RBCs resulting in haemolysis.[23,24]

All pRBCs units have a baseline haemolysis acquired during processing and storage, and this can increase during the transfusion process. Infusion pumps are one of the main factors that could potentially contribute to it.[25–28] Linear peristaltic infusion pumps are the most commonly used in clinical practice, even that, although there is no consensus over the type of pump that induces less erythrocyte damage, some authors state that this could be the type of infusion pump most susceptible to inducing RBCs haemolysis.[16,245,246] The following discussion is centred in the use of peristaltic infusion pumps.

It has been reported that peristaltic infusion pumps are capable of inducing haemolysis over human and canine pRBC units when transfused, and it is widely accepted that the main mechanism of erythrocyte damage is the direct compression of erythrocytes within the infusion tube.[16,25,27,28,245,247] The degree of erythrocyte damage depends on different factors like the storage time of the pRBC unit transfused, the blood unit´s haematocrit, the preservative solution, the transfusion line (including the filter), and pump type.[16,25,26,246,248–251] Infusion rate also influences erythrocyte damage, as increased percentage of haemolysis has been described when very low or very high transfusion rates are used.[26,248]

Haemolysis induced by peristaltic infusion pumps over human pRBC transfusion was described for the first time on 1984.[246] Since then, multiple studies have been published investigating the effect of different types of infusion pumps over human erythrocytes when transfused.[26,108,247,251–257] In a study performed by Frey et al. simulating a neonatal transfusion at 20 mL/hr, a similar speed than used in feline transfusion allowing a more accurate comparison, differences regarding integrity of human RBCs during blood transfusion using a syringe pump, a peristaltic pump, or a volumetric pump with shuttle mechanism was investigated, and they observed that the transfusion process led to statistically significant changes in RBC integrity in all three infusion pumps, with a mean increase in free HGB of 0.006 g/dl for each pump.[27]

In another study performed with human pRBCs administered at a similar speed (30ml/h), median increases in the percentage of haemolysis of 0.016% (shuttle pump), 0.026% (piston pump) and 0.241% (PIP Infusomat-Space) were described.[25] And another study with human pRBC at 100ml/h was found reporting mean haemolysis increase of 0.08%.[28]

Although erythrocyte damage induced by infusion pumps has been widely

studied in human transfusion medicine, there is still very scarce information available in veterinary transfusion medicine.[16,25–29,258] There is only one study regarding canine erythrocyte damage during transfusion. This was performed with whole blood and compared three types of PIP using free haemoglobin as a biomarker, and they reported an increase of 7% to 228% depending on the pump type tested.[258] Extrapolating this information about canine or human pRBCs to feline medicine might not be adequate, as feline erythrocytes have hemorheological differences comparing to canine or human, including smaller size and a greater increase of stiffness under hypoxic conditions, and this could result in a higher risk of haemolysis secondary to PIP when this are used for transfusion on the clinical practice.[5,259] Until now, to the authors knowledge there has been only one article published about the effect of an infusion pump over feline erythrocyte during transfusion, and they analysed the effect of a syringe infusion pump when transfusing autologous whole blood and tested red blood cell survival once transfused to the recipient, but they did not test for markers of erythrocyte damage or haemolysis.[29]

As discussed previously, haemolysis does not only lead to a loss of functional haemoglobin, but also induces the release of by-products such as free haemoglobin or potassium, which can result in induction of toxicity or exacerbation of concomitant diseases.[16,23,25] Free haemoglobin infusion has been associated with a decrease in nitric oxide, vasoconstriction, hypertension, and contribute to pro-inflammatory and pro-thrombotic states.[16] Although peristaltic infusion pumps are commonly used for feline transfusion medicine, until now, there was no evidence that supported this practice as safe.

Feline Fresh Frozen Plasma

Coagulation factor activity and stability during Feline fresh frozen plasma storage

Plasma is the aqueous component of blood that is separated from the heavy cellular component after hard-spin centrifugation of whole blood which has been collected using a closed or semi-closed system with integral transfer packs.[33,260–262] Plasma contains mainly plasmatic proteins that when in circulation are meant to maintain homeostasis, including albumin, immunoglobulins, coagulation factors, antiprotease, lipids and other biological mediators.[33,262–264] Plasma should be separated immediately after donation or alternatively, whole blood can be cooled rapidly to maintain temperature between + 20° C and + 24° C and can be held at that temperature for up to 24 hours before separating.[261]

In contrast than with whole blood, plasma can be frozen allowing it to be stored for longer periods of time and to be readily available when needed in an emergency

situation.[31,33,265] Additionally, transfusion of plasma, rather than whole blood, allows to replace haemostatic proteins without administering unnecessary cells, minimizing the risk of red cell sensitization and volume overload.[31,33,265]

After separation of pRBCs from whole blood, the remaining product is called Fresh plasma. Fresh plasma should be used in less than 24h or frozen to a temperature that adequately maintains the labile coagulation factors in a functional state. Freezing must take place using a method that allows complete freezing within one hour and ideally to a temperature of -25° C or below, with some authors recommending initial freezing temperature of between -30° C and -80° C.[32,261,266] Later, plasma can be stored at -18° C or less and is then classified as fresh frozen plasma (FFP).[31]

In a recent study, the most common indications for feline FFP were suspected coagulopathy (83%), haemorrhage (35%), and hypotension (25%).[30] The haemostatic system is compounded mainly by coagulation factors, platelets and anticoagulant proteins, and it is responsible for controlling haemorrhage and maintaining adequate blood flow.[267] Quantitative or qualitative alterations in this process can lead to abnormal haemostasis and bleeding. Plasma is a therapeutic source of labile and nonlabile coagulation factors, and as such is most commonly used for haemostatic purposes in feline patients. Its's considered the gold standard care in patients with coagulation factor deficiency due to both congenital and acquired bleeding disorders.[1,30,265,268,269]

The therapeutic efficacy of a FFP unit is determined by its coagulation factors activity levels. Therefore, proper preservation of coagulation factor activity in plasma is essential for a good therapeutic effect. The activity of coagulation factors can vary depending on donor specific variations including demographic variations, genetic and lifestyle factors, or related to FFP preparation methods, storage conditions and thawing techniques, this is why stablishing good quality control methods is essential in plasma blood banking.[269]

It is widely accepted that 1mL of FFP contains approximately 1 international unit (IU) of coagulation factor activity. During storage the stability of coagulation proteins is not flawless, and they can be depleted through time losing its therapeutic effect. In this situation dosage might need to increase to reach the desired objective. In human medicine the coagulation factors that are most labile, and thus considered critical factors for quality control of plasma derivatives are factor V and VIII, which loss has been documented in human fresh frozen plasma during storage.[32,33,261] Extrapolating from human transfusion medicine, the current veterinary convention states that when a year has passed plasma is termed as frozen plasma (FP) due to the loss of the labile clotting factors, and serves as a source of albumin, non-labile coagulation factors and globulins.[33,264,265,270,271] There are few studies in veterinary medicine that measure

coagulation factor activities in stored plasma, and all of them are performed with canine plasma. One of this studies conducted with canine plasma reported that five-year-old FP was haemostatically active, showing a significant decrease in factor VIII activity but maintaining a good stability of factor V, indicating that differences within species might exist.[272] In feline medicine it is unknown if factor V and VIII are the most labile, nor even the concentration of coagulation factors in frozen plasma and it's stability over time, and thus, extrapolating dosages, storage times and quality control techniques from human or canine transfusion medicine might guide to malpractice.[31]

In human transfusion medicine, the guidelines of the European Committee on Blood Transfusion from the council of Europe state that FFP used for clinical transfusion must contain, on average, 70% or more of the value of factor VIII in the freshly collected plasma unit and at least similar quantities of the other labile coagulation factors and naturally occurring inhibitors.[261] A minimum of 90% of the units must achieve 70 UI of factor VIII activity per 100 mL of plasma (70%) after freezing and thawing.[261]

Factors that induce loss of coagulating factor activity

There are several factors that can affect the recovery of coagulation factors, including temperature, presence of metal ions, salts, lipids or other formulation excipients, surface adsorption, pH, shaking, light exposures and packaging conditions. The factors that are considered as most relevant are the delay of separation from cells, the speed and temperature of plasma freezing, the temperature of storage and the anticoagulant solution used.[32,269] The latter depends mostly on the effect of citrate over calcium. Different concentrations of citrate during collection have been observed to affect the stability of factor VIII.[273–277] This effect is apparently non-linear and does not seem to involve the participation of proteases, therefore a careful control of the citrate and calcium concentration is necessary.[273,274,278,279]

In human medicine mainly labile factors have been studied, with most studies based in factor VIII stability.[280] Factor VIII is considered as representative of all the other proteins of the coagulation system as per the international guidelines, and thus a critical parameter for quality control and stability studies of FFP.[261,269]

Factor VIII characteristics and stability

Although species differences could be suspected regarding specific coagulation factors stability, factor VIII has been consistently reported to be the most labile coagulation factor in human and canine medicine. Thus, this has been taken as a reference for coagulation factor stability and most studies have been performed with this factor as a reference.[261,269]

Factor VIII is one of the most complex trace plasma glycoprotein and plays a

crucial role in the intrinsic blood coagulation pathway, being one of the most essential blood clotting factors known.[281–285] Together with the activated factor IX, it is essential for the formation of the tenase complex during the propagation phase that leads to the activation of factor X. Then, upon the platelet surface, factor Xa is bounded with factor Va generating a burst of thrombin and converting fibrinogen to fibrin. Fibrin is then polymerized and forms a insoluble fibrin clot.[267,280] This explains why factor VIII deficiency results in an hypo-coagulable state and bleeding, as it is required, together with factor IX and XI, for the activation of factor Xa (and subsequently thrombin) on platelet membranes and thus, in plasma blood banking it is essential to maintain adequate levels in stored plasma to obtain a desired therapeutic effect when transfusing plasma to patients with a coagulopathy.[267,280]

The particularly reduced stability of factor VIII is suspected to be secondary to its relatively large size and complexity. Human factor VIII is synthesized and released into the bloodstream by vascular, glomerular, tubular endothelium and sinusoidal cells of the liver, it is compounded by 2332 amino acids, has a molecular weight of 264,763 Da and consists of two noncovalently linked polypeptide chains: a heavy chain (HC) of 220 KD (domains A1-A2- B) and a light chain (LC) of 80 KD (domains A3-C1-C).[274,280,284–286] The factor VIII gene is one of the largest genes known, it is formed by 26 exons and 25 introns with a size range from 69 to 3106 bp and 207 to 32,400 bp respectively, and it constitutes about 0.1% of the X-chromosome.[287–289]

There are two main mechanisms for the loss of factor VIII activity; activation of coagulation and physicochemical damage.[32]

❶ Activation of the coagulation cascade

Coagulation cascade can be triggered by many stimuli produced during collection which must be avoided, including a difficult venepuncture that might produce liberation of tissue factor to the blood or slow flow during collection. The mixing of whole blood with the anticoagulant solution during blood collection is considered the most important step within collection, as insufficient mixing could also cause activation of the coagulation cascade.[290]

It is assumed that during blood handling, activation of the coagulation cascade will occur to a certain degree, therefore it is especially important to centrifugate whole blood and freeze plasma as soon as possible to stop the coagulation process at an early stage. It is considered that all the process should be completed within 24h.[32]

Platelet damage should also be avoided, as it can induce the release of proteases affecting the stability of coagulation factors. They can be activated by excessively high flow rates during collection, unsuitable sizes and shapes of the collection or transfer systems or formation of foam in the blood bag. On the other hand,

separation of platelets from plasma should be done before freezing to avoid enzyme liberation when platelets are destroyed.[32]

❷ Physicochemical losses

Media conditions can also affect factor VIII stability. Temperature and pH are two of the most important factors, as factor VIII is stable only in a narrow range of pH around 7 (between 6.2 and 7 with a variation depending on storage temperature and other components). Temperatures lower than 10° C before separation could induce coprecipitation of factor VIII with cells.[278,291–293] Factor VIII is very sensitive to temperature changes, in one study performed with human plasma at pH 9.1, factor activity decay increased three-fold per 10 °C increase in the temperature range between 17° C and 37° C.[278] Like other proteins, factor VIII unfolds at high temperatures in solution.

