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**PRELIMINARY EVALUATION OF THE QUALITY OF BLOOD
COMPONENTS FOR TRANSFUSION USE (WHOLE BLOOD,
PACKED RED BLOOD CELLS, FRESH FROZEN PLASMA) IN
CANINE, FELINE AND BOVINE BLOOD PRODUCTS AND
PREPARATION OF BLOOD COMPONENT FOR NON-
TRANSFUSION USE (PLATELET RICH PLASMA) IN DOG**

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CHAPTER 1

Foreword: Veterinary Transfusion Medicine

1. Foreword: Veterinary Transfusion Medicine

Interest in veterinary transfusion medicine has been growing over the past few decades (Gibson, 2007). Increasing interest and availability of blood products has helped to make transfusion medicine a common practice at almost every veterinary clinic (Lavenschi et al., 2001; Lucas et al., 2004; Weingart et al., 2004; Hansen, 2006).

Transfusion is defined as intravenous therapy with whole blood or blood products. Whole blood refers to blood that has not been separated into various products while blood products include blood components and blood derivatives. Blood components are blood products prepared only by physical separation (centrifugation or sedimentation) while blood derivatives are blood protein products prepared by using biochemical methods to process large pools of donor plasma. (Abrams-Ogg, 2000).

The use of blood components allows several patients to benefit from one blood donation and reduces the risk of transfusion reactions to unnecessary components. (Abrams-Ogg, 2000).

Recent introduction in Italy of guideline provided by Ministry of Health ("Linea guida relativa all'esercizio delle attività sanitarie riguardanti la medicina trasfusionale in campo veterinario", published in Supplemento Ordinario della Gazzetta Ufficiale Repubblica Italiana n.32 February 7, 2008) (Attachment n° 1) are likely to lead to further development of transfusion medicine and animal blood banking within in Italy.

Knowledge and practice of appropriate blood collection, processing, storage and administration methods is essential for ensuring the safety of the donor and recipient, as well as maximizing the use of limited clinical resources.

1.1 Donor selection

1.1.1 Canine blood donors

Canine blood donors should be healthy, good-tempered, between 1 and 8 years of age and a minimum bodyweight of 25 kg to allow collection of blood in standard human collection bags (Gibson et al., 2012). The donor should receive routine veterinary preventive health care including vaccination according to practice protocols. Dogs should not be receiving any medication at the time of donation with the exception of ectoparasite preventatives, routine worming medication and heartworm prophylaxis. Donation should not take place with 10-14 days of having received a vaccination. Any dog that has received a transfusion previously may have developed alloantibodies against different blood types and is unsuitable as a donor (Gibson et al., 2012).

The Italian guideline for the suitability of canine donors is reported in Attachment n° 1.

The health of the donor should be assessed by a veterinary by careful review of clinical history, thorough physical examination and pre donation screening blood test. Pre donation tests include determination of blood type (at a minimum a DEA 1.1 status), annual haematology and serum biochemistry profile, as well as screening for infectious diseases endemic to current or previous geographical location of the donor. Also screening of infectious agents that may have an impact on the health of the donor (for example *Dirofilaria immitis*) is important to consider. Urinalysis and faecal examination for parasites are often recommended (Abrams-Ogg, 2000).

In addition of the annual comprehensive health examination a complete donor history, physical examination and a complete blood count (CBC) should be performed prior of every donation to safeguard the health and assess the suitability of the donor (Gibson, 2007).

The Italian guideline about screening test for canine donor is present in Attachment n° 1.

1.1.2 Feline blood donors

Donor cats should be healthy large cats (> 5 kg of weight), between 2 and 8 years of age and should be up-to-date with core vaccinations (feline calicivirus, herpesvirus and panleucopenia). Donor cats should be housed indoors to minimize fight wounds and infections. Donor cats should not be on any medication other than those that prevent endo- and ectoparasites (Kohn et al., 2012) .

Italian guideline for the suitability of feline donors is present in Attachment n°1. A complete history of the donor should be taken before the donation. Annual laboratory evaluation consisting in CBC, serum biochemistry profile and infectious disease screening for feline leukemia virus (FeLV), feline immunodeficiency virus (FIV) and *Mycoplasma Haemofelis* is recommended in cats that donate regularly. Before each blood collection a CBC should be performed to safeguard the health of the donor (Kohn et al., 2012).

The Italian guideline about screening test for feline donor is present in Attachment n° 1.

1.2 Blood types

Red blood cell types are determined by species specific inherited antigens present on the cell surface. Blood typing is important in transfusion medicine because of the risk of transfusion reaction (Abrams-Ogg, 2000).

1.2.1 Canine blood types

Canine blood typing is currently classified using the Dog Erythrocyte Antigen (DEA) system. Within the DEA system there are six recognized antigen sites defined by the availability of international standardized antisera. This antigen sites are known as DEA's 1.0, 3, 4, 5 and 7 and for all the DEA groups a dog may be positive or negative (Hale, 2012). Although not fully defined or recognized there are said to be upward of 18 or more antigen specificities described. In particular DEA 6 and DEA 8 are two examples of antigens that have been identified but at this time no typing system is available.

The DEA 1.0 system describes three antigens and a null phenotype. DEA 1.1 is the most common antigen in this system and is expressed by the red blood cells (RBCs) of 62% of dogs in different countries. DEA 1.2 and 1.3 are rarely identified. (Hale, 2012)

The most antigenic blood type is DEA 1.1, however there are no naturally occurring alloantibodies against DEA 1.1. As such, most dogs will not experience a severe transfusion reaction from a first transfusion of DEA 1.1 incompatible blood. However sensitisation occurs in the DEA 1.1 negative that receive DEA 1.1 positive cells. Antibodies (strong haemolysins and agglutinins) are produced which can cause an acute haemolytic transfusion reaction following repeated antigen exposure (e.g a second transfusion with DEA 1.1 positive blood) (Gibson, 2007).

The principle of a serologic canine blood typing reaction is macroscopic agglutination to allow detection of the different RBCs surface antigens. As DEA 1.1 is the most antigenic blood type it is strongly advised that the DEA 1.1 status of both the donor and recipient is determined prior to transfusion. DEA 1.1 testing can be performed by a variety of methods (reference laboratory and in-house testing).

The card agglutination assay (RapidVet-H Canine, provided by DMS Laboratories, Flemington, NJ.) and immunochromatographic cartridge method (DME VET DEA 1.1, provided by Alvedia, Lyon, France.), performed by trained personnel, were suitable for in clinic emergency DEA 1.1 blood typing. There may be errors, particularly for samples from dogs with IMHA, and the immunochromatographic cartridge method may have an advantage of allowing typing of samples with persistent autoagglutination. However the laboratory gel-based method (bID-Gel Test Canine DEA 1.1, provided by DiaMed, Cressier-sur-Morat, Switzerland) is considered the gold standard method for canine blood typing and is used in reference laboratory (Seth et al., 2012).

1.2.2 Feline blood types

Blood groups in cats are described by the A-B system, which includes the blood types: A, B and AB. The blood types are inherited as a simple dominant trait,

with A being dominant over B. Genotypically type A cats are either homozygous a/a or heterozygous a/b, whereas type B cats are homozygous b/b. Type AB is a rare blood type in which a third allele is present, recessive to the a allele but dominant to the b allele. (Gibson, 2007). Typing of cats prior the transfusion is imperative because in cats naturally occurring antibodies are present in the plasma. These alloantibodies are isoagglutinins against the RBCs antigen that is not present in that individual cat. These antibody are found in all type A and B cats after 2 months of age and their formation does not require prior exposure through transfusion or pregnancy (Gibson, 2007).

The risk of transfusion reaction is greatest if a type B cat is given a type A blood, because almost all type B cats have high titres of anti A antibodies that result in rapid intravascular haemolysis of the donor RBCs. The transfusion reaction if a type A cat receives type B blood is usually less severe, resulting in accelerated destruction of the RBCs primarily due to extravascular haemolysis, because type A cats generally have weak anti-B antibodies that are usually of low titre.

The rare type AB cats do not possess either alloantibody. Type AB cats should ideally receive type AB blood, but when that is not available type A blood is the next best choice. (Gibson, 2007).

Most domestic short and long hair cats throughout the World are of blood group A with some geographical differences. Blood group B is more prevalent amongst particular purebred cats including the British Shorthair, Birman, Devon and Cornish Rex, Abissinian, Persian, Somali, Turkish Angora and Turkish Van (Day, 2012).

The "gold standard" of feline blood typing remains the tube or microplate agglutination test (ID Gel-Test Micro Typing System, Diamed) but for routine use exist different commercial system (Proverbio et al., 2009, Day et al., 2012).

The first in practice technology to be developed was a card-based system (DMS rapidvet-H feline, DMS Laboratories) with monoclonal anti A antibodies and triticum vulgari lectin as the B agglutinin (Proverbio et al., 2009). Most recently an immunochromatographic test (DMEVET Quick Test A + B, Alvedia) has been introduced (Day, 2012).

1.3 Blood collection

Blood can be collected from an open or closed system. A closed system is one in which the only exposure of the bag or its contents to air is when the needle is uncapped for venepuncture. For this reason closed system collection is preferred because it decreases the potential contamination of blood products and facilitates processing of blood components. Unfortunately closed systems do not guarantee a total sterility and contamination can occur from cutaneous organisms even with aseptic venepuncture technique (Abrams-Ogg, 2000).

An open system is one on which there is one or more additional sites of potential bacterial contamination during blood collection or processing. Blood collection using syringes or empty collection or transfer bags with anticoagulant added at the time of donation are all classified to be open system.

The anticoagulant-preservative solutions are important for the duration of time for which the whole blood or blood products can be stored and to support the glycolytic energy metabolism of RBCs to maintain their viability during storage. The anticoagulant solutions must often used are ACD (acid-citrat-dextrose), CPD (citrate-phosphate-dextrose) or CPDA-1 (citrate-phosphate-dextrose-adenine). Multiple bag systems with empty transfer bags and red cell preservative (like SAG-Mannitol) may be used for component processing (Gibson, 2007).

The volume of blood that may be collected safely from canine and feline donors is approximately 20% of their blood volume, every 3-4 weeks. The recommended volume limit is 18 ml/kg for dogs and 10-12 ml/kg for cats (Gibson, 2007, Spomberg, 2012).

1.3.1 Canine blood collection

Canine can safely donate approximately 15 to 20% of their overall blood volume every 3-4 weeks, equating to approximately 15-18 ml/kg. In general dogs do not requires sedation or anaesthesia and lie still for the collection process. The jugular vein is the recommended venepuncture site owing to the size and accessibility but also cephalic vein can be use in large giant dogs. Hair must be clipped from over the venepuncture site and the site must be prepare aseptically. Usually for collection of whole blood it is used commercially available collection bags (human closed system) that already contain anticoagulant and a swaged-on phlebotomy needle and are sterilized, sealed and protected in a plastic or foil overwrap. Packs are also available with "satellite bags" for preparation of blood components (Gibson et al., 2012).

Healthy dogs can safely make a donation into a 450 ml commercial blood bag with an allowable 10% variance (405-495 ml). By convention and for convenience a standard blood donation in dog with a minimum bodyweight of 25 kg is 450 ± 45 ml, which is referred to as "one canine unit" (Abrams-Ogg, 2000, Gibson et al, 2012).

1.3.2 Feline blood collection

Feline donors can safely donate 10-15% of body weight every 3-4 weeks, equating approximately 11-13 ml/kg. Unlike dogs almost all cats require sedation or anaesthesia for blood donation (for example with ketamine and midazolam or isofluorane/oxygen anaesthesia by mask). Blood is collected by jugular venepuncture with the cat restrained in either lateral or sternal recumbency (Gibson, 2007).

Closed systems for use in feline medicine are currently not available. For this reason blood is commonly collected with a 19 G winged infusion set ("butterfly") attached to a 60 ml syringe into which the anticoagulant-preservative solution has been drawn (open system). For blood banking purpose the blood is then transferred to a 100-150 ml storage bag. A standard donation in the cat results in 60 ml of anticoagulated blood (53 ml of blood and 7 ml of anticoagulant, often CPDA1) (Kohn et al., 2012).

1.4 Blood components

The optimal transfusion therapy is performed by selection of the most appropriate blood component and administration of that component in the manner most likely to avoid transfusion reaction. The principle of component therapy consists in dividing whole blood into its single elements, mainly packed red blood cells and plasma by centrifugation. (Gibson et al., 2012).

1.4.1 Whole blood

Whole blood can be used fresh (FWB) with the transfusion carries out within 8 hours of collection. In this case all blood components (red blood cells, platelets, labile and stable coagulation factors, plasma proteins) are present and functional. Whole blood can also be stored in a refrigerator at 1-6 °C for variable days depending on the anticoagulant-preservative used. The unit is then classified as storage whole blood (SWB) which will differ only form FWB by the functional reduction of labile clotting factors and platelets.

The small blood volume collected, the use of on open collection system and the difficulties in separation of blood elements make FWB and WB transfusions still the norm in feline patient (Gibson et al., 2012).

1.4.2 Packed red blood cells

The PRBCs is obtain from whole blood by separation from the plasma by centrifugation (preferably) or sedimentation. Blood component processing requires variable speed, temperature-controlled centrifuges to produce products. The precise centrifugation protocol used depends on the centrifuge but the separation of WB into PRBCs and plasma typically requires centrifugation at 5000g for 5 minute (not including deceleration time) at 4°C. (Gibson, 2007).

Once the donor unit has been centrifuged and the red cells separated from the plasma with the use of a plasma extractor a nutrient solution such as Optisol is added to PRBCs and the units are stored under refrigeration at 1-6°C. The PCV of the unit is grater than that of the whole blood because it will have been greatly concentrated (70-80% without added nutrient solution) with the volume yield dependent on the donor PCV. The storage time of PRBCs can vary from 3-6

weeks depending on the type of anticoagulant and nutrient solution use (Gibson et al., 2012).