Metal ions, especially divalent metal cations, have an essential role in structural stability and functional flexibility of protein systems such as factor VIII, playing a direct role in coagulation factor stability.[294,295] Many coagulation factors are able to bind to calcium and/or copper ions, with this interactions having a main role for its structure and function.[284,287,295,296] Some like Ca^{2+} and Sr^{2+} have a positive effect on the stability of factor VIII, while others like Fe^{2+} can be detrimental.[274,278] Calcium has a role in the association between the heavy and light chains of factor VIII, with some studies showing that 10 mM of Ca^{2+} is effective in the protection and stabilization of the protein.[274,287,295,297,298] Also, calcium chelation has shown to lead to the dissociation of factor VIII heterodimer.[274] Nevertheless, although calcium at certain concentrations seem to have a protective effect, it can also lead to destabilization of factor VIII if excessive amounts are used.[298–300]

The interaction with the blood bag can also induce factor VIII loss, as this adsorbs to a variety of surfaces, including PVC and polyethylene.[301–303] It is defined as a fast reaction, reaching an equilibrium within hours.[301,302] In addition, the amount of adsorbed factor is not directly related to concentration but depends on the surface area of exposure.[302,304] Feline plasma units have a much smaller volume comparing to human or canine and as a consequence of this, the relation between surface area and volume is much increased. This could theoretically increase the percentage of factor adhered to the blood bag and potentially affect to a higher degree the recovery of factor VIII. The use of surfactants can be effective in reducing protein surface absorption.[305]

There is evidence for only a few excipients that are able to stabilize factor VIII, including sucrose, sorbitol, mannitol, histidine, glycine and some other amino acids.[298,299,306–308] Citrate and phosphate have been described as detrimental to factor VIII during non-frozen storage and during the freeze-thaw process due to its chelation effect and buffer induced pH shift respectively.[309,310]

Light exposure could also influence the stability of plasma proteins, as light promotes the formation of free radicals that can initiate oxidative damage of proteins.[311,312]

On the other hand, high salt concentrations can induce reversible dissociation of factor VIII. Salt concentration can affect stability and solubility of factor VIII.[269,280,303] Freezing is a critical step regarding coagulation factor recovery.[269] During the freezing process pure ice is formed and solutes are concentrated in the remaining water, and when solubility of solutes is exceeded, they form crystals. The distribution of the solutes within the frozen plasma unit depends on the rate at which plasma is frozen. If slow freezing rate occurs, the diffusion of solutes is better adapted to the rate of ice formation, and solutes get increasingly concentrated at the middle of the unit, whereas if freezing rate is fast, solutes are not able to adapt and get trapped between the water crystals, making the distribution more diffuse along the unit. When freezing takes more than an hour, solutes concentrate at the middle of the unit, exposing coagulation factors to other substances like salts, which can reach high concentrations and inactivate coagulation factors.[266,269,303,313–315] To avoid this, the rate of cooling should be as fast as possible, with some authors recommending to bring the core temperature of plasma to -25° C or below within 60 minutes.[266,269,303]

To be able to improve feline plasma blood banking and use, and avoid malpractice, it is of utmost importance to optimize the production and storage techniques, and to understand the concentration and stability of clotting factors when stored in a non-physiologic state.

Hypothesis and Objectives

Hypothesis

A

A.1: Storage time in feline pRBC units leads to increased haemolysis of the stored red blood cells.

A.2: Feline pRBC units collected using a semi-closed system and using SAGM as a preservation solution will not show levels of haemolysis over 1% within 6 hours after being separated from whole blood by centrifugation at 2000 g for 15 min at 20° C (64.4° F), with 80 s of acceleration and 110 s of deceleration.

B

B.1: The use of two different peristaltic infusion pumps for the transfusion of feline pRBCs units at a speed of 25ml/h do not result clinically significant hemolysis.

C

C.1: One year of storage at a temperature between -18° C and -25° C in feline fresh frozen plasma units will induce a significant decrease in the activity of clotting factors when compared to feline fresh frozen plasma stored for less than two weeks.

C.2: To freeze feline plasma units within 24h of blood donation and to store them for less than two weeks at a temperature between -18° C and -25° C, does not lead to a decrease in the activity of clotting factors when we compare the results with a pool of non-stored plasma from healthy cats.

C.3: Recovery of factors VIII and V in feline fresh frozen plasma is significantly lower than the recovery of factors II, VII, IX, X, XI and XII after one year of storage at a temperature between -18° C and -25° C and therefore, are candidates to be considered the most thermolabile factors.

Objectives

A

To stablish the basis for feline pRBC blood banking

1. To evaluate the quality of feline pRBCs stored in SAGM for 29-35 days and for 36-42 days at 4° C by evaluating the percentage of haemolysis, and free haemoglobin.

2. To establish the maximum storage time that may be appropriate to meet the criteria established by the United States Food and Drug Administration (US-FDA) guidelines for human blood blanking.

3. To test the suitability of a semi-closed system for feline blood collection for pRBC processing and storage regarding the risk of erythrocyte damage and the risk of bacterial contamination.

4. To evaluate the need for quality control analysis before administration of feline pRBC units stored for more than 4 weeks in order to comply with the safety standards stablished by the US-FDA guidelines for human blood banking.

B

To evaluate the risk of erythrocyte damage secondary to peristaltic infusion pump use for feline pRBC transfusion

1. To evaluate the effects of two widely used linear PIPs on feline RBC integrity by measuring haemolysis biomarkers in units of pRBC stored for 35–42 days at a temperature of 4° C.

C

To investigate the activity of specific coagulation factors after freezing and during storage of the feline FFP

1. To evaluate the concentration and stability during storage time of specific coagulation factors I, II, VII, IX, X, XI and XII in feline FFP.

2. To identify the coagulation factors that are most affected by feline plasma storage and therefore must be considered critical factors for the production and quality control of feline FFP.

3. To help understand the needed volume of fresh frozen plasma to administer 10 IU/Kg of functional coagulation factors when using units of feline FFP stored for up to one year at a temperature between -18° C and -25° C.

In vitro quality control analysis after processing and during storage of feline packed red blood cells units

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Abstract

Background: During the storage of packed red blood cells (pRBC), packed cell volume (PCV), bacterial contamination and percentage of haemolysis [percentage of free haemoglobin (HGB) in relation to the total HGB] are important quality parameters. Both PCV and haemolysis are indicators of the cellular integrity of stored units. There are no published experimental studies that evaluated these parameters during storage of feline pRBC using SAGM (adenine, dextrose, mannitol and sodium chloride) as the additive solution. The present study aims to (1) evaluate the quality of feline pRBCs stored in SAGM; (2) test for the semi-closed system's suitability for use and risk of bacterial contamination; (3) establish the maximum storage time that may be appropriate to meet the criteria established by the United States Food and Drug Administration (US-FDA) guidelines for human blood banking; and (4) evaluate the need to calculate the percentage of haemolysis prior to the administration of units stored for more than 4 weeks.

Four hundred eighty nine feline pRBC units were analyzed. Bacterial culture, PCV and percentage of haemolysis were determined within 6h after processing (t0). One hundred and eighty units were re-tested for haemolysis and PCV after 29-35 days of storage (t1) and 118 units after 36-42 days (t2).

Results: Bacterial contamination was not detected in any pRBC unit. Mean PCV at t0 was 52.25% (SD: ±5.27) and decreased significantly ($p<0.001$) during storage to 48.15% (SD: ±3.79) at t1 and to 49.34% (SD: ±4.45) at t2. Mean percentage of haemolysis at t0 was 0.07% (SD: ±0.06) and increased significantly ($p<0.001$) to 0.69% (SD: ±0.40) at t1 and to 0.81% (SD: ±0.47) at t2. In addition, 13.88% and 19.49% of pRBC units exceeded 1% haemolysis at t1 and t2, respectively.

Conclusions: According to the US-FDA guidelines for human blood banking that recommend a maximum of 1% haemolysis, the results of this study show that all feline pRBC units with less than 24h of shelf life have low levels of haemolysis. However, units preserved up to 28 days can only be administered if tested for haemolysis before use, since 13.88% units exceeded the 1% limit. The semi-closed system was considered safe for use as bacterial contamination was not detected in any pRBC unit.

Keywords: blood bank, feline, haemolysis, pRBC, storage lesion, transfusion.

Background

In the last decades, feline transfusion medicine has significantly evolved, the use of packed red blood cells (pRBC) rather than whole blood was described in 15% and 47% of feline patients submitted to transfusion.[1,4]

Guaranteeing the safety of haemocomponents is essential and must be of the utmost importance for blood banks. Reducing the risks of transfusion reaction requires ensuring that the product is free of blood-borne pathogens; that there is absence of bacterial contamination; and that erythrocyte antigens are determined to avoid allogeneic immune reactions. Furthermore, the viability of erythrocytes must also be guaranteed.