1.4.3 Fresh plasma, fresh frozen plasma and frozen plasma.

Fresh plasma is plasma that has been separated from RBCs within 8 hour of collection and immediately transfused (Abrams-Ogg, 2000).

Fresh frozen plasma (FFP) is plasma that has been separated and placed at -18°C or colder within 8 hours of collection. These time restrictions are based on the deterioration of human factor VIII activity and other labile clotting factors such as factor V and vWF. Fresh plasma and FFP contain plasma proteins, albumin and globulins and have maximal activity of all coagulation factors. After 1 years storage period FFP is relabelled as frozen plasma (FP), which may be stored as such for additional 4 years at - 18°C or colder. The 1 years expiry date for FFP is also based on the deterioration of FVIII activity. FP can be also obtain if the unit of plasma has been prepared after 8 hours of collection. This product contains all the vitamin k dependent coagulation factors (II, VII, IX, X), immunoglobulins and albumin but is relatively devoid of the labile clotting factors. FP has a shelf life of 5 years after the original date of collection or 4 years after expiration of a unit of FFP (Gibson et al., 2012).

1.4.4 Platelet rich plasma (PRP) and platelet concentrate (PC)

PRP and PC may be prepared from FWB. FWB is centrifuged on "light" spin. The speed and time centrifugation are lower than those typically used to separate PRBCs and plasma, and the product is not refrigerated. The PRP and PC may be stored at 20-24 °C with gentle continuous or intermittent agitation, for 5 days when collected using a closed system. Given that the storage temperature is higher than that used for other blood products, platelet products are more susceptible to bacterial contamination, and if collected in an open system, they should be used within 4 hours of collection. Unfortunately the difficulties associated with their production often result in the use of more readily available fresh whole blood (Gibson et al., 2012).

1.5 Storage of blood components containing RBCs

RBCs stored under blood bank conditions undergo structural and functional deterioration, collectively referred to as RBC storage lesions. Most of the lesions are the result of the progressively effect RBC metabolism and are reversible in vivo. On the other hand, the structural changes and the loss of membrane are permanent and probably related to in vivo RBC survival and the adverse effects of transfusion. Although some differences in the aging profile between in vivo and ex vivo conditions have been reported, a part of stored RBCs progressively express some of the typical marks of senescence and erythrophagocytosis, like

the formation of Band 3-IgG complexes, the exposure of PS, and the spherocytosis. The increasing accumulation of aging effects in a part of the transfused RBCs contributes not only to their rapid elimination but also to the various adverse side effects of the transfusions. (Antonelou et al., 2010). In fact recent human studies have demonstrated that there may be a correlation between the number of days of cold storage of packed red blood cells (PRBC) and adverse outcomes, including mortality, among transfused patients, presumably due to structural or functional changes in RBCs that occur during storage (Vandromme et al., 2009, Napolitano et al., 2004).

Blood products containing RBCs (PRBC or SWB) have a short lifespan and are marked with a maximal expiration date (usually 28–35 days for canine RBCs), at which point storage lesions may compromise the function and viability of the cells. Due to these changes the addition of nutrient and preservative solutions is imperative to help increase red cell viability. Even with additional preservative solutions and optimal storage conditions, the quality of RBC products declines with time (Antonelou et al., 2010, Spromberg, 2012).

RBCs require oxygen and nutrient for survival and during the storage the amount of viable RBCs reduces over time. When red cells within the unit become inactive and die, they are broken and release potassium and ammonia as a by-product (Abrams-Ogg 2000). In stored PRBCs deamination of adenine (often present in preservative solutions), and plasma and intraerythrocytic protein leads to the formation of ammonia. Ammonia increase to extremely high concentration in units of canine PRBCs stored in CPD-Adsol over the accepted time period of 35 days. Besides seems that the kind of storage (small refrigerator open frequently or large blood-banking refrigerator not open more than once a day) could further affected the ammonia concentration (Waddell et al., 2001). Hyperammonemia can pose a problem for animals which underlying hepatic disease as they are unable to fully filter out ammonia and its accumulation in the hepatic patient can lead to exacerbation of the hepatic disease, CNS symptoms and hepatic encephalopathy (Gibson, 2012).

Due to the potential deterioration of activity and function of labile blood components, conditions of storage and time prior to processing are vital to the preparation of components. Delays in preparation or unsuitable conditions of storage may adversely affect quality of the final components (Gibson et al., 2012).

1.6 Aspects of red cell preservation

The anticoagulant solutions used in blood collection have been developed to prevent coagulation and to permit storage of red cells for a certain period of time. While originally designed for whole blood storage, they have also been used in blood from which components are prepared.

All of the solutions contain sodium citrate, citric acid and glucose, some of them may also contain adenine, guanosine and phosphate. Citrate binds calcium and prevents clotting of the blood. Glucose is used by the red cell during storage and each glucose molecule gives 2 molecules of adenosine tri phosphate (ATP) which is formed by phosphorylation of adenosine di phosphate (ADP) (Abrams-Ogg, 2000).

An additive solution should allow maintenance of red cell viability even if more than 90% of the plasma is removed. The use of glucose and adenine is necessary for the maintenance of red blood cell posttransfusion viability, phosphate may be used to enhance glycolysis, and other substances may be used to prevent *in vitro* haemolysis (i.e.mannitol, citrate). Sodium chloride or di-sodium phosphate may be used to give the additive solution a suitable osmotic strength (Abrams-Ogg, 2000).

The duration of storage may vary with the type of preparation (concentration of cells, formula of anticoagulant, use of additive solution, etc.) and should be determined for each type on the basis of achieving a mean 24 hours post transfusion survival of no less than 75% of the transfused red cells (Abrams-Ogg, 2000).

1.7 Contamination of blood and components

Ensuring the safety of whole blood and blood components is of the utmost importance in transfusion medicine. Source of microorganism include donors (bacteraemia and skin flora) and contaminated blood collection, banking and transfusion supplies.

Infectious screening of the donors is crucial to assure the quality of blood components but there is always the potential that the donors can have a subclinical viremia, bacteraemia or parasitemia and that the organisms could be transmitted via transfusion to a patient. Besides bacterial contamination can be iatrogen occurring during all steps to obtain and use a blood product (collection, processing, storage and administration).

The risk of transfusion-associated sepsis using human blood bag is low. The organism implicated most often in human are *Yersinia enterocolica*, *Pseudomonas* spp, *Staphylococcus* spp, *Streptococcus* spp and various Gram-negative organism (Abrams-Ogg, 2000).

Often in veterinary medicine bacterial surveillance programs are not used despite in human medicine blood transfusion therapy has emerged as a potential source of iatrogenic septicaemia. While exists a great literature about contamination of whole blood and blood components in human medicine the prevalence of microbial contamination and organism in veterinary transfusion medicine are poorly documented (Abrams-Ogg, 2000). The only two article

about contamination of blood bags in veterinary medicine regard feline units obtained with open system.

One publication (Hohenaus et al, 1997) reported the contamination of many feline units by *Serratia marcescens*. The units were contaminated during the collection via the saline flush used for sedation of feline donors and resulted in recipient morbidity and mortality.

In another study (Kessler et al, 2010) a feline PRBCs unit turned black after 22 days of storage and result positive for *Pseudomonas fluorescens*. In this study source was not found and no other contaminated units were identified.

In veterinary blood banking aseptic collection, temperature controlled storage and a visual monitoring of stored units is recommended. Discolored units should not be transfused, but examined for bacterial contamination or other blood product quality problems (Hohenaus et al, 1997, Kessler et al, 2010).

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CHAPTER 2

Evaluation of hematological parameters, ammonia concentration and microbial contamination in Canine Packed Red Blood Cells stored in CPD-SAGM for 42 days.

2. Evaluation of hematological parameters, ammonia concentration and microbial contamination in Canine Packed Red Blood Cells stored in CPD-SAGM for 42 days.

2.1 Abstract

Canine transfusion medicine practices have been growing rapidly over the past few decades and the use of specific blood components (packed red cells, plasma) has permitted to optimized the canine blood donations. The study was undertaken to evaluated the changes in RBC, MCV, Ht, RDW, WBC and ammonia concentration in canine PRBC stored in CPD-SAGM for 42 days. Also the presence of bacterial contamination was evaluated with blood culture. PRBC units were stored in a routine manner and were examined every 2-3 weeks. Hematological parameters changed significantly with increase of MCV, Ht and RDW, while WBC decreased. Also ammonia concentration increased significantly during the storage. RBC and WBC deteriorated somewhat during storage and ammonia concentration increased similar to what reported in canine and human in vitro studies. No bacterial contamination was reported. The results obtained in this study agree mostly with what previously is reported in canine and human medicine. Further studies are needed to better evaluated how the reported alterations influence viability of blood cells in canine PRBC.

2.2 Introduction

The primary purpose of transfusion therapy is to replenish the specific blood component that is diminished. Due to improvement in component preparation technology the use of stored blood components has become common practice also in canine transfusion medicine (Lavenschi et al., 2001; Lucas et al., 2004; Hansen, 2006).The use of blood components permits to optimize the blood donation and to reduce the risk of transfusion reactions to unnecessary components. In canine species blood components can be obtained by human commercially available multiple bag systems containing various additive solutions.

During storage erythrocytes undergo some complex structural, functional and metabolic alterations which are called "storage lesion" even if many effort have been made to improve the quality of RBC units and prolong their shelf life (like inline filter to remove leucocytes before storage and new additive solutions) (Zehnder et al., 2008). These storage lesions of RBCs (red blood cells) may explain why their capacity of oxygen-delivery and tissue oxygenation is reduced.

Recently in human medicine in some studies (Napolitano et al., 2004, Vandromme et al., 2009) the duration of RBC storage before transfusion may be related to morbidity and mortality in critically ill patients and after cardiac surgery.

Moreover during storage of PRBC and whole blood units also the environment within the bag undergoes changes like pH and dextrose concentration decrease, while lactate and ammonia concentration increase (Hess et al., 2000, Waddell et al., 2001).

Another important aspect related to blood components is the possible bacterial contamination that can be endogenous (transient bacteriemia of the donor) or exogenous (inadequate skin sterilization, contamination of collection bag, and anticoagulant) and that may occur during the blood collection, the preparation and storage of blood products and their administration. In human medicine, the problem of bacterial contamination is well known (Brecher et al., 2005, Lee, 2011) with an incidence between 0,002% and 1% for PRBC (D'antonio et al., 2002) while the veterinary literature is scarce and relating exclusively to the feline species (Hohenhaus et al., 1997, Kessler et al., 2010). Despite the lack of reported cases of bacterial contamination in canine transfusion medicine it is importance to use a correct protocol to prevent the problem.

The aim of this research was to investigate the safety of canine PRBC stored for 42 days in CPD-SAGM (citrate-phosphate-dextrose-saline-adenine-glucose-mannitol) and to evaluate the modification of hematological parameters and ammonia concentration during storage.

2.3 Materials and Methods

All units of whole blood were obtained with informed owner consent from dogs enrolled as donors in the community volunteer canine donor program of the Reparto Emotrasfusionale Veterinario (REV) of Veterinary Faculty of Milan.

All donors were healthy, good-tempered, 2-8 years old, and with a minimum weight of 25 kg. All donors have had annual physical examinations and vaccinations, regular flea and tick control and heartworm prophylaxis. All dogs were submitted to blood group typing (Lab Test Canine DEA 1.1 Blood Typing, Alvedia, Lyon, France) and annual screening includes a complete blood count (CBC), serum chemistry panel, urinalysis, faecal exam and testing for the following infectious diseases: *Leishmania infantum* (IFAT, Leishmania-Spot IF, Biomerieux), *Ehrlichia canis* (IFAT, Fluo Ehrlichia canis, Biopronix), *Rickettsia conori* (IFAT, Fluo Rickettsia conori, Biopronix), *Babesia canis* (evaluation on blood smear), *Dirofilaria immitis* antigen (SNAP HTWM, Ideex Laboratories) and *D. immitis* microfilariae. Dogs were excluded as donors if they had hematologic or biochemical abnormalities, evidence of systemic abnormalities on physical

examination, positive serological results ($\geq 1:80$) for *L. infantum*, *E. canis* or *R. rickettsii*, a positive test result for *D. immitis* antigen and/or microfilariae or a positive smear for *Babesia canis*. In case of doubt in the evaluation of infectious disease the result was confirmed submitted an EDTA-blood sample to PCR test. Prior to every donation recent history was taken from the owner, a physical examination was performed and a small sample of blood in EDTA was obtained to performed a CBC (Cell-Dyn 3500Plus, Abbott Diagnostics, Germany) for ensure the safety of the donor. All the dogs were fasted for up 12 hours prior to blood collection in attempt to minimized nausea and to prevent lipaemia in the collected blood.

2.3.1 Blood collection

All equipment was prepared and arranged prior to starting the donation procedure.

For all donations was used a sterile triple-pack blood collection system (CPD-SAG Mannitol Grifols Triple K.S., Grifols, Barcelona, Spain) to obtain from whole blood donation 2 different products (Packed red blood cells –PRBC – and Fresh Frozen Plasma – FFP-) and a blood scale and shaker (Delcon Hemotek 2, Italy) which rocks the blood during gravity collection and clamps the tubing when the bag reaches a desired weight. The donation session required the participation of 3 people (minimum 2 vets) – one phlebotomist and two adequately trained personnel to restrain and monitor the donor, as well as to assist with handling of the collection equipment during the donation.

The dog was restrained securely and comfortably on a table to facilitates comfortable restraint for the period of approximately 10-15 minutes required for the blood collection. The venipuncture site corresponding to the cephalic vein was shaved and prepared aseptically with ethanol (ethanol 94°, ACEF S.p.a.). The volume of blood collected was 450 ml, with an allowable 10% variance (405-495 ml).