Reported recommendations for storage of feline pRBC using additive solutions and citrate-based anticoagulants vary between 30 and 42 days at 2-6ºC.[10,316,317] However, there are no experimental reports aiming to evaluate haemolysis or bacterial growth in feline pRBC units stored in such conditions. There is just one publication that tested for haemolysis and bacterial growth in 27 feline fresh WB units, using an open collection system.[318]

During storage, blood cells maintain their metabolic activity, releasing byproducts to the media and suffering from biologic and immunologic changes, which may affect red blood cells (RBCs) function and survival. These changes are known as storage lesions, and may cause adverse effects on the recipients.[10–15] Haemolysis, as one result from these processes, may be regarded as an indicator of storage lesions. Haemolysis percentage is considered the standard in human medicine to determine pRBC's shelf life.[21]

Released byproducts, mainly produced by leucocytes and platelets, contribute to RBC haemolysis and are an important cause for transfusion reactions; most of them of the febrile non-haemolytic type.[319,320]

Units' haemolysis is highly influenced by the availability of ATP, produced mainly via anaerobic glycolysis through the Embden-Meyerhof pathway, catalyzed by phosphofructokinase (PFK). ATP is essential to maintain erythrocyte function and stability.[321–323] During storage, hydrogen ion activity inhibits PFK, and it increases with time as lactic acid accumulates due to anaerobic glycolysis.[324,325] As the lack of energy sources becomes critical, the RBC's metabolic activity fails to maintain normal functionality, its membrane elasticity reduces, intracellular viscosity increases, and morphological changes occur, resulting in haemolysis.[10,11,63–67,326]

Furthermore, these changes also affect the RBCs capacity of oxygen distribution and CO_2 removal from tissues.[11] Once in circulation, transfused RBCs either reassume the original biconcave shape within 24h or they will be removed

by the reticuloendothelial system, thus reducing their survival time on the recipient.[8,327,328]

By adding saline, dextrose, adenine, and mannitol to the additive solution used to store RBCs, it is possible to delay the loss of ATP and increase the pRBC lifetime up to 44 days.[36,53]

Mechanical and environmental factors may also affect the RBCs viability.[8,9] Haemolysis is highly influenced by the processing, storage and administration protocols, including the delay between collection and separation, centrifugation speeds, sterility of the units, intravenous tubing gauge, occluded needles, storage temperature, and the units' PCV.[9,113,329–331]

Therefore, it is important that such factors are optimized, standardized and monitored, and that quality controls are periodically done to ensure that the units are not damaged and the procedures are safe and effective.[113]

It is important to ensure that the transfused units are minimally haemolyzed, not only to ensure that the transfused RBCs are functional, but also because free haemoglobin, resulting from haemolysis, may be an important cause of transfusion reaction, mainly of the nonimmune-mediated haemolytic type.[112] Acute fatal or life-threatening transfusion reactions associated to the administration of haemolysed pRBCs in dogs have been described, with clinical signs similar to an acute haemolytic reaction.[14] When free HGB surpasses plasma and cellular binding capacities, it acts as an important vasoactive and redox active protein.[109,110] It is also important to notice that free HGB is potentially toxic for the vascular, myocardial and renal systems, the toxicity depending on exposure time and concomitant diseases such as renal insufficiency.[110,143,144,159,181,332–335]

PCV reduction, bacterial contamination, and haemolysis are important pRBC quality parameters that allow for addressing the cellular integrity of stored units, and are limiting factors for the shelf-life of stored red blood cells.[219] The Council of Europe and the U.S. Food and Drug Administration (US FDA) guidelines for human blood banking recommend that, at the end of storage, no more than 0.8% and 1% haemolysis, respectively, is surpassed to ensure that no haemolysed units are transfused to patient.[219,220,336] Similar recommendations, however, lack in veterinary medicine.

The present study aimed to (1) evaluate the quality of feline pRBCs stored in SAGM; (2) test for the semi-closed system's suitability for use and risk of bacterial contamination; (3) establish which maximum storage time may be appropriate to meet the criteria established by the United States Food and Drug Administration (US-FDA) guidelines for human blood blanking; and (4) evaluate the need for quality control analysis before administration of units stored for more than 4 weeks.

Materials and methods

From all units collected between 2014 and 2016 at the Animal Blood Bank in Spain and Portugal (Banco de Sangre Animal, Barcelona, Spain\ Banco de Sangue Animal, Porto, Portugal), one out of each 5 units was randomly selected for quality control analysis. Thus, a total of 489 feline fresh whole blood (FWB) units were analyzed. All donors were indoor healthy cats weighing 4-9 Kg that had been vaccinated, dewormed, tested for Feline Immunodeficiency Virus, Feline Leukemia virus (Uranotest FeLV-FIV, Uranovet, El Prat de Llobregat, Barcelona), *Mycoplasma haemofelis, Candidatus Mycoplasma haemominutum and Candidatus Mycoplasma turicensis* (PCR analysis by Genevet, Algés, Portugal). Complete blood counts and chemistry profiles prior to the collection procedures were within normal reference ranges.

Whole blood units were collected using a specific feline semi-closed system without leukocyte depletion filters, consisting of a 50 ml syringe and a primary blood bag collection attached to the syringe with a sterile connector (CompoDock, Fresenius SE, Hesse, Germany). The collection system was sealed, sterilized with Ethylene Oxide (EtO), and 8 mL of CPD (tri-sodium citrate, sodium phosphate and dextrose) were added as anticoagulant to the syringe, under sterile conditions using a laminar flow hood (Cruma FL-1, Diantech Solutions S.L., Barcelona, Spain).

After a complete physical examination, an intravenous catheter was placed on the cephalic vein, and mild sedation was applied intravenously using ketamine and diazepam. The use of NMDA receptor antagonist and benzodiazepine combination is commonly reported in the bibliography.[19] A combination of tiletamine and zolazepam has been reported safe for feline blood donation.[337] Once sedated, donors were placed in sternal recumbency, and the puncture area over the jugular vein was shaved and aseptically prepared using chlorhexidine and alcohol. Jugular venipuncture was performed and blood was withdrawn applying negative pressure by gently pulling manually the syringe plunger. A maximum of 10-12 ml/kg was collected.[19] During collection, the syringe was gently stirred to allow proper contact of the blood with the anticoagulant. The collected blood was then transferred to the blood bag through the sterile connection ensuring the maintenance of a closed environment. After that, the tubing was sealed (Composeal, Fresenius Kabi, Hesse, Germany), units were stored at room temperature (22±2ºC) and processed within 24h. The volume of pRBC units was calculated on the basis of their weight, considering that 1mL of pRBC weights 1.085 g.[338]

Units were gently mixed and placed in the centrifuge cups (Megafuge 40R, Thermo Scientific, Massachusetts, USA) eliminating void space by using manufactured plastic adaptors. Weight differences under 0.3 g between opposite

cups were tolerated. Whole blood units were centrifuged at 2000 g for 15 minutes at 20°C (64.4°F), with 80 seconds of acceleration and 110 seconds of deceleration.

Plasma was then expressed into a secondary transfer bag using a sterile connection of polyvinyl chloride tubing (CompoDock, Fresenius SE, Hesse, Germany), and 10 mL of SAGM (adenine, dextrose, mannitol and sodium chloride) were added to the pRBC unit under a laminar flow hood (Cruma FL-1, Diantech Solutions S.L., Barcelona, Spain).

For sampling purposes, pRBC units were gently mixed by inversion, and a 3 mL aliquot was aseptically collected using a sterile connection with a sample bag (Macopharma, Mouvaux, France), and analyzed (t = 0) for PCV, total HGB and supernatant HGB.

PCV was measured by microhaematocrit centrifugation.[339] Total HGB was measured using a specific analyzer (Hb 201 System, HemoCue Inc., California, USA), according to the manufacturer's protocol. After centrifugation (Centrifuge IEC Centra CL3R, Thermo Scientific, Massachusetts, USA), supernatant HGB was determined by spectrophotometry using an analyzer for low values of HGB (Plasma Low Hb, HemoCue Inc., California, USA), according to the manufacturer's protocol. The percentage of haemolysis was obtained using the following formula:[113]

$$\% \ haemolysis = Supernatant \ HGB \ (g/L) \ x \ (100\text{-}PCV) \ / \ Total \ HGB \ (g/L)$$

Bacterial cultures were performed in all units at t0 by adding, under sterile conditions, 2,5 mL of pRBC to aerobic culture bottles with specific growth medium (Bact/Alert PF, Biomerieux, Marcy l'Etoile, France), followed by incubation at 37°C (98.2°F) and continuous examination for 14 days using a specific analyzer (Bact/Alert 3D, Biomerieux, Marcy l'Etoile, France).

Packed RBCs units were stored at 4°C in a dedicated refrigerator (Medika 250, Fiocchetti, Luzzara, Italy). One hundred and ninety-one units were used for clinical purposes, and 298 units were retested for haemolysis and PCV after storage.

Data was grouped, according to storage times, in 3 groups; group 1 evaluated within 6h after processing (t0); group 2 re-tested after 29-35 days of storage (t1); group 3 re-tested after 36-42 days of storage (t2).

Results were analyzed with statistical software (SPSS, version 22.0.0, IBM, Illinois, USA). Normal distribution of data was assessed with the Kolmogorov-Smirnov test. ANOVA F de Snédècor and Tukey's *post hoc* Test were used to assess for haemolysis, PCV, or total HGB differences between evaluation moments. The relationship between the number of units that exceeded 1% of haemolysis and the storage duration was assessed using Chi Square. Values were considered significant at $p < 0.001$.

Results

A total of 489 pRBC units were tested for bacterial contamination, PCV and haemolysis at t0, 180 were retested for PCV and haemolysis after 29=35 days of storage (t1) and 118 units tested for these same parameters after 36-42 days of storage (t2). The remaining units were used before retesting.

Data for all variables were normally distributed. Haemolysis, PCV and total HGB values from pRBC units are displayed in **Table 1**. There were significant PCV and haemolysis differences between the evaluation moments ($p< 0.001$) **(Table 1)**.

Table 1. PCV, Total Haemoglobin and haemolysis values at t0, t1 and t2.

	t0		t1		t2		F	p
	M	SD	M	SD	M	SD		
PCV (%)	52.25	5.27	48.15	3.79	49.34	4.45	55.078	<.001
Total Haemoglobin	11.15	8.49	10.13	7.87	10.24	8.04	2.069	.127
Haemolysis (%)	.07	.06	.69	.40	.81	.47	578.540	<.001

t0: units evaluated within 6h after processing; **t1:** units re-tested after 29-35 days of storage; **t2:** units re-tested after 36-42 days of storage; **M:** mean value; **SD:** standard deviation; **PCV:** Packed cell volume; **F:** F-value; Significant value $p< 0.001$.

The mean percentage of haemolysis at t0 was 0.07%, (SD: ±0.06), with all units below the maximum of 1% allowed by the US FDA, and the mean PCV was 52.2% (SD: ±5.27). At t1, the mean percentage of haemolysis was 0.69% (SD: ±0.4), with 25 (13.88%) units surpassing 1% haemolysis, and the mean PCV was 48.15% (SD: ±3.79). At t2, the mean percentage of haemolysis was 0.81% (SD: ±0.47), with 23 (19.49%) units presenting more than 1% haemolysis, and the mean PCV was 49.34% (SD: ±4.45) **(Table 2)**.

Table 2. Differences between t0, t1 and t2.

	Group	Dif	SE	p
PCV (%)	t0-t1	4.107	.419	<.001
	t0-t2	2.915	.492	<.001
Haemolysis (%)	t0-t1	-.620	.023	<.001
	t0-t2	-.743	.028	<.001
	t1-t2	-.123	.032	<.001

t0: units evaluated within 6h after processing; **t1:** units re-tested after 29-35 days of storage; **t2:** units re-tested after 36-42 days of storage; **Dif:** differences between mean values of each group; **SE:** standard error; **PCV:** Packed cell volume; Significant value $p< 0.001$.

Results show that PCV decreased between t0 and t1, and between t0 and t2, while haemolysis increased between t0 and t1, between t0 and t2 and between t1 and t2. There was no statistical difference in PCV between t1 and t2 **(Figures 1, 2 and table 3)**. There was a statistically significant relation between the evaluation moments and the number of units exceeding 1% of haemolysis ($\chi^2(2) = 46.694$; $p < 0.001$).

Table 3. Number of packed red blood cells (pRBC) units exceeding 1% of haemolysis at t0, t1 and t2.

	≤ 1% (n = 739)	> 1% (n = 48)
t0 (n = 489)	100% (n = 489)	0%
t1 (n = 180)	86.12% (n = 155)	13.88% (n = 25)
t2 (n = 118)	80.51% (n = 95)	19.49 (n = 23)

$\chi^2 (2) = 87.778$; p < .001.

t0: units evaluated within 6h after processing; **t1:** units re-tested after 29-35 days of storage; **t2:** units re-tested after 36-42 days of storage; **≤ 1%:** units not exceeding 1% of haemolysis; **>1%:** units exceeding 1% of haemolysis; **n:** number of units.

Figure 1. Progression of haemolysis during storage of feline pRBC units.
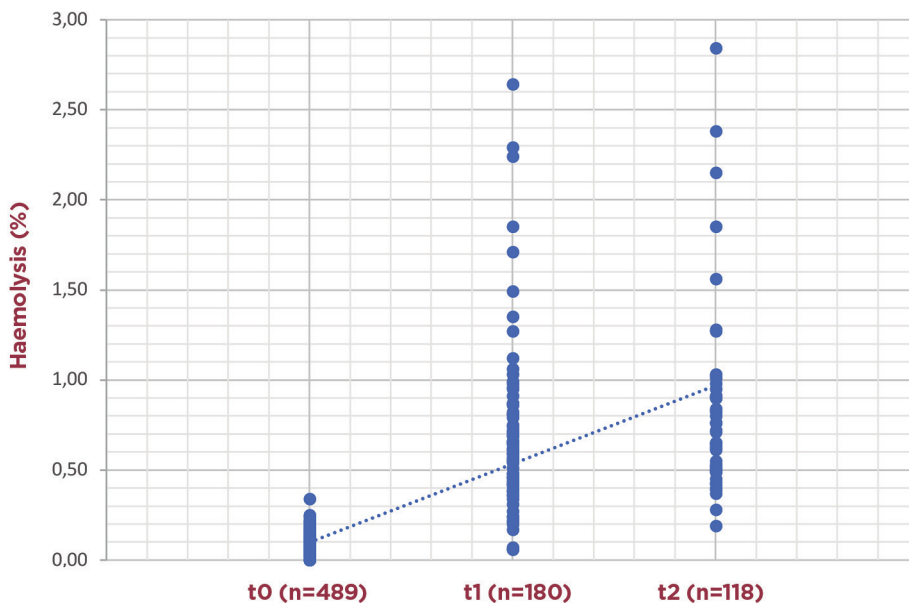
t0: units evaluated within 6h after processing; **t1:** units re-tested after 29-35 days of storage; **t2:** units re-tested after 36-42 days of storage.

Figure 2. Progression of PCV of feline pRBC units during storage.

PCV: Packed cell volume; **t0:** units evaluated within 6h after processing; **t1:** units re-tested after 29-35 days of storage; **t2:** units re-tested after 36-42 days of storage.

Discussion

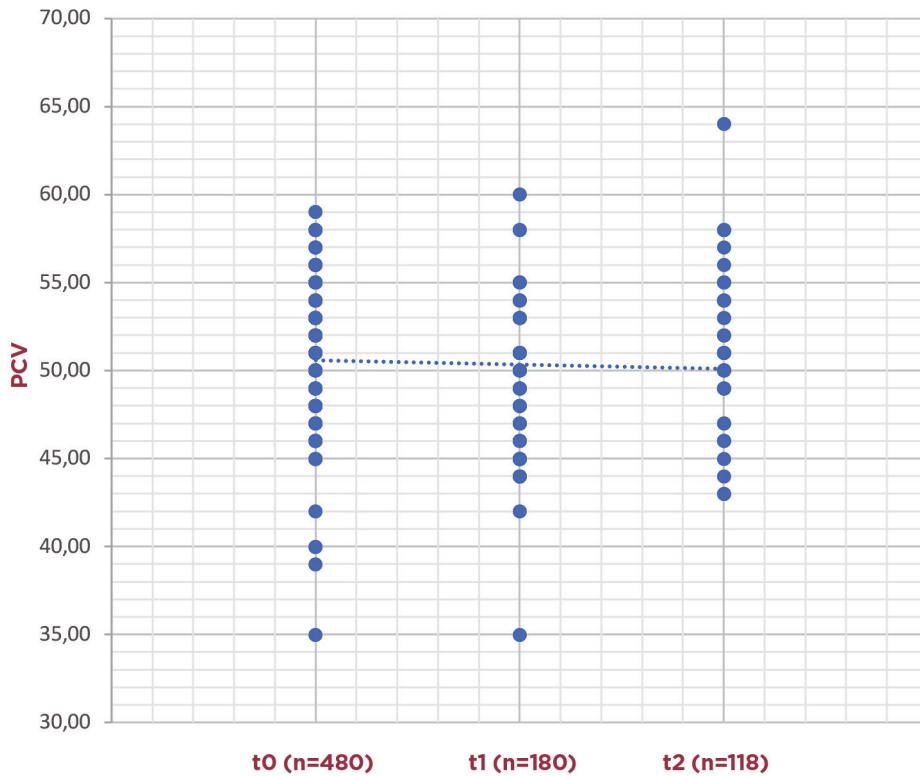Percentage of haemolysis is the mostly used parameter to establish the viability of pRBC manufacturing and conservation protocols.[21] It is influenced by blood cells metabolic activity and release of substances into the supernatant, but also by environmental factors such as temperature, or collection and processing protocols. As an indicator of storage-related cell damage, it is also an important parameter for the transfusion safety. The United States Food and Drug Administration guidelines for human blood banking recommend a maximum of 1% haemolysis to ensure that no haemolytic products are transfused to patients, and use haemolysis as one of the main parameters to approve additive solutions and conservation protocols.[17,340]

Only two recent studies aimed to evaluate storage lesions in feline pRBCs.[15,21] Significant supernatant increases of lactate, ammonia, sodium and chloride, and decreases of glucose and potassium levels were described.

To the authors' knowledge, there are no previous publications on the quality control of feline pRBC that reported their haemolysis after collection and during storage with SAGM or other preservative solutions. Similar to studies performed in human and canine pRBC units, there was an increase in the haemolysis levels over storage time. We can assume that this might be due to the progressive depletion of ATP and the effect of the proinflammatory substances produced mainly by leucocytes and platelets.[10,322,341,342] Our results indicate that after 29-35 days of storage, nearly 14% of the units suffered more than 1% haemolysis, and such value increased to almost 20% after 36-42 days. Hence, it could be postulated that units preserved for more than 28 days should only be administered once tested for haemolysis to ensure that its value does not compromise safety and efficacy of the transfusion.

In one previous study, 164 human units were analyzed after 42 days of storage in a hospital-based transfusion service, and 13.4% exceeded 0.8% of haemolysis.[341] In our series, haemolyzed units after similar storage times were more common, possibly due to higher difficulties in the collection processes associated to the need for negative pressure, smaller vein diameters, and a shorter lifespan of feline pRBC (77 days).[343]

Previous studies of our group in dogs concluded that 6% of the units surpassed 1% haemolysis at 35 days, although this number increased to 51% at 42 days of storage.[344] Interestingly, feline pRBCs showed a higher proportion of haemolysed units at fifth week of storage, but much lower than canine at sixth week (18.57% *vs* 51%). The explanation for such difference may reside in the distinct RBC metabolism between species or be due to differences in the feline blood bags that, being smaller and with a higher surface-to-volume ratio, allow for a higher capacity for gas exchange and faster temperature homogenization.

In blood banks and clinical practices, pRBC units should always be checked for visible alterations indicating haemolysis before its use. Although clinically useful, it is considered a non-reliable and subjective method, as it often overestimate the haemolytic status of the pRBC units since even as little as 0.09% of haemolysis causes the appearance of an evident pink discoloration of the supernatant.[17,340] HaemoCue has been compared to the gold standard tetramethylbenzidine spectrophotometric method and reported to be a reliable objective method to measure plasma HGB for routine quality control and validation process, being a faster, easier and reliable system.[340]

In our series, mean PCV was maintained during storage, with a slight decrease over time, from 52.2% (SD: ±5.27) at t0 to 48.15% (SD: ±3.79) at t1, and to 49.34% (SD: ±4.45) at t2. These results contradict those previously reported in canine or human pRBC. Canine and human pRBC PCV increased during storage, a phenomenon explained by the influx of water into RBCs caused by cell

membrane damage during storage and the osmotic effects of the supplementary solutions.[342,344]

The differences between other mammalian and cats may be attributed to physiologic and metabolic particularities of the latter that may lead to different morphologic changes during storage. One other possible cause for these differences could be the smaller size and surface to volume ratio of the feline pRBC that may result in a lower RBC osmotic fragility and consequently a reduced capability of swelling, when compared to canine RBCs, leading to a membrane destruction earlier in the swelling process, thus not allowing for PCV to increase.[345] These hypotheses warrant future studies on the morphologic changes of feline erythrocytes during storage.

Closed collection systems are not always available in feline transfusion medicine. The alternatives are semi-closed or open systems, which are used in many blood banks and veterinary hospitals, but the latter preclude storage due to the high risk of bacterial contamination, leading some blood banks to use semi-closed collection systems.[19] The semi-closed system, used in this study, was considered safe since no bacterial contamination was detected in any pRBC unit and haemolysis (mean 0.07%, SD ±0.06) was under 1% in all units before storage, similar to the results of canine collections using close systems (0.09%, SD ±0.06).[344]

To the authors' knowledge there are no previous reports aiming to validate the semi-closed collection systems for use in blood banking. In previous studies, contamination by *Serratia* spp. and *Pseudomona* spp. have been described in feline WB and pRBC units using open collection systems, and one pRBC unit collected with a semi-closed system was tested positive to *Pseudomonas fluorescens* after colour changes were noted.[9,42,346] Moreover, two studies performed blood cultures in feline pRBC collected with open systems, testing 10 feline pRBC at day 32 of storage in one study and 6 units at day 42 of storage on the other. Both studies reported negative blood cultures at the end of storage.[20,21]

One limitation of this study was that blood culture was performed only 24h after collection, and not repeated after the storage period, although contamination during storage is considered unlikely, sensitivity for bacterial contamination during processing might be higher after a long storage period. However, no units showed signs of bacterial growth (e.g. dark purple to black or green discoloration) or visible signs of clotting or fibrin in the blood bag at any time during storage, although the absence of these indicators does not preclude de possibility of bacterial contamination.[346,347]

Another limitation was that units were not re-tested for haemolysis before 29 days, which was because feline pRBC are a valuable resource, and the design of the study was thought to optimize the availability of the pRBC for clinical use.

Other quality parameters that were not analyzed in our study but might have been necessary for a complete quality control analysis include, as routinely performed in human medicine, biochemical measurements and RBC morphology analysis, including red cell shape, size, cell surface markers, glucose utilization rates, lactate production rates, and ATP levels and utilization rates. Our aim, by analyzing the storage times and processing methods in regard to haemolysis and PCV, was to describe representative indicators for red blood cell storage damage.

Conclusions

Considering the U.S. FDA guidelines for human blood banking that recommend a maximum of 1% haemolysis to ensure that no haemolytic products are transfused to patients, our results evidenced that all pRBC units with less than 24h of shelf life have negligible haemolysis. However, units preserved for more than 28 days can only be safely administered if tested for haemolysis before its use, since 13.88% units exceeded the 1% limit at 35 days of storage. Furthermore, our results indicate that the semi-closed collection systems, when manipulated in sterile conditions, are reliable for feline blood banking. Further studies are needed to assess storage lesions and erythrocyte morphologic changes in feline RBCs during storage of pRBC.