2.3.2 Care at the donor after donation

The dog was observed for 15-20 minutes for weakness, pale mucous membrane, weak pulse and other signs of hypotension. Dog was offered water and food during the post donation observation period. The owners were advised to avoid exercising the dog excessively for several days.

2.3.3 PRBC preparation

RBCs were separated from plasma within 6 hour of collection using a blood centrifuge (Rotixa 50 RS –Hettich, Germany). The blood centrifuge was set at a temperature of 4° C, 3500 RPM with initial acceleration (run up 9) and free deceleration (run down 0) for 25 minute. At the end of centrifugation the

plasma had been extracted with a manual plasma extractor (Separation Stand Teruflex ACS-201) and put into the first empty satellite bag. To prepare PRBC the seal of the preservative solution bag (SAG-M) was opened and the solution was transferred for gravity into the primary bag containing the RBCs. Once the preservative has transferred completely the bag was gently rocked back and forth to ensure adequate mixing. Using a manual stripper the blood remaining in the tubing was stripped into the bag, the bag was gently manually mixed and compressed to refill the tubing. This procedure was made 3 or 4 times to have a correct distribution of PRBC into the tube. The tubing was sealed with a thermal sealers (Hemoweld B-Delcon, Italy) to provides 6 aliquots of about 0.5 ml (tubing 10 cm long) which were used to performed test.

2.3.4 Storage of PRBCs

All bags were labelled with the date, the type and volume of the product, identification number of the donor, blood type, collection and expiration date. One aliquot of PRBC was taken immediately before the storage to performed hematological test and measured ammonia concentration at day 0 (D0) and one aliquot was taken the day before for blood culture. The units were stored in a specialized blood storage refrigerator with built-in temperature alarm at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (Emoteca 250, Fiocchetti, Luzzara, Italy). The bags were posed vertically to reduce the risk of damage and to maximize oxygen diffusion in to, and carbon dioxide diffusion out of, the bag. To ensure a uniform distribution of the anticoagulant solution and increase the viability of red blood cells the bags were manually massaged and gently mixed three - four times per week.

2.3.5 Hematological parameters and ammonia concentration

Every 2 weeks starting from D0 and until the expiry at D42 an aliquot of PRBC was used to perform test.

Before performing tests the aliquot was manually mixing for 1 minutes, after this the sample was take with a 27 gauge needle and syringe and put in an eppendorf tube. The eppendorf tube was gently mixed 2-3 times before measure ammonia with an automated tool (Ammonia Checker II model AA-4120, Menarini, Italy) and a special test strip (Ammonia Test Kit II, Menarini Italy). The remaining sample was put on a blood rocker for minimum 5 minutes before performing CBC (Cell-Dyn 3500 PLUS, Abbott Diagnostics, Germany). The same procedure was made at D14, D28 and D42.

2.3.6 Blood culture

During a period of 6 months 10 PRBC units were randomly chosen for blood culture. The culture was performed at D1, D21 and D42 using for each culture an aliquot of stored PRBC. The culture was performed using a modified and

adapted standard microbiological procedures (Carter et al., 2004). An amount ranging from 200 to 500 μ L of blood was seeded in sterile way in a culture broth (Tryptic Soy Broth, TSB, Oxoid, Italy) in a 1:10 ratio. The tube was then incubated at 37°C for 24 hours; if after this period, the broth was clear, with packed red cells on the bottom, the tube was again incubated for 24 hours up to a maximum of 72 hours; over this period the sample was indicated as sterile. On the contrary in case of turbidity of the culture medium, index of positivity, 100 μ L of sample was transplanted on solid medium agar-blood, which is obtained by adding defibrinated sheep blood to base medium Tryptic Soy Agar (TSA) (Microbiol, Italy). This solid culture medium allows to obtain colonies with the macroscopic morphology typical of the various bacterial species. Then the plate was incubated at 37 ° C also for 24 hours, after which it was proceeded to the identification of bacteria by macro-and microscopic examination (evaluation of macroscopic morphology and execution of the Gram stain), construction of simple biochemical tests and transplantation on selective and differential culture medium (Quinn et al., 2002; Carter et al., 2004; Poli et al., 2005; Jones, 2006). Also visual inspection of color of the PRBC contained in the bags was made to research possible signs of contamination.

2.4 Statistical Analysis

Data analysis was performed using a commercial statistical software (MedCalc Software, Mariakerke, Belgium). Data were tested for normality using the Kolmogorov-Smirnov test and mean ammonia concentration, means of Red Blood Cells count (RBC), White Blood cells count (WBC), Mean Cell volume (MCV), Hematocrit (Ht), Red cell Distribution Width (RDW) at each sampling point were compared using a one way repeated measures ANOVA. Correlation of MCV and Ht was evaluated by Spearman's Coefficient. A p value of < 0.05 was considered statistically significant.

Hemoglobin has not been considered in the statistical analysis because the automated hematology analyzer used in this study, CELL-DYN 3500 PLUS, lyses red blood cells to determine the amount of hemoglobin. In this way the analyzer can not distinguishing among the hemoglobin present in the plasma and resulting from the normal in bag hemolysis and the hemoglobin contained inside the red blood cells. Because MCH and MCHC are calculated by the analyzer starting from hemoglobin also these 2 parameters are excluded by the statistical analyses.

2.5 Results

A total of 33 canine PRBC units were subject to evaluation of hematological parameters at D0, D14, D28 and D42. Mean and standard deviation of all parameters are reported in Table 1.

There was no significant change in RBC during storage period ($p = 1$) (Graph 1). All other parameters evaluated showed statistically significant differences during the time points. In particular there was:

- a significant increase in Ht between D0 and D 42 ($p = 0.0003$) and between D14 and D42 ($p = 0.002$) (Graph 2)

- a significant and progressive increase of MCV during all time points, in particular between D0 and D14 ($p = 0.0353$), between D0 and D 28 ($p = 0.0002$), between D0 and D 42 ($p < 0.0001$), between D 14 and D28 ($p = 0.0001$), between D14 and D 42 ($p < 0.0001$) and between D28 and D42 ($p = 0.0011$) (Graph 3)

- a significant increase of RDW between D0 and D42 ($p < 0.0001$), between D14 and D42 ($p < 0.0001$) and between D28 and D42 ($p = 0.0005$) (Graph 4).

Our analyzer (Cell-Dyn 3500 Plus, Abbott Diagnostics) permits to obtain WBC with 2 different methods: impedance measurement (WIC) and optical counting (WOK) of leukocytes. The WBC values measured with both methods have showed extremely different counts during all storage time. In particular WIK showed an increase in WBC during the storage and WOK a decrease during storage. After the evaluation of some smears obtained from the same PRBC units at D0, D14, D28, D42 we have considered more reliable the laser count (WOC). In fact analyzing blood smears the leukocytes were reduced in number with the passage of time and appeared often as lysed cells (Figure 1).

Statistical analysis of WBC (optical count with laser) showed a significant decrease of WBC between D0 and D14 ($p < 0.0001$), D0 and D28 ($p < 0.0001$), D0 and D42 ($p < 0.0001$), D14 and D28 ($p = 0.0001$), D14 and D42 ($p = 0.0004$), but not significant between D 28 and D42 ($p = 1$) (Graph 5).

Also a statistically significant and positive correlation was evident between Ht and MCV at D0 ($\rho = 0.420$, $p = 0.0150$), at D28 ($\rho = 0.658$, $p < 0.0001$) and at D42 ($\rho = 0.628$, $p = 0.0002$).

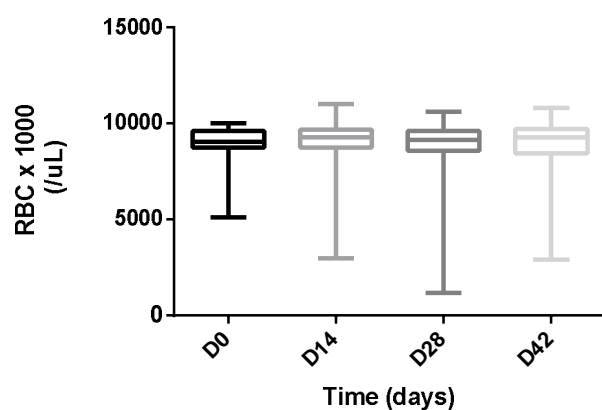
A total of 30/33 units of PRBC were subjected to determination of ammonia concentration at D0, D14, D28, D42. Mean and standard deviation are reported in table n°1. Statistical analysis showed a significant and progressive increase of ammonia during all time points ($p < 0.0001$) (Graph 6).

No PRBC unit of 10 randomly chosen showed bacterial contamination or changes in color at D0, D21 and D42.

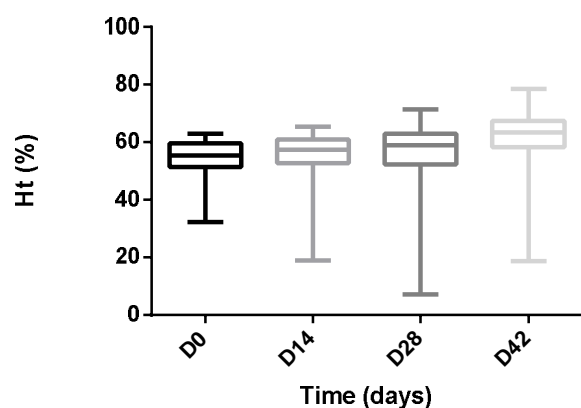
Table 1: RBC, Ht, MCV, RDW, WBC (in 33 PRBC units) and ammonia concentration (in 30 PRBC units) in canine PRBC stored in CPD-SAGM for 42 days. Mean \pm standard deviation

Time points	RBC count (x 10 ³ /μL)	Ht (%)	MCV (fL)	RDW (%)	WBC count (/μL)	NH3 (μg/dL)
D0	9030,6667 \pm 168,6423	54,8667 \pm 1,1069	60,8000 \pm 0,5111	15,6152 \pm 0,2032	11701,5625 \pm 798,7550	39,7296 \pm 6,0139
D14	9074,3333 \pm 251,0002	55,9800 \pm 1,5609	61,9758 \pm 0,5753	15,9364 \pm 0,2005	2928,0938 \pm 423,7766	260,7921 \pm 12,5268
D28	9016,0000 \pm 215,7380	56,7797 \pm 2,3123	64,6879 \pm 0,7599	16,0303 \pm 0,2215	782,6563 \pm 96,0858	342,4568 \pm 16,0945
D42	9002,6667 \pm 250,9462	61,7267 \pm 1,9488	68,1879 \pm 0,8813	17,9030 \pm 0,4375	855,8312 \pm 234,2504	423,8443 \pm 12,6180

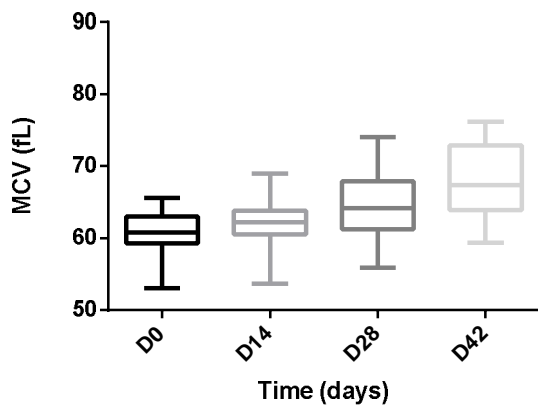
Graph 1: RBC (x 1000/μL) in canine PRBC stored in CPD-SAGM for 42 days.



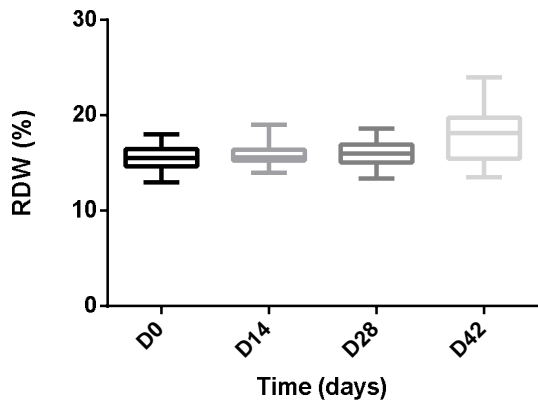
Graph 2: Ht (%) in canine PRBCs stored in CPD-SAGM for 42 days.



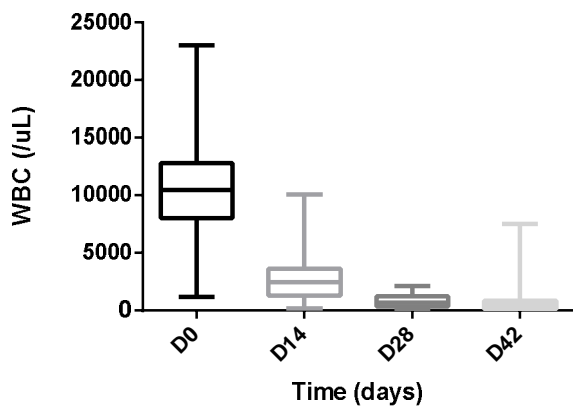
Graph 3: MCV (fL) in canine PRBC stored in CPD-SAGM for 42 days.



Graph 4: RDW (%) in canine PRBC stored in CPD-SAGM for 42 days.



Graph 5: WBC in canine PRBCs stored in CPD-SAGM for 42 days (laser count).