Quantitative assessment of infusion pump-mediated haemolysis in feline packed red blood cell transfusions

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Abstract

Objectives: Haemolysis caused by the use of peristaltic infusion pumps (PIP) has been described in human and canine packed red blood cells (pRBC). The aim of this study was to evaluate the effects of two different linear PIP on hemolysis of feline pRBC units stored for a long duration.

Methods: Feline pRBC units stored with SAGM (adenine, dextrose, mannitol and sodium chloride) were manufactured.

After 35 to 42 days of storage at 2-4°C, a line administration system with 180 µm filter (Infusomat Space Line, Braun) was attached to every pRBC bag, the system was drained by gravity alone (8 drops/min) and a 1.3 ml sample was collected (G). A NIKI V4 pump was then used at a flow rate of 25 ml/h, the flow was stopped when the infusion system was filled with blood coming from the infusion pump, and another 1.3 ml sample was collected (NK). Finally, an Infusomat FmS pump was evaluated, collecting another 1.3 ml sample (IM).

Packed cell volume (PCV) was measured in all samples by microhaematocrit centrifugation, total haemoglobin (HGB) was measured using a specific haemoglobin analyzer and, after centrifugation, free HGB was determined by spectrophotometry. The percentage of haemolysis was calculated.

The Friedman test was used to compare the samples.

Results: AFifteen feline pRBC units were evaluated. Average degree of haemolysis for sample G (gravity-assisted) was 1.12%. Comparison of the degree of gravity-assisted haemolysis with haemolysis in PIP NK (1.13%) and IM (1.14%) samples revealed no significant differences, with just a difference of 0.01% and 0.02% respectively.

Conclusions and relevance: The results of this study demonstrate that the use of two common peristaltic infusion pumps in veterinary hospitals do not produce levels of haemolysis that are significantly different than that caused by gravity alone during transfusion of feline pRBC at a rate of 25 ml/h.

Introduction

Transfusion of packed red blood cells (pRBC) is a common practice in the treatment of feline anaemia. Percentage of haemolysis and serum free haemoglobin in pRBC are markers of red blood cell integrity that are used as a safety and quality-related characteristic.[25] All units have a baseline haemolysis level which can increase during administration to a patient, and the use of infusion pumps could potentially contribute to this haemolysis.[259]

Haemolysis leads not only to a functional reduction in haemoglobin, but also to the release into the circulation of cell components such as potassium or free haemoglobin, which have the potential to exacerbate or provoke renal or cardiovascular complications.[16,25,348] Free haemoglobin transfusion to the recipient could also be associated with a decrease in nitric oxide, vasoconstriction, hypertension, and contribute to pro-inflammatory and pro-thrombotic states.[16]

Packed RBCs may be administered by allowing the fluid to flow by gravity through the administration system, or by using infusion pumps. The latter are devices that regulate administration rates by applying positive pressure to the system.[16] In feline medicine, the need for low transfusion rates, particularly in patients at high risk of volume overload, hampers infusion by gravity due to the difficulty associated with calculating and maintaining at a constant rate an adequately slow flow. Because of this, there may be advantages to using positive pressure pumps. Peristaltic infusion pumps (PIP) are widely used for transfusion of feline pRBC, but their effects on red blood cell integrity are unknown.

Haemolysis caused by the use of PIP has been described with both human and canine pRBC.[16,25,28,245] The degree of haemolysis depends on factors such as infusion rate, blood haematocrit, preservative solution and administration catheter diameter.[16,348] Increased levels of haemolysis have also been observed when very low or high rates are used, and the storage time of pRBC may also have a major effect on pump-induced haemolysis.[26,248]

Feline red blood cells have haemorheological differences compared to dog red blood cells, including smaller size and a greater increase of stiffness under hypoxic conditions.[5,246] This loss of elasticity could potentially result in a higher cell fragility and subsequent haemolysis when PIP's are used for transfusion of feline RBCs. The effect of PIP on feline erythrocytes is unclear, and consequently it is unknown whether their use in the species is safe.

The aim of this study was to evaluate the effects of two widely used linear PIPs on feline red blood cell integrity by measuring haemolysis biomarkers in units of pRBC stored for 35-42 days.

Materials and methods

Animals

All donors were indoor healthy cats weighing 4–9 kg that had been vaccinated and dewormed. Complete blood counts and chemistry profiles prior to the collection procedures were within normal reference ranges. No animals were specifically recruited for this study, and all data was obtained from the routine standardized procedures performed at the Animal Blood Bank (Banco de Sangre Animal, Barcelona, Spain). All units included in this study were due to be discarded due to expired storage life and stock excess. Plasma units were used for clinical purpose. All units of blood were collected after a signed informed owner consent. This study was conducted according to European legislation on the Protection of Animals Used for Experimental and Other Scientific Purposes (86/609/EU).

Blood collection and processing

Whole blood units were collected by using a specific feline semi-closed system without leukocyte depletion filters, consisting of a 50 ml syringe attached to a 20-gauge needle with an extension set and a primary blood bag attached to the syringe with a sterile connector (CompoDock, Fresenius SE, Hesse, Germany). The collection system was sealed, sterilized with Ethylene Oxide (EtO), and 8 mL of CPD (tri-sodium citrate, sodium phosphate and dextrose) was added as anticoagulant to the syringe, under sterile conditions using a laminar flow hood (Cruma FL-1, Diantech Solutions S.L., Barcelona, Spain).

After a complete physical examination of each donor cat, an intravenous catheter was placed and mild sedation was administered using ketamine and diazepam.[19,337] Once sedated, donors were placed in sternal recumbency, and the puncture area over the jugular vein was clipped of hair and aseptically prepared using chlorhexidine and alcohol. Jugular venipuncture was performed and blood was withdrawn by gently manually pulling the syringe plunger. A maximum of 10 ml/kg (donor weight) was collected.[19] During collection, the syringe was gently agitated to allow proper contact of the blood with the anticoagulant. The collected blood was then transferred to the blood bag through the sterile connection ensuring the maintenance of a closed environment. The tubing was then sealed (Composeal, Fresenius Kabi, Hesse, Germany), units of whole blood were stored at room temperature (20-22 °C) and processed into separate units of pRBCs and plasma within 24 h.

Units were gently mixed and placed in centrifuge cups (Megafuge 40R, Thermo Scientific, Massachusetts, USA) eliminating void space by using manufactured plastic adaptors. Weight differences under 0.3 g between opposite cups were

tolerated. Whole blood units were centrifuged at 2000 g for 15 min at 20° C (64.4° F), with 80 s of acceleration and 110 s of deceleration. Plasma was then expressed into a secondary transfer bag by using a sterile connection of polyvinyl chloride tubing (CompoDock, Fresenius SE, Hesse, Germany). Finally, 10 mL of additive solution SAGM (adenine, dextrose, mannitol and sodium chloride) were aseptically added to the pRBC units, after one last sterile connection.

The volume of each pRBC unit was calculated on the basis of its weight, considering that 1 mL of pRBC weighs 1.085 g.[338]

Storage and haemolysis biomarker measurement

After 35 to 42 days of storage at 2-4°C in a dedicated refrigerator (Medika 250, Fiocchetti, Luzzara, Italy), units were allowed to achieve room temperature over 30 minutes. A line administration system with a 180 µm filter (Infusomat Space Line, B.Braun) was attached to every pRBC bag, the system was drained by gravity (approximately 8 drops per minute) and, after discarding the first 3 ml, a sample of 1.3 ml was collected (G). This sample was used to assess the percentage of haemolysis and free haemoglobin of the unit without the effect of any PIP.

A NIKI V4 (NIKI V4, Everest, Molins de Rei, Spain) infusion pump was then attached to the system and set to deliver 25 ml/h. The authors had previously measured the volume of the line and the flow was stopped when the transfused volume exceeded this volume by at least 3 ml, to ensure that the infusion system was filled only with blood coming from the infusion pump. Subsequently, another sample of 1.3 ml was taken (NK). This sample was used to assess the percentage of haemolysis associated with the NIKI V4 pump. Finally, an Infusomat FmS (Infusomat FmS, B.Braun, Melsungen , Germany) pump was applied, and the same procedure was performed, taking a third sample of 1.3 ml (IM) . This sample was used to assess the percentage of haemolysis associated with the Infusomat FmS pump. The entire process from connection of the line administration system to collection of the final sample was completed within 96 minutes.

Packed cell volume (PCV) was measured in all samples by microhaematocrit centrifugation, total haemoglobin (HGB) was measured using a specific analyser (Hb 201 System, HemoCue Inc.) and, after centrifugation, free HGB was determined by spectrophotometry (Plasma Low Hb, HemoCue Inc., Ängelholm, Sweden). The percentage of haemolysis was obtained using the following formula:

$$\textit{\%haemolysis = free HGB(g/L) x (100-PCV) / Total HGB(g/L)}^{16,348}$$

As n = 15 (<30), non-parametric tests were chosen, and thus, normality of the distribution was not checked. The Friedman test is a non-parametric test suitable for comparing between paired samples, and was used to compare % haemolysis, total haemoglobin, free HGB and PCV of the samples at G, NK and IM in our study. A p-value ≤ 0.05 was considered statistically significant.

Results

Fifteen feline pRBC units were included. Five units were stored for four weeks, six units for five weeks and four units for six weeks.

The mean ± SD haemolysis level in sample G (administration by gravity) was 1.12 ± 0.84%, while mean haemolysis in sample NK and sample IM (the two PIP) was 1.13 ± 0.78% and 1.14 ± 0.84%, an increase of 0.01% and 0.02% respectively. When comparing the percentage of haemolysis in gravity-administered samples with samples administered by PIP infusion, there were no significant differences between methods.

The mean free HGB level in sample G (administration by gravity) was 0.336 ± 0.29 g/dl, while mean free HGB in sample NK and sample IM (the two PIP) was 0.340 ± 0.29 g/dl and 0.345 ± 0.31 g/dl, an increase of 0.004 g/dL and 0.0087 g/dL respectively. When comparing free HGB in gravity-administered samples with samples that were administered by PIP infusion, there were no significant differences between methods.

The mean total haemoglobin and PCV levels in sample G (administration by gravity) was 15.32 ± 2.13 g/dl and 45.47 ± 7.37% respectively, while mean total haemoglobin in sample NK and sample IM (the two PIP) was 15.24 ± 2.25 g/dl and 15.38 ± 2.27 g/dl, and mean PCV was 45.20 ± 7.63 % and 45.40 ± 7.5 % respectively. When comparing the mean total haemoglobin and PCV levels in gravity-administered samples with samples that were administered by PIP infusion, there were no significant differences between methods. In six out of 15 units (40%), levels of haemolysis after gravity-assisted administration were higher than 1%, reaching a maximum level of 3.3% in one unit. From these six units that had more than 1% basal haemolysis level, four had been stored for 6 weeks, one for 5 weeks and one for 4 weeks. All results are shown in **Table 1**.