Graph 6: Ammonia concentration in canine PRBCs stored in CPD-SAGM for 42 days

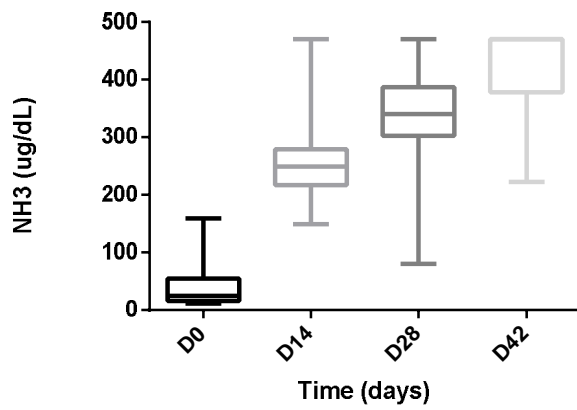
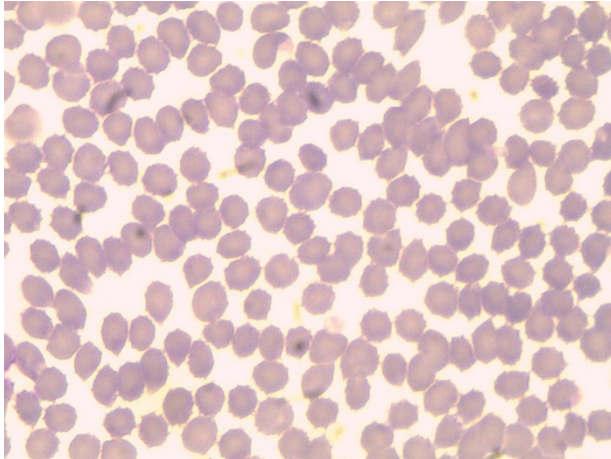
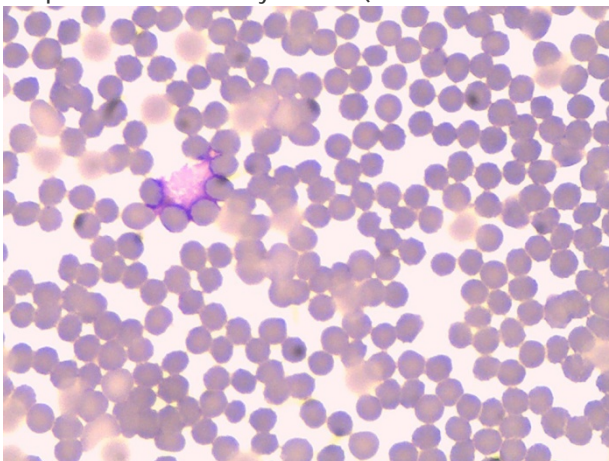


Figure 1: Blood smear of canine PRBC in CPD-SAGM at D0 and D42 (x1000 magnification)
1a: presence of some macrocytes at D0



1b: presence of some lysed cell (red blood cells and one leukocyte) at D42



2.6 Discussion

Our study has investigated the effects of storage in canine PRBC preserved in CPD-SAGM solution for 42 days on some hematological (RBC, Ht, MCV, RDW, WBC) and biochemical (ammonia concentration) parameters. In veterinary medicine few studies have evaluated hematological parameters in stored whole blood or stored PRBC cells (Wardrop et al., 1997, Mudge et al., 2004, Ekiz et al., 2012).

In our study RBCs did not show significant differences during the storage, like reported in canine (Ekiz et al., 2012) and in human studies (Garritsen et al., 2003). Probably this is due to the fact that the majority of red blood cells maintained their integrity during the storage.

All the other hematological parameters evaluated in our study showed significant difference during the storage.

The significant increase of Ht during the storage time is comparable to what reported in a recent study (Ekiz et al., 2012) on canine PRBC stored in SAGM but it is different from what reported from other studies in animals (Wardrop et al., 1997, Mudge et al., 2004). In particular Wardrop et al. (1997) evaluated canine PRBC stored in 2 different solution (CPDA 1 and Nutricel) for 35 days and did not show significant increase of Ht during storage time. Similar data were reported in a study in which Ht of equine blood stored with ACD, CPD and CPDA1 were relatively constant throughout the storage (Mudge et al., 2004). Moreover the mean value of Ht in our PRBC units at D0 (54,86%) is lower respect what reported by Wardrop et al. (1997) in canine PRBC (63% in Nutricel PRBC, 74% in CPDA1PRBC) and higher respect what reported by Ekiz et al. in canine PRBC (2012) (38,89% in SAGM PRBC). The Ht of PRBC units may vary depending on the Ht of the donor, diluting effects of the additive solutions, and the amount of plasma volume in the bag.

In our study also MCV is statistically increased during storage like reported in other studies (Tuner et al. 1987 , Arduini et al., 2007, Ekiz et al., 2012,). In particular Ekiz et al. (2012), showed a significant increase in MCV during storage of canine PRBC with SAGM starting to a MCV of 66,67fL and arrived at D28 to a value of 69.93 fL. In our study the increase in MCV was higher with a difference between D0 and D42 of 7.4fL. A little change in MCV is also reported in human blood stored for 42 days in CPDA1 (Turner et al, 1987). In this study the authors report that this "swelling " phenomenon is presumably due to the accumulation of CPDA1 metabolites and that the incubation of the samples for 24 h in fresh plasma (and presumably reinfusion in vivo) apparently reverses the effects of this secondary cell swelling phenomenon and results in the production of shrunken cells.

Also in another human study conducted by Arduini et al. (2007) erythrocytes preserved in SAGM present an increase of MCV during storage. The MCV

increase is correlated in part to the use of SAGM that produce RBC swelling (Zehnder et al., 2008) and in part it is the consequence of substances products by leukocytes, which are known to affect erythrocyte membrane properties. In fact Ekiz et al. (2012) reported that MCV was lower in leukoreduced canine PRBC units.

However our data is different from what reported by Mudge et al. (2004) in which MCV did not vary consistently with time of storage for equine blood stored with ACD, CPD and CPDA1. The differences of our study with the study conducted by Mudge et al. (2004) is probably due in part to the different species and in part to the different anticoagulant-preservative used.

In our study, Ht and MCV increased steadily during storage and was highlighted a positive correlation between these two parameters. In fact, since RBC remained constant during storage the increase of Ht is correlated to the increase in the volume of red blood cells (MCV). The same correlation was also highlighted by a study of human medicine conducted by Garritsen et al. (2003) on 2 different kind of packed red cells in SAGM.

In our study also RDW value is statistically higher at the end of the storage like reported in Ekiz et al. (2012). This increase during storage might be an indicator of red blood cells changes during storage (Photo 1). In fact the RBC morphology shifted towards echinocytosis during storage (Zehnder et al., 2008). In this case our value of RDW are similar to what reported from Ekiz et al. (2012).

Finally, in our study, WBC (laser count) has undergone a rapid and statistically significant reduction in storage. It is in fact known that the very short duration of life of granulocytes does not allow the storage and transfusion of this product (Kohn et al., 2012). Our data concerning WBC does not agree with those reported by Ekiz et al. (2012). In the study of Ekiz et al. in canine PRBC without leukoreduction WBC count increased from 15930/ μ L at D0 to 16200/ μ L at D35 with a statistically significant difference. However in the study of Ekiz et al. (2012) the type of automatic analyzer used for the counts is not reported. The analyzer used in our study (Cell-Dyn 3500, Plus) allows two methods to count leukocytes, impedance and laser. The first method gave us an increase of WBC during the storage while the second method gave us a decrease of WBC during storage. In our study on the basis of what has been shown by some smears obtained from the PRBC units the laser count has been considered to be closer to reality (characterized by the lysis of the majority of white blood cells during storage). Based on this we can not compare our data with the study of Ezik et al. (2012).

Unfortunately, our automated analyzer did not allow us to quantify correctly the Hb and consequently the parameters calculated from it (MCH and MCHC). Furthermore, the amount of sample remaining after CBC and measurement of

ammonia was not sufficient to measure the free hemoglobin on supernatant after centrifugation. Percentage of hemolysis during storage is an important parameter for the evaluation of the quality of stored whole blood or PRBC units (Arduini et al., 2007, Garretsen et al., 2003) and will be evaluated in further studies.

In our study we also measured the concentration of ammonia that is increased to extremely high concentrations like reported by Waddell et al. (2001) that evaluated ammonia of canine PRBC in CPD-Adsol over the period of 35 days. The ammonia concentration in our study is similar at D0 ($39,73 \pm 6 \mu\text{g/dL}$) and D14 ($260,79 \pm 12,5 \mu\text{g/dL}$) to those reported by Waddell et al. (2001) for PRBC units stored properly (respectively 23 ± 8 and $276 \pm 56 \mu\text{g/dL}$) while at D28 our ammonia concentration result much lower ($343,445 \pm 16,1 \mu\text{g/dL}$ in our study, $466 \pm 30 \mu\text{g/dL}$ in the study of Waddell et al., 2001). This difference is probably due to the different kind of additive solution which have different composition, characteristics and storage times. Finally, the fact that the standard deviation of ammonia concentration present in our study is fairly small (between 6 and 16) is probably due to a correct preservation of the units. In fact, the ammonia is a very sensitive parameter which, as highlighted by Waddell et al. (2001), suffers much of an improper storage. In fact, in the study conducted by Waddell et al. (2001) there is a large difference in ammonia concentration between units of PRBC stored in small refrigerator open frequently (that showed higher ammonia values) and PRBC stored in large blood-banking refrigerator that was not opened more than once a day for the entire 35 days of storage. Unfortunately in our study was not possible to obtain the real final ammonia concentration because our kit arrives to measure a concentration of ammonia maximum of $470 \mu\text{g/dL}$.

Finally, in our study, we also evaluated the possible bacterial contamination of PRBC units. No PRBC unit has showed positive culture at D0, D21 and D42. We decided to evaluate PRBC not only at D0 and D42 but also at D21 because a study estimated that, at storage temperatures of blood bags ($4 \pm 2 \text{ }^\circ\text{C}$), the quantity of bacteria capable of inducing sepsis in the recipient patient is reached around the nineteenth day of storage (Hess, 2004). In the author's knowledge there are no studies about bacterial contamination of canine blood units and in veterinary literature there is only the description of two contaminating bacteria, *Pseudomonas fluorescens* and *Serratia marcescens*. Both cases are reported in feline transfusion medicine characterized by the use of open systems to collected blood (Hohenhaus et al., 1997; Kessler et al., 2010).

Despite the negative result of culture on our PRBC units we believe that is fundamental pay close attention to possible bacterial contamination during all steps of PRBC preparation (evaluation of the donor, blood collection, centrifugation, preparation and storage of the bags and proper storage) and that the bags must be visually evaluating 3-4 times per week looking for changes in

color or texture (signs of probable contamination) to reduce at minimum the risks of contamination.

Based on our results we can say that the blood collection, preparation and storage of PRBC canine units analyzed in our study, provides a final product safe for the absence of bacterial contamination.

As in other species the ammonia concentration increased steadily during storage. In the absence of studies regarding the effect of this alteration in subjects with hepatic disease we avoid transfusion with older units in these subjects. In fact already at D14 of storage most of the units show values of ammonia far above the canine normal value.

As regards the evaluation of hematological parameters the results obtained by us agree mostly with what is reported in canine and human medicine. Like reported in man, dog and horse would be interesting to evaluate the permanence of transfused red blood cells in the recipient at different time of storage and to perform the evaluation of further parameters that characterize the quality of the product like potassium, glucose, pH value, entity and percentage of free hemoglobin, red blood cell hemolysis index entity, content of ATP and 2,3 DPG, molecules essential for cell survival.

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CHAPTER 3

The safety and quality of feline whole blood units collected with an open system and the effect of storage on haematological parameters and ammonia concentration

3. The safety and quality of feline whole blood units collected with an open system and the effect of storage on hematological parameters and ammonia concentration

3.1 Abstract

The veterinary transfusion medicine is constantly in progress but still now feline blood donation, collection, and conservation of whole blood and blood products present some problems. Feline whole blood collected with open system and stored in CPDA1 for 35 days at 4°C was evaluated for hematological parameters, ammonia concentration and sterility during the storage period. Statistical analysis resulted in significant increase in ammonia concentration and decrease of WBC. No other significant changes resulted in hematological parameters (RBC, Ht, MCV, RDW). No units presented bacterial contamination during storage. The use a standardized protocol during blood collection, preparation and storage of feline whole blood permit to obtain a product without microbial contamination, minimum changes in haematological parameters but with a very high ammonia concentration.

3.2 Introduction

The progress recently made in veterinary medicine, particularly in intensive care and emergency medicine, have increased the interest to veterinary transfusion medicine. There are different diseases that can require transfusion, as acute loss of large quantities of blood, coagulation disorders and chronic non-regenerative anemia (Weingart et al., 2004). In canine transfusion medicine it can used whole blood (WB) or blood components: packed red blood cells (PRBC), fresh frozen plasma (FFP) frozen plasma (FP), platelet rich-plasma (PRP) and cryoprecipitate (Lucas et al., 2004). These components permit to optimize every donation from which can be obtain two different components and, at the same time, increase the effectiveness of transfusion therapy and reduced transfusion reactions caused by no necessary blood components.

In feline transfusion medicine, however, is mainly used fresh whole blood or WB. There are different problems related to feline blood donation, collection and conservation of blood products: the frequently need of anesthesia of the donor during blood collection, to use of open collection systems (as is not possible use human blood bag), the difficult to separate whole blood into its components due to the smaller amount of blood that constitutes the sample (unit of whole blood is about 50-60 mL) (Weingart et al., 2004).

The purpose of this study is to evaluate the quality of feline whole blood obtained with an open system, preserved with anticoagulant CPDA-1 (citrate-phosphate-dextrose with adenine) and storage for 35 days at 4°C. Four parameters were chosen as indicators of the quality of the bag at the end of preservation: sterility, ammonia concentration and hematological parameters.

3.3 Materials and methods

All units of whole blood were obtained from owned cats and cats belonging to a closed colony enrolled as donors in the volunteer feline donor program of the Reparto Emotrasfusionale Veterinario (REV) of University of Milan. All cats were healthy large cats (> 5 kg of weight), 2-8 years of age, correctly vaccinated with core vaccinations (feline calicivirus, herpesvirus and panleucopenia). For all cats blood typing (Lab test A+B, Alvedia), clinical visit and screening with complete blood count (CBC), chemistry panel and infectious disease test were performed. The test for infectious diseases were the following: feline leukemia virus antigen and feline immunodeficiency virus antibody (Snap FeLV/FIV Combo Plus Test; Idexx Laboratories, Hoofddorp, the Netherlands) and *Mycoplasma haemofelis* on blood smear. In case of doubt or positivity for *M. haemofelis* at blood smear the result was confirmed submitting an EDTA-blood sample to PCR test. Cats were excluded if any retroviral or PCR test resulted positive or if they had hematological, biochemical or physical abnormalities.

3.3.1 Donor preparation and blood collection

All equipment is prepared and arranged prior to starting the donation procedure. The donation session required the participation of at least 3 people (minimum 2 vets) – one phlebotomist, one adequately trained personnel to restrain and monitor the donor, as well as to assist with handling of the collection equipment during the donation and a third person to aspirate blood and rock the syringe during the collection. All cats are 12 hours fasted and required anaesthesia. Cats were anaesthetised with a combination of tiletamine and zolazepam (Zoletil 100; Virbac, Milan, Italy) at a dose of 12 mg/kg given intramuscularly in the thigh muscles, based on body weight.

A 20-22 G catheter was aseptically placed into the cephalic vein for the purpose of administering saline solution during and after blood collection.

Three 20 ml syringes were loaded immediately before the blood collection with 2,3 ml of CPDA 1 taken every time by a new and closed human single collection bag (Terumo single blood bag, 350 ml with 49 mL di CPDA) to obtain a feline unit of whole blood corresponding to 53 ml of whole blood and 7 ml of CPDA1.

The area over the jugular vein was shaved and prepared aseptically with ethanol (ethanol 94°, ACEF S.p.a) prior to venepuncture and the cat was put in lateral or sternal recumbency.

Blood was collected with a 19G winged infusion set (butterfly) attached to the 20 ml syringe with CPDA1 (open system) (Photo 1). The syringes were gently rocked during blood collection to ensure adequate mixing of blood and anticoagulant. Blood collection was typically completed in 5-10 minutes and vital signs were monitored through the procedure. Routine monitoring during and post anaesthesia was performed. Also the blood pressure was monitored with high definition oscillometry (Memo Diagnostic - High Definition Oscillometry) before, during and after the blood donation. The protocol for the fluid therapy provided subcutaneous infusion of 90 ml of physiological solution (NaCl 0.9%) before the blood collection and intravenous infusion of 60 ml of the same solution starting half way through the donation was performed. (Kohn et al., 2012). After blood donation cat was monitored (mucous membrane colour, pulse rate and strength, respiratory rate) and returned to the owners when fully awake.

3.3.2 Blood bag units preparation

At the end of the donation 1 ml of whole blood was put on a sterile empty test tube maintained at room temperature and another 1 ml in a sterile empty tube maintained at 4°C. The remained blood was transferred to a 150 ml storage bag (Teruflex Transfer Bag) (Photo 2). The whole blood was gently rocked back and forth to ensure adequate mixing. Using a manual tube stripper the blood remaining in the tubing was stripped into the bag, the bag was gently manually mixed and compressed to refill the tubing. This procedure was made for 3 or 4 times to have a correct distribution of whole blood into the tube. The tubing was sealed with a thermal sealers (Hemoweld B-Delcon, Italy) to provides 4 aliquots of about 0.3 ml (tubing 5 cm long) which were used to performed tests (Photo 3).

3.3.3 Whole blood units storage

All bags were labelled with the date, the type and volume of the product, identification number of the donor, blood type, collection and expiration date (Photo 4).

One aliquot of whole blood was taken immediately before the storage to performed laboratory test at day 0 (D0). The bag was then stored in a specialized blood storage refrigerator (Emoteca 250, Fiocchetti, Italy) with built-in temperature alarm at 4°C ± 2°C. The bags was posed horizontally to reduce the risk of damage and to maximize oxygen diffusion in to, and carbon dioxide diffusion out of, the bag. To ensure a uniform distribution of the anticoagulant

solution and increase the viability of red blood cells the bags were manually massaged and gently mixed three - four times per week. During these manualities the bags were submitted to visual inspection of color and texture to search clot or fibrin (signs of possible contamination)

3.3.4 Blood culture

24 hour after the blood collection the 2 samples of blood, one maintained at room temperature and one at 4°C, were subjected to culture. A second aliquot of whole blood was taken at the expiry date (D35) to perform culture and laboratory test.

The blood culture was performed using a modified and adapted standard microbiological procedures (Carter and Wise, 2004). The culture was made on a room temperature sample and a refrigerated sample at D0 and on a refrigerated sample at D35. The double culture at D0 had the objective to verify the presence of bacteria inhibited by refrigeration temperatures. An amount ranging from 200 to 500 μ L of blood was seeded in sterile way in a culture broth (Tryptic Soy Broth, TSB, Oxoid, Italy) in a 1:10 ratio. The tube was then incubated at 37 ° C for 24 hours; if after this period, the broth was clear, with packed red cells on the bottom, the tube was again incubated for 24 hours up to a maximum of 72 hours; over this period the sample was indicated as sterile.

On the contrary in case of turbidity of the culture medium, index of positivity, 100 μ L of sample was transplanted on solid medium agar-blood, which is obtained by adding defibrinated sheep blood to base medium Tryptic Soy Agar (TSA) (Microbiol, Italy). This solid culture medium allows to obtain colonies with the macroscopic morphology typical of the various bacterial species. Then the plate was incubated at 37 ° C also for 24 hours, after which it is proceeded to the identification of bacteria by macro-and microscopic examination (evaluation of macroscopic morphology and execution of the Gram stain), construction of simple biochemical tests and transplantation on selective and differential culture medium (Quinn et al., 2002; Carter et al., 2004; Poli et al., 2005; Jones, 2006). Also visual inspection of color and texture of all blood units was made to search clot or fibrin, signs of a possible contamination.

3.3.5 Ammonia concentration and Hematological Parameters

Before performing tests the tube segment was manually mixing for 1 minutes, after this the sample was taken with a 27 gauge needle and syringe and put in an eppendorf tube. The eppendorf tube was gently mixed 2-3 times before measure ammonia with an automated tool (Ammonia Checker II model AA-4120, Menarini, Italy) and a special test strip (Ammonia test KIT II, Menarini, Italy). The remaining sample was put on a blood rocker for minimum 5 minutes before

performing CBC (Cell-Dyn 3500 PLUS, ABBOTT Diagnostics). The same procedure were made at D0 and D35.

Photo 1: Collection of blood through the jugular vein using three 20 ml syringes pre loaded with anticoagulant CDPA-1



Photo 2: Transfer of collected blood from 3 syringes into a sterile empty storage bag of 150 ml.



Photo 3: Units of feline whole blood at the end of the preparation. Small aliquots use to perform evaluation of hematological parameters, ammonia concentration and blood culture are visible on the top of the bags.



3.4 Statistical Analysis

Data analysis was performed using a commercial statistical software (MedCalc Software, Mariakerke, Belgium). Data were tested for normality using the Shapiro-Wilk. Minimum, maximum, mean and standard deviation (SD) of ammonia concentrations, RBC, WBC, Ht, MCV, RDW were compared using a Student t test. Number and percentage of contaminated bags were reported. A p value < 0.05 was considered statistically significant.

Hemoglobin had not been considered in the statistical analysis because the automated hematology analyzer used in this study (CELL-DYN 3500 PLUS, Abbott Diagnostics) lyses red blood cells to determine hemoglobin concentration so the authors can not distinguishing among the hemoglobin present in the plasma and resulting from the normal in bag hemolysis and the hemoglobin contained inside the red blood cells. Because MCH and MCHC are calculated by the analyzer starting from hemoglobin values also these 2 parameters were excluded from the statistical analyses.

3.5 Results

3.5.1 Blood culture

During the study period (year 2012) a total of 26 units of feline whole blood (53 ml of whole blood and 7 ml of CPDA-1) were obtained and stored. The blood culture was performed in 26 samples at D0 and 25 samples at D35.

A D35 one sample was not subjected to blood culture because of insufficient amount of sample.

All bags resulted without microbial contamination at the beginning (both for the samples stored at room temperature that for samples at refrigeration temperature) and at the end of preservation. No bag showed variations in color and no bag presented fibrin clots and macroscopically detectable.

3.5.2 Ammonia concentration

The ammonia was evaluated in 12 samples at D0 and 16 samples on D35, the results obtained are shown in Table 1. For technical reasons (due to unavailability of the instrument and reagents) in some cases it was not possible to determine the ammonia. It should be noted that the ammonia automated tool used in this study permits to evaluate a maximum ammonia value of 465 µg/dl, and therefore that for 15/16 samples the ammonia concentration obtained is not the true value, but the maximum value detected by the instrument.

There was a statistically significant increase of ammonia concentration during the storage period ($p < 0,0001$) (Graph 1).

3.5.3 Hematological parameters

Complete blood count was performed on 25 samples at D0 and 20 samples at D35 of blood storage. For all samples were investigated the number of red blood cells (RBC), hematocrit (Hct), the red cells distribution width (RDW), the mean cell volume (MCV), and finally the number of white blood cells (WBC). The results obtained are shown in Table 2.

The mean value of RBC, Ht and MCV decrease during the storage without a statistically significant difference (respectively $p = 0,4$; $p = 0,3$; $p = 0,8$) (Graph 2, Graph 3, Graph 4).

The mean RDW showed a little increase during the storage time but with no statistically significant ($p = 0,5$) (Graph 5).

Only WBC count show a statistically difference between D0 and D35 with a drastic reduction of white blood cell in the storage whole blood ($p < 0,0001$) (Graph 6).

Our analyzer (Cell-Dyn 3500 Plus, Abbott Diagnostics) permits to obtain WBC with 2 different methods: impedance measurement (WIC) and optical counting (WOK) of leukocytes. The WBC values measured with both methods have showed extremely different counts during all storage time. In particular WIK

showed an increase in WBC during the storage and WOK a decrease during storage. After the evaluation of some smears obtained from the same unit at D0 and D35 we have considered more reliable the laser count (WOC).

Table 1: Ammonia values obtained in feline whole blood bags at D0 and D35.

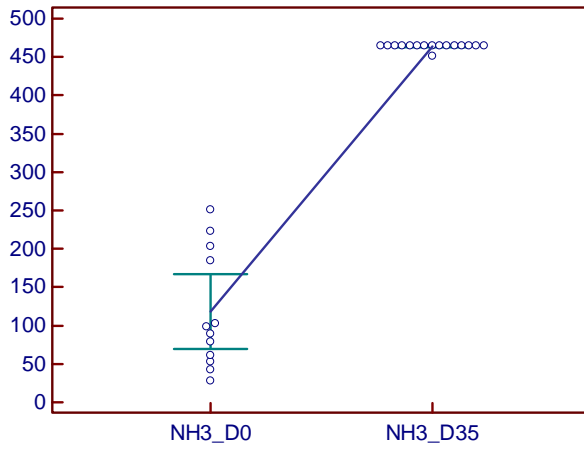
Whole Blood Bag	NH3 D0	NH3 D35
	µg/dL	
G08/12	28,23	465
G09/12	ND	465
G10/12	ND	465
G12/12	223,5	465
G13/12	184,7	465
G14/12	42,35	465
G15/12	78,82	465
G16/12	89,41	465
G17/12	61,17	ND
G18/12	102,35	465
G19/12	52,94	465
G21/12	ND	465
G22/12	ND	451,76
G23/12	ND	465
G30/12	250,6	465
G08/12	99	465
G32/12	203	465
Mean± SD	118,2 ±76,6	464,2 ±3,3
MIN –MAX	28,3 250,6	451,8 465
p value	<0,0001	

NH3 = ammonia, ND = not determined (for technical reasons).

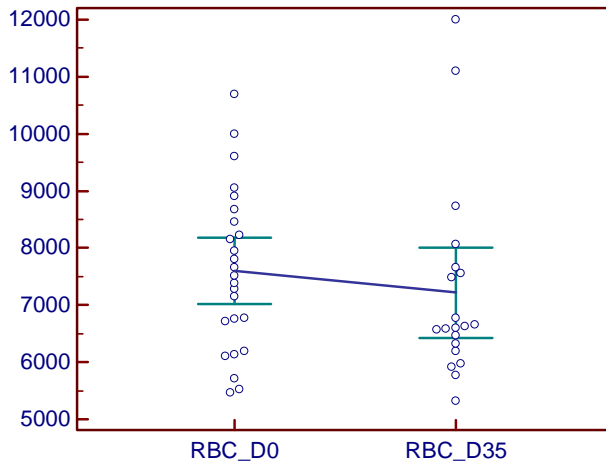
Table 2: RBC, Hct, RDW, MCV and WBC obtain from 26 units of feline whole blood ad D0 and D35.