Discussion

This study investigated red blood cell damage produced during simulated feline pRBC transfusion using two different peristaltic infusion pumps compared to gravity-assisted administration. Peristaltic infusion pumps can be classified based on their pump mechanisms (e.g. linear peristaltic, rotary peristaltic, reciprocating piston or piston-actuated diaphragm pumps). There is no consensus on the type of pump that induces less red blood cell damage. Some authors state that linear peristaltic pumps might be the most susceptible to producing haemolysis, although they remain the most commonly used pumps in practice.[16,245] Linear PIPs have been found to increase the free haemoglobin concentration (a marker of RBC damage) in human pRBC, possibly due to direct compression of erythrocytes within the intravenous tube.[27,247]

Table 1. Comparison of indices of haemolysis after administration of feline pRBC by gravity and use of two different PIP (NIKI V4 and Infusomat FmS).

	Gravity	NIKI V4		Infusomat FmS	
	Mean ± SD (Median)	Mean ± SD (Median)	NK-G	Mean ± SD (Median)	IM-G
Haemolysis (%)	1.12 ± 0.84 (0.96)	1.13 ± 0.78 (0.92)	0.01	1.14 ± 0.84 (0.85)	0.02
Free haemoglobin (g/dl)	0.336 ± 0.29 (0.26)	0.34 ± 0.29 (0.30)	0.004	0.345 ± 0.31 (0.26)	0.009
Total haemoglobin (g/dl)	15.32 ± 2.13 (15.5)	15.24 ± 2.25 (15.1)		15.38 ± 2.27 (15.6)	
PCV (%)	45.47 ± 7.37 (46)	45.2 ± 7.63 (46)		45.4 ± 7.5 (46)	

SD, standard deviation; **NK − G**, difference between mean value for NIKI V4 and mean value for Gravity; **IM − G**, difference between mean value for Infusomat FmS and mean value for Gravity. No statistically significant differences were observed between the 3 groups regarding haemolysis, $p = .356$; **PCV**, $p = .380$; total haemoglobin, $p = .618$; and free haemoglobin, $p = .507$.

Two widely used linear PIPs in veterinary medicine were chosen for this study, the NIKI-V4 and Infusomat-FmS.

Multiple biomarkers of red blood cell damage have been described in human and veterinary transfusion medicine research, including free haemoglobin, percentage heamolysis, band-3 protein, CD47 and phosphatidylserine exposure, potassium, lactate deshydrogenase, alanine aminotransferase and aspartate aminotransferase concentrations, microvesicle release, osmotic fragility and changes in RBC morphology.[16,34,62] Free hemoglobin and hemolysis were analyzed in the present study because they are considered good markers of red blood cell integrity and are widely used in human research. Free HGB is the biomarker most present in human publications investigating the relation between Infusion pumps and red blood cell damage in transfusion, and percent haemolysis is considered to be the standard in human medicine and is used as a marker for failure of RBC processing or administration techniques.[16,17] Potassium concentrations are another widely used biomarker of red blood cell damage, but their use is not recommended when aged pRBC are tested, as in our study.[28]

A clinical situation with potential maximum risk for haemolysis was simulated by selecting pRBC units that were near or at the end of their recommended storage life (35-42 days). During storage, blood cells continue to exhibit metabolic activity. When ATP reserves decrease, metabolic activity begins to fail, membrane elasticity is reduced, and morphological changes occur, predisposing to increased cell fragility.[16,258,348] Additionally, because of ethical reasons, units

nearing or at expiration dates were used, and only when a high number of units were available.

In addition, there are different studies in which, using PIP, a higher degree of haemolysis was observed at low velocities such as those used in cats.[27,245,258] Exposure time and stress levels are two main parameters that determine the degree of RBC damage due to shear stress, which can result in hemolysis and fragmentation of erythrocytes.[349] At lower flow rates, exposure time to mechanical shear force increases, and this may induce higher levels of RBC hemolysis.[245] Veterinary textbooks recommend transfusion rates of 2-10 ml/kg, and a maximum rate of 10 to 20 ml/kg/h to avoid circulatory overload.[350,351] In a previous study, rates of approximately 10 ml/kg/h for normovolaemic cats showed no complications attributable to the rates used.[37] A flow rate of 25 ml/h was chosen in this study to cover those of common feline transfusions, matching calculated rates for cats weighing 2.5 kg at 10 ml/kg, or 5 kg at 5 ml/kg.

Although mean increases of free HGB in the range of 0.004 and 0.0087 g/dl were observed when using NIKI-V4 and Infusomat-FMS PIPs respectively, the differences were considered non-significant when compared to administration by gravity. These results are similar to a previously published study using human pRBC, in which a mean increase in free HGB of 0.006 g/dl was observed when using different types of PIP.[27] In human medicine, an increase of less than 0.06 g/dl of free HGB is considered not to be clinically significant.[245]

In another study conducted with human erythrocytes administered at 30 ml/h, median increases in the percentage of haemolysis of 0.016% (shuttle pump), 0.026% (piston pump) and 0.241% (PIP Infusomat-Space) were described.[25] Our results (0.017% NIKI-V4 and 0.022% Infusomat-FMS) were comparable to those reported in the previous human study with non-peristaltic pumps, but were associated with far less haemolysis than associated with the use of the PIP Infusomat-Space in human erythrocytes. This difference could be explained by the fact that an anti-siphon valve was applied when testing the PIP Infusomat-Space in the human study, or by other variables such as morphological and metabolic variability between species, pre-transfusion blood storage times, or dissimilar unit PCV. Another study performed in human pRBC that tested two different linear PIP at 100 mL/h and 300mL/h infusion rates found that there was a mean haemolysis increase of 0.08% at 100 ml/h.[28] No anti-siphon valve was used in this second study, but infusion was performed at higher speeds than in our study.

In veterinary medicine there is only one study performed with canine whole blood, using free HGB alone as a biomarker and comparing three types of PIP. Free haemoglobin increases of 7% to 228% were observed depending on the pump type used.[258] In our study, a mean free HGB increase of 1.19% was

detected when using the NIKI-V4 and 2.68% when using the Infusomat-FMS pump. To the authors' knowledge, the only published comparable study in feline medicine used syringe infusion pumps and autologous whole blood, and tested post-transfusion survival of red blood cells in the recipient, but not erythrocyte damage or haemolysis.[29]

Six out of 15 units on this study had haemolysis levels that were higher than the FDA human medicine "recommended limit" of 1%, even when administered by gravity.[352] A previous study reported that 13.88% and 19.49% of feline pRBC units have higher than 1% haemolysis levels after 28 to 35 and 35 to 42 days of storage, respectively.[348] In our study the percentage of units with over 1% haemolysis was higher than previously reported, with 40% of the units (6/15) surpassing the recommended limit. For our study, the initial degree of haemolysis was not considered to be an exclusion criteria. This higher proportion of haemolysed units was probably due to the differences in storage durations between the units tested. Four of the 6 units with more than 1% haemolysis had been stored for 6 weeks, the maximum currently accepted time limit for storage. We cannot rule out a contributing effect of the filter and infusion system used on haemolysis levels, although in human medicine, needle gauge, tubing length and tubing diameter appear to have had no effect on haemolysis.[249]

Although the FDA-recommended haemolysis limit is 1% in human transfusion medicine, it has been reported that much higher levels of haemolysis can be tolerated by humans without apparent untoward effects.[353,354] The pathophysiologic effects of exposure to such levels of free haemoglobin in feline patients have not been evaluated. In one study performed in healthy dogs, kidney injury was reported when an infusion of 4 g/kg of canine HGB was administered.[333] To administer the same quantity of free haemoglobin to a cat, and considering the mean PCV and total haemoglobin of the present study, a unit with 7.12% of haemolysis (and 2 g/dL of free HGB) would have to be administered at a dosage of 20 ml/kg.[333]

This study had some limitations that are inherent to an *in vitro* investigation. We studied laboratory markers of haemolysis which, while they are considered to be good markers of erythrocyte damage, might not be representative of post-transfusion *in vivo* erythrocyte circulating half-life. Additional *in vivo* studies are warranted. Another limitation was the fact that only units that had been stored for a long duration were investigated. Old units may have the highest risk of shear stress haemolysis due to a lower viscosity and loss of membrane integrity. Multiple articles performed in human pRBC, however, show no difference in pump-induced haemolysis between units stored for short or long periods.[25,355] The impact of duration of storage has not been yet demonstrated in feline pRBC.

Regarding the study design, a crossover study including different groups with different PIP order would have been preferable. In our study, the IM pump was

always evaluated last, and this could have affected results due to different times of exposure to non-refrigerated temperatures before transfusion. The longer blood sits at room temperature, the higher the risk of increasing hemolysis levels.[356] To avoid different total exposure time to room temperature, samples from each pRBC unit were held until all were collected and tested at the same time. In our study, no statistical difference was noted between methods, and thus it is unlikely that this lack of crossover would have had a significant effect over the results. Another limitation was that no power analysis was done, and as such the study may be underpowered to detect a difference. Therefore, a failure to detect statistically significant differences in this study does not necessarily imply that there is no difference. A minor limitation was that no basal sample was collected before blood was passed through the infusion set, so the full transfusion process could have also been studied, including the effect of the infusion system, but this was not the objective of this study. The study was designed to evaluate the effect of the infusion pumps on RBC, not the effect of the infusion set and filter.

In conclusion, our study has demonstrated that the level of haemolysis of pRBC units caused by the use of two different linear PIPs (Infusomat Fms-B.Braun and NIKI V4-Everest) at a rate of 25 ml/h, after 35-42 days of storage at 4° C, is not significantly different than that seen with gravity infusion.

Stability of coagulation factors on feline fresh frozen plasma intended for transfusion after one year of storage

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Abstract

Background: The most common use of plasma transfusion is for haemostatic purposes, but coagulation factor activities in stored feline plasma are unknown.

Hypothesis/Objectives: Concentration and stability of coagulation factors I, II, V, VII, VIII, IX, X, XI, XII in feline fresh frozen plasma (fFFP) stored for 1 year were studied.

Animals: Fifty-five indoor healthy blood donor cats.

Methods: Fifty-five fFFP units were collected. Twenty-one units were stored for less than two weeks and 34 were stored for one year. After completion of storage, specific coagulation times for factors II, V, VII, VIII, IX, X, XI, XII were tested using modified one-stage activated partial thromboplastin or prothrombin time assays. Fibrinogen was determined using the von Clauss Method.

Results: Parametric Student's T test with alpha of .05 were used. Mean±SD specific activity for factor II, V, VII, VIII, IX, X, XI and XII were 101.94±19.06%, 71.94±24.14%, 102.78±24.69%, 77.52±30.39%, 84.86±29.35%, 96.24±25.10%, 88.76±22.73% and 89.50±21.85% respectively at T0, and 73.23±39.06%, 97.87±62.33%, 60.08±38.17%, 50.32±23.8%, 71.37±22.23%, 83.91±49.54%, 66.28±22.20% and 55.46±23.18% respectively at T1. Fibrinogen was 2.76 g/l (±1.09) at T1. Significant T0 vs T1 differences were found for factors II, p=.0008; V, p=.0458; VII, p=.0003; VIII, p=.0015; XI p=.0011; and XII p=.000003.

Conclusions and clinical importance: Although a decrease in most coagulation factors activities was noted, one-year-old fFFP was haemostatically active *in vitro*. The most suitable factors for quality control of fFFP are factor VII and VIII. Approximately 13 to 20ml of fFFP are required to administer a minimum of 10IU/Kg of all coagulation factor's activity.