	RBC D0	RBC D 35	HCT D0	HCT D35	RDW D0	RDW D 35	MCV D0	MCV D 35	WBC D0	WBC D 35
SACCA	X 10 ³ /μL		%		%		fL		/μL	
G07/11	7800	5320	29,3	18,1	20,8	21,9	37,6	34	10800	1480
G08/11	7150	6190	28,9	24,6	17,4	16,9	40,4	39,8	11200	1200
G01/12	7660	ND	32,1	ND	17,3	ND	41,9	ND	6570	ND
G02/12	8150	ND	29,3	ND	20,4	ND	36	ND	12500	ND
G03/12	8910	ND	35,6	ND	18,6	ND	40	ND	17300	ND
G04/12	6760	ND	21,9	ND	23,5	ND	32,4	ND	6300	ND
G05/12	8680	6570	36	26,4	18	17,1	41,5	40,2	6510	559
G06/12	8450	8730	31,6	32,2	18,9	21,7	37,4	36,9	8100	833
G07/12	9050	6660	35,8	26	17,9	17,5	39,6	39,1	6140	1020
G08/12	9610	6320	36,2	24,8	16,1	17,2	37,7	39,2	13400	1320
G09/12	ND	7660	ND	30,7	ND	19	ND	40,1	ND	435
G10/12	6190	5980	23,5	22,9	18,4	18,4	38	38,4	9070	4030
G11/12	7950	ND	35,3	ND	17,4	ND	44,4	ND	18000	ND
G12/12	10000	7560	38,4	29,3	19,6	17,6	38,4	38,7	8720	608
G13/12	7510	5910	29,9	23,4	18,2	17	39,8	39,5	5070	1110
G14/12	7380	8070	27,5	29,9	18,5	18,7	37,3	37,1	7500	1340
G15/12	8230	12000	29,8	41,8	19,4	23,9	36,2	34,7	13400	822
G16/12	6130	6620	24,5	25,9	16	17,9	40	39,1	10400	370
G17/12	6710	ND	27,3	ND	16,6	ND	40,7	ND	8890	ND
G18/12	5470	ND	20,6	ND	18	ND	37,6	ND	15200	ND
G19/12	5710	6460	21,8	25,2	15,5	17,5	38,2	39	7620	483
G21/12	6110	6600	23,8	28,6	17,5	17,9	38,9	43,3	7980	931
G22/12	7280	7490	26	26,6	19,3	22,1	35,7	35,5	11600	763
G23/12	5530	5770	20,6	22,2	18,3	18,8	37,3	38,5	9710	2320
G30/12	10700	11100	44,6	43,5	17,6	19	41,8	39,2	5010	997
G08/12	ND	6770	ND	25,8	ND	17,5	ND	38,2	ND	209
G32/12	6770	6590	27,5	27,7	17,5	15,9	40,7	42	3550	123
Mean± SD	7595,6	7218,5	29,5	27,8	18,3	18,7	38,8	38,6	9621,6	1047,6
	±1423,2	±1784,3	±6,2	±6,0	±1,7	±2,1	±2,5	±2,2	±3631,3	±860,4
MIN - MAX	5470 - 10700	5320 - 12000	20,6 - 44,6	18,1 - 43,5	15,5 - 23,5	15,9 - 23,9	32,4 - 44,4	34 - 43,3	3550 - 18000	123 - 4030
p value	0,4		0,3		0,5		0,8		<0,0001	

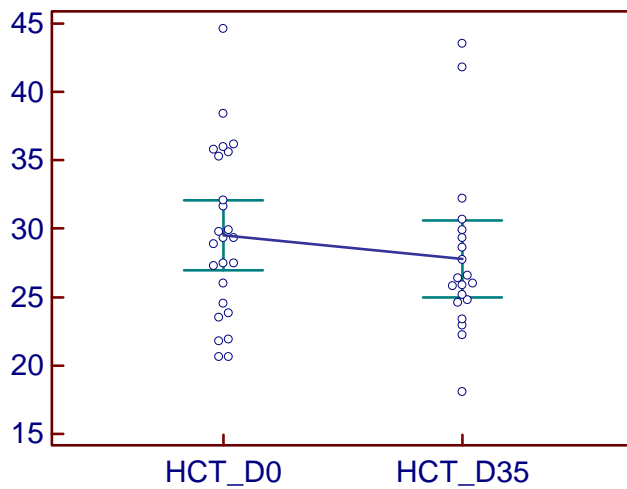
Graph 1: ammonia concentration in bags of feline whole blood with CPDA1 stored for 35 days. Ammonia values are expressed in $\mu\text{g}/\text{dL}$.



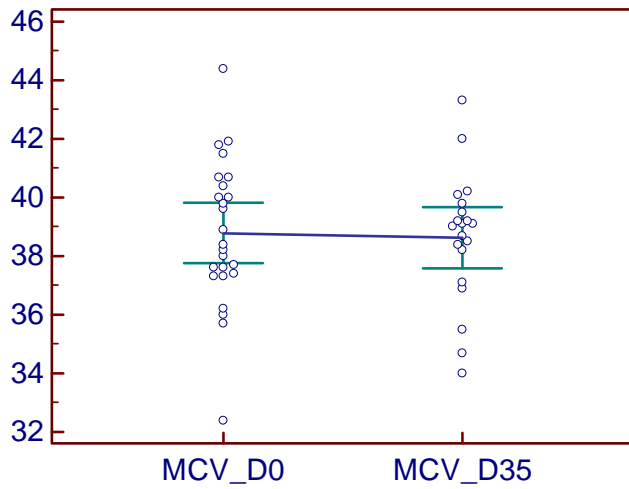
Graph 2: RBC in bags of feline blood with CPDA1 stored for 35 days (RBC $\times 10^3/\mu\text{L}$).



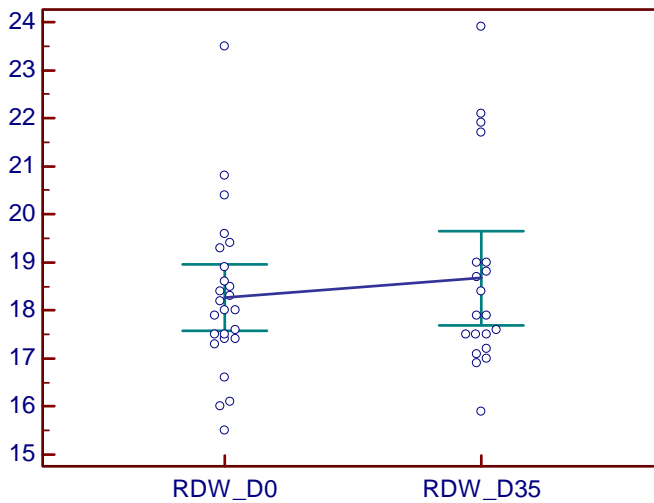
Graph 3: Hematocrit in bags of feline blood with CPDA1 stored for 35 days (%).



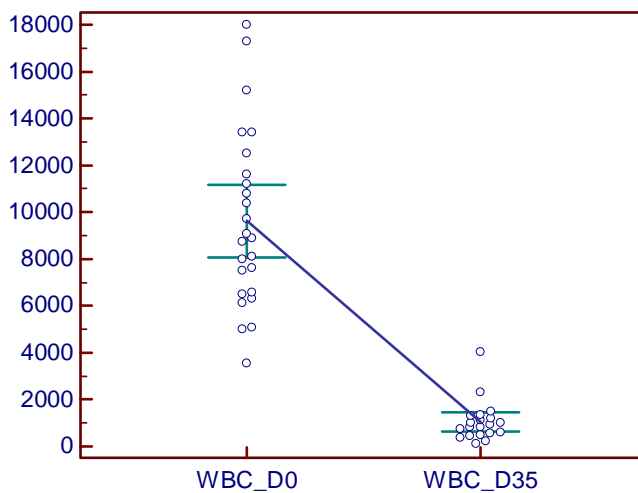
Graph 4: MCV in bags of feline blood with CPDA1 stored for 35 days (fL)



Graph 5: RDW in bags of feline blood with CPDA1 stored for 35 days (%)



Graph 6: WBC in bags of feline blood with CPDA1 stored for 35 days (μ L)



3.6 Discussion

All bags of feline whole blood in our study were visually checked 2-3 times a week in order to highlight possible alteration of color, presence of hemolysis or turbidity of the plasma fraction, presence of clots or fibrin, all signs of possible bacterial contamination (Kessler et al., 2010). The ocular inspection carried out on our bags stored did not detect any macroscopic alteration. The bags of stored feline whole blood of our study were also analyzed for bacteriological research at D0 on a sample maintained at room temperature and on a refrigerated sample and also at D35 on a stored sample. All samples submitted to culture result negative for bacterial contamination.

In human medicine, 1/2000 unit of blood bag collected is contaminated (Yomtovian et al., 2006), but fortunately, thanks to storage at low temperatures, many of these bacteria do not survive during storage (Hess, 2010). There are numerous studies in human medicine that described the presence of bacterial colonies on stored blood (Black et al., 1967; Blajchman et al., 1979; D'Antonio et al., 2002; Sharma et al., 2004; Blajchman et al., 2005; O'Brien et al., 200). The bacteria involved in the contamination of human blood products are numerous, the main and most frequent among the Gram-positive bacteria are staphylococci, streptococci, *Clostridium perfringens* and *Bacillus cereus*; among Gram-negative bacteria are mainly represented by *Yersinia enterocolitica*, *Serratia marcescens* and *Serratia liquefaciens* (Hogman et al., 1998).

The veterinary literature reports only the description of two contaminating bacteria, *Pseudomonas fluorescens* and *Serratia marcescens*, both cases are reported in the context of transfusion feline medicine (Hohenhaus et al., 1997; Kessler et al., 2010). This demonstrates that the open system use in feline blood collection is an important risk factor for bacterial contamination.

We decided to analyze blood units at D0 and D35 because blood is an excellent microbial growth medium and the amount of bacterial colonies present at D0 may increase during storage (Hohenhaus et al., 1997; D'Antonio et al., 2002). In fact analyzing the bag only on D0 the amount of bacteria present could be less than the detection capability of the method used for the bacteriological investigation (Kessler et al., 2010), and a bag can be incorrectly labeled as "sterile". It is also known that the risk of microbial contamination increases during the conservation and it reaches its peak in the nearest days to maturity of the product (Hogman et al., 1998). It is estimated that, at storage temperatures of blood bags, the quantity of bacteria capable of inducing sepsis in the recipient patient is reached around the nineteenth day of storage (Hess, 2004).

The results of our study show that, although operating with an open system, it is possible to obtain a totality of blood units without bacterial contamination both at the beginning and at the end of storage period.

This result was achieved thanks to a scrupulous collecting system and to a high attention to eliminate or limiting all possible sources of contamination (for example skin flora) (Scott, 1984; Muller et al. 2001). It is also important to maintain for all blood collections a standardized procedural scheme to ensure uniformity of results. In fact a study shows that only thanks to the repeatability of the work we can be able to find the source of contamination (Hohenhaus et al., 1997).

The literature provides conflicting data regarding the percentage of contamination of the blood bags (Engelfriet et al., 2000) and is even more difficult to get statistics in veterinary medicine because fewer studies have been done about it.

For this reason is difficult to determine whether the result on bacterial contamination in our study really reflects the general situation or if it represents our reality only.

The ammonia concentration in units of feline whole blood in our study was assessed on D0 few hours after blood donation and at D35. Our result detects that the ammonia concentration grows during the period of storage. In fact there is a statistically significant difference ($p < 0.0001$) between the means of the samples on D0 (118 $\mu\text{g/dL}$) and D35 (464.2 $\mu\text{g/dL}$). The values obtained in D35 have reached in 15/16 samples the maximum value detected by the instrumentation. We can therefore say that the ammonia concentration on D35 is greater than that detected, even if it is not possible to know the exact magnitude of the final ammonia value.

The scientific literature about feline medicine report few studies about ammonia concentration. A work of Ogilvie et al. (1985) measured the ammonia concentration in feline plasma stored at -20°C for 9 days. This study shows a statistically significant difference between the values measured at day 0 and at day 9, showing an increase of the value of 13 times. However, the different storage temperature, the different sample and the different time interval makes it difficult to make a direct comparison between the 2 studies. In addition to this work, there are also studies, more similar for time and mode of observation to our study conducted in human medicine both on whole blood and PRBC and in canine medicine to PRBC which results are perfectly integrated with data obtained from our study (Prytz et al., 1970; Waddell et al., 2001). In fact, even in these works, is highlighting a statistically significant difference in ammonia at the start and at the end of storage.

On D0 of our study the ammonia concentration show a large SD that indicates the wide range of values of baseline ammonia concentration. The measurements, in fact, vary from a minimum of 28 $\mu\text{g/dl}$ to a maximum of 251 $\mu\text{g/dl}$ with a SD of 76.6 $\mu\text{g/dl}$. This result has also been seen in studies of human medicine (Prytz et al., 1970) and demonstrate how this parameter is sensitive to any variation in

sample preparation. Furthermore this variability of baseline ammonia to D0 could reflect a different concentration of ammonia in the blood of the donor, that evaluation is not performed in our study.

The study of Waddell et al. (2001) on the evaluation of ammonia in canine unit of PRBC performs measurements on days 0, 7, 14, 21, 28 and 35 of storage showing a linear correlation between increased concentration of ammonia and storage time. The above cited work of canine medicine has been carried out on samples of PRBC, while the samples of our study come from whole blood. However it is possible a comparison between these two studies since it has been demonstrated that the increase of ammonia, which takes place in whole blood, occurs almost entirely by the red blood cells (Conn, 1966). In fact, similar results are obtained by measuring the concentration of ammonia in human whole blood and PRBC. (Prytz et al., 1970; Waddell et al., 2001).

All whole blood units of our study were evaluated at the beginning and at the end of the storage for the following blood parameters: RBC, Ht, MCV, RDW, WBC. The statistical analyzes performed on the considered parameters showed a substantial stability of the parameters during the storage in anticoagulant CPDA-1 for 35 days.

The red cell distribution width (RDW) is the only parameter to be increased, slightly, during storage. RDW rises from 18.3% at D0 to 18.7% to D35. This data shows a slight increase of the variability in the size of red blood cells. These results are similar to what is shown in a study of Niinistö et al. (2008) where no statistically significant difference was detected in the cell count at D0 and D35 in equine red blood cells stored in SAG-Mannitol.

The analysis of white blood cells at day 0 and on day 35 instead shows a marked decrease in the number. Leukocytes pass from a mean value of 9621.6/ μL at D0 to 1047.6/ μL at the end of storage, with a highly statistically significant difference ($p < 0.0001$). The analysis of blood parameters has rarely reported in the scientific literature, and to the authors knowledge there are no studies about feline species. However, it is possible to draw some conclusions from human medicine. Interesting is the comparison with the work of human medicine of Garritsen et al. (2003) in which were analyzed the differences of blood parameters in units of packed cells obtained from placental blood after 35 days of storage in SAG-Mannitol. From this study there is a substantial stability in RBC but a statistically significant difference between Ht at D0 and D35 ($p < 0.05$) (Garritsen et al. 2003). The difference with our study may be due both by the different preservative solution and the diverse species.

Red blood cell indices are studied also by Turner et al. in 1987. In this study human packed red blood cells shows a stability during the 42 days of observation with regard to the cell volume and the average concentration of hemoglobin; however, the authors attribute this result not so much to a real cell stability

during storage but to a "masking" effect of CPDA-1 solution. In fact, if the RBC stored for more than 21 days are introduced into a solution of fresh plasma at 37 ° C, situation that better mimics the placing in the circulation for 24 hours, it can notice a change in cells characteristics with a statistically significant decrease in cell volume and a significant increase of the average concentration of hemoglobin (Turner et al., 1987). The statistically significant decrease of WBC occurred during the storage reflects the results obtained in other studies. From the literature it emerges that in feline species the amount of WBC in stored blood is significantly decreased ($p < 0.01$) already after the first 72 hours of storage (Grenn et al, 1976) and in human medicine after 35 days of storage the WBC comes to be equal to 0 (Garritsen et al, 2003). These results, however, do not come unexpected. It is in fact known that the very short duration of life of granulocytes does not allow the storage and transfusion of this product (Kohn et al., 2012).

Analysis of the literature and the results obtained in our study probably reveal a good stability during storage of red blood cells, even if is not clear if this stability is real or only apparent. Much of this stability is probably attributable to the type of anticoagulant used, CPDA-1. This preservative and anticoagulant solution contains dextrose which is a substrate for glycolysis in red blood cells, citrate which prevents coagulation by inhibiting the calcium-dependent coagulation cascade, and phosphate which results in a high production of ATP during storage. Adenine finally improves ATP synthesis of red blood cells. In fact erythrocytes can not synthesize adenine, but they can use it together with glucose to form ATP and AMP. For all these features the CPDA-1 is the anticoagulant-preservative of choice even in feline transfusion medicine (Kohn et al., 2012). In conclusion the open system used in this study to collect feline whole blood with CPDA1 permits to stored feline blood for 35 days without bacterial contamination, with few changes of hematological parameters but with an high concentration of ammonia.

3.7 References

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CHAPTER 4

Preliminary Evaluation on the Stability of Protein in Bovine Fresh Frozen Plasma

4. Preliminary Evaluation on the Stability of Protein in Bovine Fresh Frozen Plasma

4.1 Abstract

The aim of this study were to evaluating preliminary stability of glucose, urea, total protein and protein fractions in bovine fresh frozen plasma (FFP). Blood was collected into human sterile double-pack blood collection system containing citrate-phosphate-dextrose-adenine (CPDA) and after centrifugation the plasma units were stored within 8 hours from blood collection at – 19°C obtaining FFP. The analysis of biochemical parameters were performed on fresh plasma after centrifugation of whole blood unit and on thawed FFP after 1 and 6 months of storage. Pre and post storage results were compared using a one way repeated measures ANOVA. Seven FFP were obtained from 7 different whole blood units. There was no significant changes in the concentrations of glucose, urea, total protein and protein fraction during the entire period of storage. This preliminary study showed that during 6 months of storage no significant changes were appeared in the evaluate biochemical parameters in bovine FFP.

The development of blood banking and component processing in veterinary medicine has resulted in more frequent use of blood components like Packed Red Blood Cells (PRBC) and Fresh Frozen Plasma (FFP). In particular plasma is most commonly used for the proteins it contains, such as albumin, immunoglobulins, and hemostatic proteins particularly in human (Benjamin et al., 2012) and canine medicine (Gibson et al., 2012). FFP is indicated for use in animal with acquired or inherited coagulopathies (inherited factor deficiencies, vitamin K deficiency or antagonism, disseminated intravascular coagulation, severe liver disease), with hypoproteinemia (Gibson et al., 2012) or to give passive transfer of immunoglobulin (Prettie et al., 2004, Green, 2012)

Even if bovine blood and blood products are often used for the production of biopharmaceuticals (e.g. Vaccines) sometimes also in bovine medicine is necessary for the practitioner use whole blood or blood product. In literature are reported single cases in which fresh whole blood transfusion has been used for particular disease like a bovine neonatal pancytopenia (Bernier Gosselin et al., 201) or idiopathic immune-mediated anemia (Lallemant et al., 2006). Others studies report the use of bovine plasma or hyperimmune bovine plasma in newborn calves for the management of failure of passive transfer (Turgut et al., 1996, Selim et al., 1995).

In bovine species blood collection and donation can be difficult to perform in practice, are time-consuming, expensive, and stressful to the animal (Hunt et al., 1999) but in contrast to the whole blood from which it is derived, plasma can be stored frozen, making it a convenient product to keep in most veterinary hospitals or veterinary blood bank. To be labeled as "FFP," the plasma must be placed in a -18°C or lower freezer within 6 to 8 hours of phlebotomy (Benjamin et al., 2012) . Maximum storage times for animals have been adapted from studies on human plasma. Based on it FFP maintains for 1 year all the components of a fresh plasma (albumin, globulin, hemostatic factors) even if a canine study (Wardrop et al., 2001) report statistically significant changes in some coagulation factor during the storage.

The aim of this study is to assess the stability of glucose, urea, total protein, albumin and globulins in bovine FFP obtained from centrifugation of whole blood in CPDA and stored within 6-8 hours from phlebotomy at -19°C.

The plasma was obtained at the Reparto Emotrasfusionale Veterinario (REV) of Faculty of Milan from bovine whole blood collected using human sterile double-pack blood collection system (CPDA double bag, 450 ml, Grifols, Barcelona, Spain) To obtain FFP all samples were submitted with 8 hours of blood collection to centrifugation and separation using a blood centrifuge (Rotixa 50 RS – Hettich, Germany).

The blood centrifuge was set at a temperature of 4° C, 3500 RPM with initial acceleration (run up 9) and free deceleration (run down 0) for 15 minute and it had permitted to obtain a clear bovine plasma without red blood cell contamination.

Plasma was separated from RBCs using a manual plasma extractor (Separation stand Teruflex ACS-201). When the plasma had been extract almost completely the tubing just above the plasma bag was clamped. The satellite bag containing plasma and the anticoagulant was separated from the other bag (containing mainly red blood cells) with the use of thermal sealer. The tubing was sealed with the thermal sealers to provides 4 aliquots of about 2 ml.

One aliquot of fresh plasma was taken immediately before the storage to performed all tests. Fresh plasma was submitted to determination of glucose, urea, total protein (TP) and electrophoretic separation using agarose gel (AGE) to obtain the protein fractions and albumin. The glucose was quantified with a enzymatic colorimetric determination (GOP-POD-PAP), urea with UV kinetic method, TP with a spectrophotometric determination using a Biuret Method on an automatic system for blood chemistry (Cobas Mira Classics Roche). The protein fractions were evaluated in concentration (g/dl) and also for percentage (determination of peak-area of the electrophoresis scanning pattern). Also albumin to globulin ratio (A/G) was calculated. AGE of plasma samples was

performed using a semi-automated agarose gel electrophoresis system (Hydrasis, Sebia PN 1210, Issy-les-Molineaux, France).

The bags and the aliquots of fresh plasma were immediately stored in a specialized blood storage freezer with built-in temperature alarm at -19°C . to obtain FFP. After 1 and 6 months of storage one aliquot of FFP was thawed at room temperature and submitted to the evaluation of the same parameters evaluated on the fresh aliquot at day 0.

Data analysis was performed using a commercial statistical software (MedCalc Software, Mariakerke, Belgium). Mean glucose, urea, TP, AGE and A/G at each sampling point were compared using a one way repeated measures ANOVA. Test significance was set at $P < 0.05$

A total of seven fresh frozen plasma units obtain from 7 different whole blood units were subjected to evaluation of glucose, urea and proteins at D0, before storage, and after 1 and 6 months of storage.

The values registered in each sample for all evaluated parameters, the mean values and standard deviation of pre- and post-storage samples are reported in table 1 and table 2.

The ANOVA analysis of all parameters evaluated do not show statistically significant modification during the storage period (all $p > 0.05$).

The results of this study represent a preliminary findings in the stability of plasma components in bovine FFP. Data obtain on bovine FFP in a 6 months period storage seems to demonstrate that glucose, urea and plasma proteins are stable during storage at -19°C .

Stability of serum and plasma protein after storage has been reported in the veterinary literature (Thorensen et al., 1995, Reynolds et al., 2006, Cray et al., 2009, Yaxley et al., 2010) while significant differences between pre- and post-storage values has been noted in canine FFP for coagulation factors VIII, IX, and X, and vWf (Wardrop et al., 2001). Based on our preliminary results, it can be assumed that stored bovine FFP could be used in bovine medicine for example in case of necessity for the transfer of passive immunity in calves that have not taken properly colostrum.

The present study does have some limitations. The first is the small number of samples evaluated, the second the short evaluated storage time. Moreover our study has evaluated only glucose, urea, total protein and protein fraction without evaluating the others important constituents of plasma like coagulant factors.

However, additional studies are needed to gain more information about the stability of bovine FFP evaluating longer freeze periods and other plasmatic factors.

Table 1: Total protein concentration (biuret method), concentration and percentage of albumin, α_1 , α_2 , β_1 , β_2 , and γ globulin fractions (agarose gel electrophoresis) and calculated albumin/globulin (A/G) ratios in fresh plasma samples (D0) and fresh frozen plasma samples (D1 and D6) stored and then thawed from 7 different bovine FFP.

Sample	TP	TP	TP	Alb	Alb	Alb	Alb	Alb	Alb	α 1	α 1	α 1	α 2	α 2	α 2	β 1	β 1	β 1	β 2	β 2	β 2	γ	γ EF	γ EF
	D0 fresh	D1 frozen	D6 frozen	g/dl	g/dl	g/l	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl	EF g/dl
1	7,5	8	8,8	2,9	3,2	2,9	2,84	2,8	3,23	0,27	0,31	0,33	0,41	0,44	0,5	0,28	0,3	0,34	0,44	0,5	0,53	3,27	3,66	3,86
2	7,4	7,8	8,1	3,3	3,3	3,1	3,13	3,16	3,39	0,16	0,2	0,28	0,33	0,49	0,37	0,26	0,32	0,3	0,51	0,64	0,57	3,02	2,99	3,19
3	7,5	8,6	7,6	3,5	3,5	3,2	3,18	3,72	3,15	0,25	0,29	0,24	0,37	0,33	0,34	0,26	0,28	0,28	0,53	0,65	0,62	2,92	3,4	2,97
4	7	7,2	6,7	3,5	3,5	3,3	2,78	3,82	2,97	0,41	0,35	0,25	0,58	0,34	0,68	0,32	0,32	0,58	1,02	0,62	0,81	1,9	2,46	1,4
5	7,2	7,9	7,5	3,5	3,6	3,7	3,28	3,89	3,39	0,27	0,28	0,29	0,37	0,31	0,68	0,28	0,29	0,67	0,55	0,29	0,98	2,45	2,54	1,5
6	6,9	6,7	7,1	3,4	3,5	3,5	3,06	3,26	3,29	0,27	0,26	0,22	0,36	0,3	0,37	0,28	0,27	0,26	0,55	0,5	0,56	2,39	2,3	2,4
7	7	6,7	6,8	2,8	2,8	3	2,59	2,59	2,45	0,27	0,25	0,24	0,36	0,32	0,32	0,27	0,25	0,22	0,59	0,6	0,49	2,93	2,7	3,08
Mean	7,21	7,56	7,51	3,27	3,34	3,24	2,98	3,32	3,12	0,27	0,27	0,26	0,4	0,36	0,47	0,28	0,29	0,38	0,6	0,54	0,65	2,7	2,86	2,63
SD	0,1	0,27	0,28	0,11	0,10	0,11	0,1	0,2	0,12	0,028	0,018	0,014	0,03	0,03	0,06	0,01	0,01	0,1	0,08	0,05	0,07	0,18	0,19	0,34

Sample	Alb % EF	Alb % EF	Alb % EF	α 1 % EF	α 1 % EF	α 1 % EF	α 2 % EF	α 2 % EF	α 2 % EF	β 1 % EF	β 1 % EF	β 1 % EF	β 2 % EF	β 2 % EF	β 2 % EF	γ % EF	γ % EF	γ % EF	A/G	A/G	A/G
	D0	D1	D6	D0	D1	D6	D0	D1	D6	D0	D1	D6	D0	D1	D6	D0	D1	D6	D0	D1	D6
	fresh	frozen	frozen	fresh	frozen	frozen	Fresh	frozen	frozen	fresh	frozen	frozen	fresh	frozen	frozen	fresh	Frozen	frozen	fresh	frozen	frozen
1	37,9	35	36,7	3,6	3,9	3,8	5,4	5,5	5,7	3,7	3,7	3,9	5,8	6,2	6	43,6	45,7	43,9	0,61	0,54	0,58
2	42,2	40,5	41,8	2,1	2,6	3,5	4,5	6,3	4,6	3,5	4,1	3,7	6,9	8,2	7	40,8	38,3	39,4	0,73	0,68	0,72
3	42,4	43,2	41,5	3,3	3,4	3,1	4,9	3,8	4,5	3,5	3,3	3,7	7	7,5	8,1	38,9	38,8	39,1	0,74	0,76	0,71
4	39,7	48,3	44,3	5,8	4,4	3,8	8,3	4,3	10,2	4,6	4	8,7	14,5	7,9	12,1	27,1	31,1	20,9	0,66	0,93	0,8
5	45,5	49,2	45,2	3,8	3,5	3,8	5,2	3,9	9,1	3,9	3,7	8,9	7,6	7,5	13	34	32,2	20	0,83	0,97	0,82
6	44,4	47,3	46,4	3,9	3,7	3,1	5,2	4,4	5,2	4	3,9	3,6	7,9	7,3	7,9	34,6	33,4	33,8	0,8	0,9	0,87
7	37	38,6	36	3,9	3,7	3,5	5,1	4,8	4,7	3,8	3,7	3,3	8,4	8,9	7,2	41,8	40,3	45,3	0,59	0,63	0,56
Mean	41,3	43,16	41,7	3,77	3,6	3,51	5,51	4,71	6,29	3,86	3,77	5,11	8,3	7,64	8,76	37,26	37,11	34,63	0,71	0,77	0,72
SD	1,21	2	1,53	1,1	0,55	0,31	0,48	0,34	0,89	0,14	0,1	0,95	1,1	0,32	1	2,16	1,96	3,92	0,03	0,06	0,04

Table 2: Glucose and urea concentration in fresh plasma samples (D0) and fresh frozen plasma samples (D1 and D6) stored and then thawed from 7 different bovine FFP.