Introduction

The main objective of blood banks is to produce safe and efficacious blood products and to establish quality standards that provide even safer and more efficient derivatives for transfusion.[260] Fresh Frozen Plasma (FFP) is a blood product obtained either from whole blood or from plasma collected by apheresis.[33,260] Nowadays feline plasma storage is common practice in blood banks and veterinary practices. Using plasma products rather than whole blood allows for longer storage times, increased availability, lower costs, and minimizes the risk of transfusion reactions.[33,265] The most common use for feline FFP (fFFP) transfusion is for haemostatic purposes, and is considered the gold standard care in patients with coagulopathies.[30,265]. In a recent study, the most common indications for fFFP were suspected coagulopathy (83%), haemorrhage (35%), and hypotension (25%).[4]

Coagulation factor activities in fFFP are unknown and thus, in many cases, plasma is given to effect. In a recent article, cats were significantly less likely to be coagulopathic posttransfusion with a median dose of fFFP of 6 mL/kg[4]. However, the optimal dose of fFFP to replace coagulation factors in cats has not been investigated. Coagulation factor activities are used as a standard to define blood plasma products. A general guideline is that 1ml of fresh plasma contains approximately 1 international unit (IU) of each coagulation factor activity.[31]

Current veterinary convention is that FFP is termed frozen plasma (FP) when it exceeds the recommended 1 year storage time, and it is theoretically characterized by a reduced content of labile coagulation factors. [33,265,7] Quality control of human FFP is based on the measurement of coagulation factors V and VIII, which are considered the most labile.[32,33] A canine plasma study conducted in 2013 demonstrated that five-year-old FP is haemostatically active, with a significant decrease of factor VIII activity but with a good stability of factor V, indicating that differences within species might exist.[9] Nowadays, recommended storage times for feline plasma have been adapted from human medicine, in which many authors consider that plasma can be only classified as FFP within one year of storage based on the loss of factors V and VIII.[265] However, to the authors´ knowledge, there are no studies evaluating the stability of feline haemostatic proteins in plasma during storage.

The main aim of this study was to evaluate the concentration and stability over time of coagulation factors during storage of fFFP. The secondary aims were to identify the coagulation factors that are most affected by storage in feline plasma, aiming for them to be considered the critical factors for production and quality control of fFFP, and to help understand the optimal volume of fFFP needed to administer 10 IU/Kg of functional coagulation factors when administering units stored up to 1 year.

Materials and methods

In this prospective study, whole blood was collected from fifty-five indoor healthy cats weighing 4–9 Kg that had been regularly vaccinated and dewormed. Complete blood counts and chemistry profiles were performed, and cats were included only if the results were within normal reference ranges. *Feline Immunodeficiency Virus, Feline Leukemia Virus* (Uranotest FeLV-FIV, Uranovet, El Prat de Llobregat, Barcelona), *Mycoplasma haemofelis, Candidatus Mycoplasma haemominutum and Candidatus Mycoplasma turicensis* (PCR analysis by Genevet, Algés, Portugal) also tested negative in all cats.

No animals were directly involved for the purpose of this study. All samples were obtained from the routine procedures of the Animal Blood Bank in the production of packed red blood cells (pRBC) and fFFP; therefore, no unnecessary procedures were performed on the donors. All blood samples were collected after signed informed owner consent. This study was conducted according to the European legislation (86/609/EU).

Whole blood units were collected using a specific feline semi-closed system as described elsewhere.[348] Whole blood units were gently inverted and placed in the centrifuge cups (Megafuge 40R, Thermo Scientific, Massachusetts, USA) eliminating void spaces by using manufactured plastic adaptors. Weight differences under 0.3 g between opposite cups were tolerated. Units were centrifuged at 2000 g for 15 min at 20 °C (64.4 °F), with 80 s of acceleration and 110 s of deceleration. Fresh plasma was then separated from erythrocytes and expressed into a secondary transfer bag using a sterile connection of polyvinyl chloride tubing (CompoDock, Fresenius SE, Hesse, Germany). Ten mL of SAGM (adenine, dextrose, mannitol and sodium chloride) were added to the pRBC unit under a laminar flow hood (Cruma FL-1, Diantech Solutions S.L., Barcelona, Spain).

Once separated from the pRBC, fresh plasma was frozen at -80ºC and maintained at this temperature during 24h. Then the fFFP units were stored at a temperature between -18ºC and -25ºC. The pRBC units were used for medical purposes.

The fFFP units were then separated into two groups, T0 and T1. The former was stored for less than two weeks before testing, and the latter was stored for one year before testing. All units were stored at a temperature between -18ºC and -25ºC.

After the completion of their designated storage times, all units were thawed at room temperature and 1.5 ml samples were separated and refrozen at -80ºC within 30 minutes. Analysis of the coagulation factors in the latter samples was performed the following day.

Specific coagulation times for factors VIII, IX, XI and XII were tested using a modified one-stage activated partial thromboplastin time assay and coagulation times for factors II, V, VII and X with the modified one stage activated prothrombin time assay (diagnostic stago ST4, France), with human specific factor deficient plasma. Fibrinogen was determined using the von Clauss method. Each factor activity was measured twice per unit and repeated if more than a 10% discrepancy was observed. A 4 dilutions curve of pooled fresh plasma from 10 different healthy cats was used to derive standard curves for all factors.

The variables under study were shown to follow the normal distribution using the Kolmogorov-Smirnov test. Thus, the parametric Student's T test for two independent samples was used to compare specific coagulation times for all analyzed factors between groups T0 and T1. An alpha of 0.05 was used. Results were analyzed with statistical software (SPSS, version 21.0., IBM, Illinois, USA).

Specific factor recovery was calculated for each factor by dividing the observed plasma specific factor activity after one year of storage (group T1) with the observed plasma specific factor activity at the beginning of storage (group T0).

Results

A total of 55 units were included in the study, with 21 units being stored for less than two weeks (T0) and the remaining 34 stored for one year (T1).

Within the T0 group, the mean activity for each specific coagulation factor was higher than 70%. When comparing the values obtained from the groups T0 and T1, significant specific activity decreases were observed for factors II, VII, VIII, XI and XII after one year of storage, with mean decreases of 28.71%, 42.7%, 27.2%, 22.48% and 34.04% respectively from the specific factor activity at T1 comparing to T0. An increase in specific factor activity was observed only for Factor V (25,95% increase). Although a reduction in most of the coagulation factor activities was identified, the majority of units after one year of storage (T1) were still within the reference ranges (>50%).

The factors with lowest mean activities at T1 were factor VIII (50.32%), factor XII (55.46%) and factor VII (60.08%). All specific factor activities of the T0 and T1 groups, the variation between both groups and the percentage of recovery are detailed in **Table1**.

Table 1.

Coagulation Factor % of activity	T0 <2 weeks		T1 One year				Reference interval	% decrease T0 to T1	% recovery from T0 at T1
	Mean	SD	Mean	SD	T	p			
Factor I (g/l)	–	–	**2.76**	1.09	–	–	1.5 – 3	–	–
Factor II	**101.94**	19.06	**73.23**	39.06	3.578	.001	>50%	-28,71	71,84
Factor V	**71.94**	24.14	**97.89**	62.33	-2.061	.046	>50%	25,95	136,07
Factor VII	**102.78**	24.69	**60.08**	38.17	4.943	<.001	>50%	-42,7	58,45
Factor VIII	**77.52**	30.39	**50.32**	23.80	3.628	.001	>50%	-27,2	64,91
Factor IX	**84.86**	29.35	**71.37**	22.23	1.895	.064	>50%	-13,49	84,10
Factor X	**96.24**	25.10	**83.91**	49.54	1.195	.236	>50%	-12,33	87,19
Factor XI	**88.76**	22.73	**66.28**	22.20	3.544	.001	>50%	-22,48	74,67
Factor XII	**89.50**	21.85	**55.46**	23.18	5.293	<.001	>50%	-34,04	61,97

Statistically significant T0 vs T1 differences were found for factors II with t (50) = 3.578; p = .001, V with t (41) = -2.061; p = .046, VII with t (51) = 4,943; p <.001, VIII with t (51) = 3,628; p = .001, XI with t (51) = 3,544; p = .001 and XII with t (51) = 5,293; p <.001.

Discussion

This study was designed to evaluate the concentration of coagulation factor activities in fFFP and their stability over one year of storage. At the beginning of storage, the mean activity for all specific coagulation factors was higher than 70% and, although after one year of storage a decrease was identified for the majority of factors, most of the units were still within the reference ranges, and thus, it was not likely to be clinically relevant.

When specific coagulation factor stabilities were evaluated over time, our study showed a significant decrease of factors II, VII, VIII, XI and XII, when comparing plasma units stored for one year with those stored for less than two weeks, most likely due to their deterioration during storage.

The factors with the lowest mean activities after one year of storage were factor VIII (50.32%), factor XII (55.46%) and factor VII (60.08%). These three factors were the only with a recovery, after one year of storage (group T1), of less than 70% of the factor activity results when comparing them to the results at the beginning of storage (group T0).

In one study of canine stored FFP, the factors with the lowest activities were factors VIII, X and IX, the latter being lower than 50% after 6 months of storage.[31] In our study, changes in factor IX activity were considered non-significant (84.86% at T0 vs 71.37% at T1). This difference is probably due to species differences, test

variability (10-15%), differences in sample size (with only 7 units tested in the dog study), or the fact that factor IX mean activity was initially 61% before storage in the canine study. In another study performed in dogs, a good conservation of factor IX was observed during 5 years of storage at -30º C.[272] Although a small decrease of factor X activity was observed in our study, the difference was not statistically significant. In humans, factors V and VIII are considered the most labile; however, a factor V decrease was not detected in our study, nor was it detected in another study performed in canine plasma.[32,272] Conversely, a factor VIII decrease has been commonly observed in canine and human FP, similar to the feline FP in our study.

In the present study, factor V was the only specific factor showing an increased activity after one year of storage. Factor V is stored in platelets complexed with a polymeric alpha-granule protein multimerin.[357] The physiology of feline platelets have been shown to be different than those of humans or canines. Therefore, a possible explanation for the factor V increase during storage could be that feline platelets may contain higher concentrations accumulated, liberating it to plasma during storage. On the other hand, in human medicine a thrombophilia named factor V resistance has been described, in which mutations to factor V makes it resistant to inactivation by activated protein C, leading to the preservation of a higher specific factor activity.[358] In our study, T1 group contained several units with factor V activity over 150%. A similar alteration to the one described in human factor V resistance could explain the high factor activity at T1, although this has not been yet described in cats. Another possibility could be that a technical error occurred during the analysis, however thorough double-checking techniques were in place for every measurement. Furthermore, this phenomenon was not observed in any other specific factor activity measurements other than factor V.

The factors most affected by storage were factor VIII, XII and VII, with factor VIII presenting the lowest mean activity and factor VII the lowest recovery when comparing to initial values. To decide on the critical factors for fFFP processing and storage, we must also consider their clinical relevance and the need for transfusion in coagulopathic patients. This is especially significant in regards to factor XII. When factor XII is activated to factor XIIa, it participates in an enzymatic amplification loop referred to as the contact pathway, one of its functions being to initiate the intrinsic pathway via activation of Factor XI.[359] While the rate of *in vitro* clot formation in the activated partial thromboplastin time (APTT) depends on this contact pathway activation, the contact system has no defined role in current models of in vivo hemostasis.[359] In human medicine it has been observed that deficiencies of the contact system proteins, including Factor XII, prekallikrein, and high molecular weight kininogen, were not associated with clinical signs of disease.[360] In fact, the major physiologic role of

FXII remains to be established.[359,360] In one study 25 cats with Factor XII deficiency were identified and none of them had experienced spontaneous hemorrhage or abnormal bleeding. Twenty of these cats had undergone an ovariohysterectomy or castration and none experienced hemorrhagic complications.[361] Thus, factor XII deficiency is not considered an essential reason for transfusion in bleeding patients.

In view of this information, factor VIII and factor VII were considered the best candidates for being considered critical factors in the production and quality control of fFFP intended for transfusion in coagulopathic patients.

At the beginning of storage, the mean activities for all specific coagulation factors were higher than 70%. The requirements and quality control of human products establish that FFP must contain at least 70 IU of factor VIII per 100 mL (70% of specific factor activity) and similar quantities of the other coagulation factors and their natural inhibitors.[260] The council of Europe states that 90% of FFP units must contain at least 70% of specific factor VIII activity in the first month of storage.[261] Other guidelines, like the ones of the British Society for Haematology, state that 75% of the units must contain at least 70% of factor VIII activity.[362] In our study, only 60% of units had more than 70% of specific factor VIII activity in the first month of storage (group T0). This could be due to variability between species (between human and feline plasma) or due to differences in the stablished storage temperatures.

Two studies from human transfusion centres addressed this fact: the first, published in 1992 and performed in 6 different transfusion centres, reported that only 40.9% of FFP units had values of factor VIII over 70%, while the second study, published in 2018, reported that >70% of factor VIII activity was observed in 95% of the FFP units.[363,364] Both studies were performed in different countries, but the authors hypothesise that this could also reflect storage and management improvements through time, as 16 years separate both studies. The same improvements may be observed in feline plasma blood banking in the future, as more knowledge is gathered allowing to the improvement of storage protocols.

Multiple factors may influence specific coagulation factor activities in fFFP, but those that are considered most relevant are the temperature of storage, the delay until separation from cells, the anticoagulant solution and the speed and temperature of plasma freezing.[32]

Freezing is a critical step in the preservation of some plasma proteins. When the freezing rate is slow, solute diffusion adapts to the rate of ice crystal formation, that are formed starting at the periphery of the unit and progressing to the interior, increasing the solute concentration in the centre of the unit. This phenomenon includes not only the coagulation factors, but also other solutes

like salts, reaching high central concentrations that, after prolonged contact, can inactivate Factor VIII. If the freezing rate is high, ice formation overtakes solute displacement, forming small clusters of solutes trapped homogeneously in the ice, avoiding prolonged contact between highly concentrated salts and factor VIII molecules.[303]

FFP must be frozen within 24 hours of extraction, and to achieve the highest concentration of coagulation factor activities, rapid freezing must be applied allowing for complete freezing within 1 hour at a temperature lower than -25/30° C.[260,303] In our study all units were processed and frozen at -80°C within the first 24h of extraction.

Plasma coagulation factors stability is also highly dependent on storage temperature.[31,32,260,261,365,366] In a study of human FFP, the stability of Factor VIII was tested by comparing four different temperatures during 6 months of storage.[365] Temperatures of -10° C, -17° C, -20° C and -30° C were applied to different samples, and a significant decrease of the Factor VIII activity was observed on units stored at -10° C and -17° C when compared with the units stored at lower temperatures.[365] In another study performed in human FFP stored at -40° C, loss of Factors V and VIII activities was 0.6% and 9% respectively over three years, showing that the recovery of coagulation parameters in FFP was not reduced below 70% of the starting activity and remained in acceptable ranges.[366]

The Council of Europe recommendations version of 2017 are that human FFP units maintained under -25° C can be stored up to 36 months, but only three months are recommended if the temperature is maintained between -18° C and -25° C.[261] In the 2020 edition of the American Association of Blood Banks guidelines, the recommended temperature was lower than -30°C and the World Health Organization states that the optimal storage temperature is below -25°C.[261,367]

In our study, following the veterinary recommendations for one year of storage, whilst also trying to demonstrate what practitioners are able to do when storing plasma in their own practice, the temperature was maintained between -18° C and -25°C.[31,368] Decreasing the storage temperature of feline plasma units could improve the conservation of coagulation factors, as previously observed in human plasma storage and thus, improve the therapeutic effect.[365] However, further studies are needed to confirm this hypothesis.

Recognition of coagulation factors that are most labile in feline plasma stored up to 1 year could help to understand the optimal volume of fFFP needed to administer 10 IU/Kg of functional coagulation factors. The effective control of active bleeding in dogs with Factor IX deficiency using a dose of 10U/kg of factor IX has been described in one study. In another study were an increase of Factor

VIII activity was tested in non-bleeding dogs, a transfusion of approximately 15 U/kg of Factor VIII in German Shepherd Dogs with hemophilia A resulted in a Factor increase of more than 30%.[31,369] To the authors' knowledge there is currently no corresponding published information in feline medicine.

One ml of fresh plasma is considered to contain approximately 1 international unit of each coagulation factor activity.[31] The loss of activity during storage could mean that the volume of stored FFP needed for transfusion may need to be larger than that of fresh plasma to attain the same therapeutic effect.

Considering that 10 IU/Kg could be a target therapeutic dose for most cats with factor deficiency (information extrapolated from dogs), and on the basis of specific factor activities in the present study, it was calculated that approximately 10 to 15 ml/kg of fFFP stored up to one year could be necessary to provide a minimum of 10 IU/Kg of factors II, V, IX, X and XI; 10 to 17 ml/kg for factors VII and XII; and approximately 13 to 20 ml/kg of fFFP for factor VIII, depending on the storage times (less than 2 weeks or one year, respectively). In every case there are differences in target concentration, factor yield and possible individual differences in pharmacokinetics, and thus these dose recommendations must be solely considered as guidelines for the purpose of initial dosing.

These recommended dosages are similar to those that have been reported in humans, in which 10 to 20 ml/kg are recommended depending on the consulted guidelines.[370–373] In a retrospective study in dogs, the reported mean dosages used were 13.9 ml/kg in small dogs and 5.1 ml/kg in large dogs. On the other hand, another study shows a median of 16 ml/kg was administered to every dog regardless of its size.[374,375] Veterinary textbooks recommend initial doses ranging from 6-20 ml/kg without specifying the species.[265,376]

In a recent retrospective feline study, the FFP doses commonly used (mean 6 ml/Kg) were lower than the ones reported for coagulopathy in dogs and humans.[30] This could make us hypothesize that lower doses of functional plasma might be needed to control bleeding, although the same study concluded that small initial doses of plasma were associated with repeated FFP transfusions. This leads the authors to believe that larger initial volumes may result in less repeated transfusions and faster resolution of clinical signs. It must be noted, however, that only 33% of the receptors were bleeding at the time of transfusion in the mentioned study, so most of the transfusions were performed with a prophylactic objective.[30] In another recent study, doses of 2.15–10.85 ml/kg were effective for the treatment of prolonged prothrombine time (PT) and/or APTT in cats. There is controversy on the usefulness of PT and APTT to predict bleeding. In humans, they are not considered predictive of haemorrhage and little differences have been reported in bleeding complications when they are increased, regardless of the administration of prophylactic FFP.[271,372,377] Thus, PT and APTT might

not be good markers of treatment response. Further *in vivo* prospective studies evaluating different fFFP dosages are warranted.

This study had some limitations inherent to an *in vitro* study and secondary to its design. The measurement of specific coagulation times have been reported to be quite variable, with inter-assay coefficients of variation (CVs) for factors VII and IX surpassing 10% reported in a previous study with canine plasma.[31] Thus, the exact magnitude of their loss was difficult to define. In an attempt to attenuate this, each factor activity was measured twice on every unit and repeated if more than 10% discrepancy was observed, and a single lot of reagents was used, as different reagents and substrate plasmas could contain different residual levels of coagulation factor activity, affecting the linearity of standard curves.[378]

Another limitation was that when calculating the recommended dosage, information of IU required for clinical effectiveness was extrapolated from canine publications and calculated with *in vitro* results of the present study. Further feline *in vivo* studies should be performed to confirm our dosage recommendation.

Additionally, for testing purposes, all units were thawed at room temperature and 1.5 ml samples were taken and refrozen at -80ºC within 30 minutes. We have to consider that this additional freeze–thaw cycle procedure may have affected haemostatic stability. In a previous article performed with feline and canine plasmas, a cycle of thawing and refreezing within 1 hour demonstrated to have no deleterious effect on hemostatic protein activity including fibrinogen and factors II, VII, VIII, IX, X, XI, and XII.[379] Furthermore, the authors of the present study performed a similar test using pooled plasma from 10 healthy cats and compared results with direct testing after thawing and after a cycle of thawing and re-freezing. These results showed no differences, therefore it is considered unlikely that the freeze–thaw cycle had any major influence on the results of the present study.

In conclusion, a decrease was identified for specific coagulation factor activity for factors II, VII, VIII, XI and XII after one year of storage at a temperature between -18ºC and -25ºC, although most were still within the reference ranges. Considering the results of this study, the most suitable factors for quality control of fFFP are factor VII and factor VIII, the latter having the lowest values after one year of storage at a temperature between -18ºC and -25ºC, with its recovery reduced to 65% of the starting activity. Taking this into account, a dosage of approximately 13 to 20 ml/kg, depending on the storage time, is needed to ensure the administration of at least 10 U/Kg of each specific coagulation factors tested in this study.

Thesis conclusions

1 Considering the U.S. FDA guidelines for human blood banking that recommend a maximum of 1% haemolysis to ensure that no haemolytic products are transfused to patients, our results evidenced that all pRBC units collected with a specific semi closed system and separated from whole blood centrifugation at 2000 g for 15 min at 20° C (64.4° F), with 80 s of acceleration and 110 s of deceleration, and with less than 24 h of shelf life have negligible haemolysis levels.

2 Feline pRBC units preserved for more than 28 days can only be safely administered if tested for haemolysis before its use, since 13.88% units exceeded the 1% limit at 35 days of storage in our study.

3 Semi-closed collection systems, when manipulated in sterile conditions, are reliable for feline blood banking when evaluated for bacterial contamination.

4 The level of haemolysis of pRBC units caused by the use of two different linear PIPs (Infusomat FmS [B.Braun] and NIKI V4 [Everest]) at a rate of 25 ml/h, after 35–42 days of storage at 4° C, is not significantly different than that seen with gravity infusion.

5 Regarding feline FFP, a decrease was identified for specific coagulation factor activity for factors II, VII, VIII, XI and XII after one year of storage at a temperature between -18° C and -25° C, although most were still within the reference ranges.

6 The most suitable factors for quality control of feline FFP are factor VII and factor VIII, the latter having the lowest values after one year of storage at a temperature between -18° C and -25° C, with its recovery reduced to 65% of the starting activity.

7 A feline FFP dosage of approximately 13 to 20 ml/kg, depending on the storage time, is needed to ensure the administration of at least 10 U/kg of each specific coagulation factors tested in our study.

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