Sample	Glu	Glu	Glu	Urea	Urea	Urea
	D0 fresh	D1 frozen	D6 frozen	D0 fresh	D1 frozen	D6 frozen
1	339	455	430	30	32	28
2	645	535	585	39	42	39
3	253	430	465	31	28	26
4	860	495	445	23	20	22
5	525	445	510	32	25	23
6	525	510	580	25	25	25
7	550	440	550	24	22	25
Mean	528.1429	472.8571	509.2857	29.1429	27.7143	26.8571
SD	74.9223	15.2307	24.2857	2.1317	2.8006	2.1539

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CHAPTER 5

Foreword: No-Transfusion Blood Components And Platelet Rich Plasma

5. Foreword: No-Transfusion blood components and Platelet-Rich Plasma

The use of non-transfusion blood components starts in human medicine in the 1998 with the first publication of Marx et al. about the use of platelet concentrate (also called platelet rich plasma) in odontoiatric medicine. In the years a lot of production methods and different application are been proposed in various spheres of Medicine and Surgery.

The most important non-transfusion blood component is the Platelet Rich Plasma (PRP).

PRP is an autologous concentration of "human" platelets in a small volume of plasma. Because it is a concentration of platelets, it is also a concentration of the 7 fundamental protein growth factors (GF) proves to be actively secreted by platelets to initiate all wound healing. These GF include the 3 isomeres of platelet-derives growth factors (PDGF $\alpha\alpha$, PDGF $\beta\beta$, PDGF $\alpha\beta$), 2 of the numerous transforming growth factors β (TGF β 1, TGF β 2), vascular endothelial growth factor and epithelial growth factor. Because these platelets are concentrated in a small amount of plasma it also contains extra-platelets component that also contribute to regeneration. In particular fibrin, fibronectin and vitronection, proteins know to act as cell adhesion molecules for osteoconduction and as a matrix for bone, connective tissue and epithelial migration (Marx et al., 2004).

For these reasons the rational for the therapeutic use of platelet is to make platelet-derived factors locally available for tissue to be healed, to induce or to accelerate the healing process (Mazzucco et al., 2009).

For definition platelet concentration in PRP is above baseline whole blood platelet concentration (200000 platelet/ μ L in human medicine). Platelets are natural providers of platelet derived factor therefore PRP is a concentration with the "right" factors in the "right" proportions necessary for the healing process. The human arbitrary target of platelet concentration of 800000-1000000 platelets/ μ L that is three to six folds platelet concentration in whole blood has been proved as clinically effective by different studies, both in vitro that in vivo (Marx et al., 2004, Mazzucco et al., 2009). Because most individuals have a baseline blood platelet count of 200000 \pm 75000/ μ L a platelet count of 1 million/ μ L as measured in the standard 6 ml aliquot has become the human benchmark for "therapeutic PRP" (Marx et al., 2004).

The SIMTI (Società Italiana di Medicina trasfusionale e Immunoematologia) reported in the "Raccomandazioni SIMTI sugli emocomponenti per uso non trasfusionale" that PRP has a specific platelet concentration in a variable volume of plasma depending from method of production and use. For the SIMTI for

definition PRP contain 1 million platelet/ $\mu\text{L} \pm 20\%$ while the concentration of red and white blood cells depending to the preparation method.

Studies have shown that an "excess" of platelet concentration does not necessarily determine a greater cellular response but paradoxically an inhibitory effect (Weibrich et al., 2004).

The technique of preparation of PRP can be divided into four phases: collection, adding anticoagulant, centrifugation and product activation.

The original technique of Marx et al. (1998) included the presence of specialized technicians, a blood collection of 400-500 ml in CPD performed by central venous catheter, a cell separator (type Eletromedics 500 of Medtronic) that, using a gradient of density collects and concentrates platelets during surgery. Such machine by 2 centrifugations allowed to obtain platelet-poor plasma (PPP, subsequently reinfused to the patient), platelet rich plasma (PRP) corresponding to the intermediate layer rich in platelets and white blood cells and a layer of red blood cells (packed red blood cells, PRBC, subsequently reinfused into the patient). Marx has also observed that the upper layer of the PRBC band (1-3 mm) contains the youngest and largest platelets. So in PRP the first mm of the erythrocyte fraction has started to be include. Then considering the difficulties and risks associated with a so great blood collection the original technique has been modified (maintaining the principles of sedimentation and division of whole blood in PPP, PRP and PRBC) until to obtain PRP starting to a smaller whole blood quantitative (5-40ml) (Pacifci et al., 2002, Graziani et al., 2007).

Actually the PRP can be prepared from blood donation using a specific blood collection system, from apheresis, from blood sample using a specific device (commercial kit) or using a manual procedure (home made). Several commercially available methods to obtained PRP concentrate are currently used in the clinical setting and there are many types of kits, centrifuges, and vials available.

There is still no universally accepted protocol for the production of PRP (Graziani et al., 2007). In literature are reported several discrepancies regarding not only the speed but also the number of centrifugations (1 or 2).

Various kinds of anticoagulant are use in PRP preparation: heparin, citrate, acid citrate dextrose (ACD), citrate-theophylline-adenosine-dipyridamole (CTAD) (Lei et al., 2009). It is important to seek an appropriate anticoagulant to avoid spontaneous activation in samples, in fact if platelets are activated during the PRP production process, growth factors in platelets will be released into plasma most of which is discarded during PRP preparation, which consequently decreases the efficacy of PRP when applied (Lei et al., 2009). A recent study (Lei et al., 2009) showed that ACD and CTDA, compared with heparin and citrate, maintained platelet integrity for a longer time, reduced platelet spontaneous activation, increase the amount of growth factor released from PRP.

Initially the PRP is presented in liquid form and for some kind of utility, such as orthopedics or dentistry, must be activated to produce platelet degranulation and clot formation. The substance used in activation are bovine thrombin (not present in Italy and associated with the formation of antibodies against factors V, XI and antithrombin), autologous thrombin (obtained during the same phases of centrifugation for the production of PRP), recombinant thrombin or calcium (Graziani et al., 2007).

The application of PRP (often PRP gel) covers many clinical settings including skin, bone, dental care, maxillofacial surgery, diabetic foot and leg, vascular and cardiac surgery, tympanic lesions, ocular and corneal lesions, nerve lesions, spinal fusion, burns, aesthetic surgery and lifting (Borzini et al., 2007).

The use of PRP has been investigated in several studies, the majority reports success in promoting regeneration while others found no additional benefit (Everts et al., 2006, Redler et al., 2011, Borzini et al., 2007, Sampson et al., 2008). Therefore the clinical usefulness of PRP remains controversial (Graziani et al., 2006). In human medicine among hundreds of published papers about the clinical use of platelet derivatives, very few belong to the class of the prospective randomized clinical trials or to the category of retrospective clinical trials. The great majority belongs to the category of the case report or to that of the pilot study (Borzini et al., 2007). The methods of preparation of PRP and consequently the concentration of growth factors that are achieved are variables and that may explain the different clinical results that are obtained (Graziani et al., 2006). In particular it is still uncertain which concentration of PRP are optimal in promoting enhanced wound healing and regeneration. (Graziani et al., 2006).

The extreme heterogeneity of clinical protocols presents in the literature and associates with discordant results have contributed to the confusion in the scientific community with the lack of a unanimous opinion easily interpretable (Marx et al, 2004, Freymiller et al, 2004, Redler et al, 2011.). Moreover, the absence of a unique protocol of preparation has created concerns about the validity of all the methods proposed.

Even if the platelet concentration is an important factor in the definition on PRP the platelet growth factor content is quite variable among individuals and it is not necessarily proportional to the platelet count. Activation and release of growth factor also occur during platelet processing. Hence the sole platelet count cannot be predictive of the growth factor content in individual PRP preparation (Mazzuco et al., 2009). Some authors reported a correlation between such parameters (Epply et al., 2004) some other did not (Weibrich et al, 2002). In theory several factors might contribute to this lack of correlation: manipulation-induced platelets stress, variable susceptibility of platelets to stress, and microaggregates affecting an accurate platelet count. Furthermore, it cannot be

excluded that growth factor-absorbing proteins (fibrin monomers, thrombospondin and other proteins release from platelet during high speed centrifugation phase) might affect both platelet count and growth factor measurement (Mazzucco et al., 2009).

Platelets are extremely sensitive to any kind of process-induces stress, from blood collection to PRP gel production. Thus the amount of platelet derived growth factor available at the end of the manipulation process depend on cumulative effects over platelets starting to phlebotomy and ending with gel formation (Mazzucco et al., 2009).

Pets (especially laboratory rats, rabbits and dogs) have been involved in a number of experimental studies aimed at providing models for human medicine (Table 1 and 2). In some of these studies, platelet growth factors have proved capable of promoting the healing of different tissues (Kim et al., 2002, You et al., 2007, Rai et al., 2007) but not in others (Jensen et al., 2004, Casati et al., 2007, Rabillard et al., 2009).

In veterinary medicine some studies have been conducted to study the potential therapeutic effects of PRP in wound healing and in the repair lesions at tendons and ligaments of the horse (De Rossi et al., 2009; Torricelli et al., 2011) (Table 3).

The results obtained were encouraging, even if carried out on a limited number of subjects.

With regard to the studies carried out on dog most have been performed to study a model for humans (Table 1). One study (Kim et al., 2009) reported the successful healing of a single chronic skin lesion unresponsive to standard therapy. Two other studies (Rabillard et al., 2009, Souza et al., 2012) reported the application of PRP for the treatment of critical size ulnar gap and a radial gap ostectomy with different results.

Finally in veterinary literature there are only 2 studies (Ferraz et al., 2007, Silva et al., 2012) (Table 1) that attempt to standardize the method for obtaining the PRP autologous, so as to define a method unambiguous and reproducible, since there are no guidelines reliable for the production of a PRP effective. Often for the production of PRP in animals home made methods are used because of rapid implementation and low cost. These methods differ greatly in amount of blood used, speed, number and time of centrifugation, inclusion or not of the buffy coat and platelet concentration reached (Table 1). Moreover the methods of production are not explained carefully thus making it very difficult a possible reproduction.

Table 1: Articles relating to canine PRP prepared with home made methods (Articles with positive outcome are highlighted in green, Articles with negative outcome are highlighted in red)

Author	Journal	Whole blood (ml) and anticoagulant	Subjects (n°)	Centrifugation (n°)	Speed 1° centrifugation	Speed 2° centrifugation	Platelet concentration in PRP	Amount of PRP obtained (ml)	Conclusion
Kim et al., 2002	J Oral Maxillofac Surg	10 ml divided in 2 tubes (5ml) containing 10% of trisodium citrate	10	2	1000 rpm for 10' at room temperature	1 mL of the PPGF from each 5-mL tube was discarded. The remaining plasma was collected and centrifuged at 1500 rpm for 10'.	1735000/mm ³ (range of 1520000 to 2005000/mm ³ , increase of 392%).		"These results suggested that bone defects around titanium implants can be treated successfully with DBP and that PRP may improve bone formation."
Choi et al., 2004	Int. J. Oral Maxillofac. Surg	60 ml	10	2	2400 rpm for 10'	3600 rpm for 15'	124.2 ± 24.3 x 10 ⁴ /μL		"The results showed that the viability and proliferation of alveolar bone cells were suppressed by high PRP concentrations, but were stimulated by low PRP concentrations (1–5%). These in vitro results support the view that variations in the PRP concentrations might influence the bone formation within the PRP-treated bone grafts."
Jensen et al., 2004	Journal of Orthopaedic Research	9 ml in EDTA * 12 tubes	8	1	4000 rpm for 20' (it considered PRP the buffy coat and the adjacent 1 mm	-	1884000/μL, mean increase 670%	Approximately 0.6 ml for each 9 ml test tube. PRP from all 12 samples were	"We found no significant effect of PRP alone ore mixed with bone allograft on implant fixation or bone formation."

					of PPP and erythrocytes			pooled	
Casati et al., 2007	Int. J. Oral Maxillofac. Surg.	Three 5 ml tubes with 0.5 ml of sodium citrate	10	2	1200 rpm for 10'	1200 rpm for 15'	460350 / μ L (320%)		"it was concluded that platelet-rich plasma alone did not enhance bone regeneration for peri-implant defects"
Tae-Min You et al, 2007	Oral Surg Oral Med Oral Pathol Oral Radiol Endod	20 ml	6	2	2400 rpm for 10'	3600 rpm for 15'	1380000/ μ L (range: 1010000 to 2230000).		"when PRP is used as an adjunct to Bio-Oss in the repair of bone defects adjacent to titanium dental implants, PRP may decrease periimplant bone healing."
Rai et al, 2007	J Oral Maxillofac Surg	10 ml of heparinized blood	8	2	2400rpm for 10' (PRP and PPP portions again centrifuged)	3600rpm for 15'	485 x 10 ³ \pm 110/ μ L	1.3-1.4 ml	"This pilot study suggest the efficacy of PCL-TCP scaffolds together with PRP from the treatment of critical-sized defects of the canine mandible. "
Ferraz et al., 2007	Braz. J. vet. Res. anim. Sci., São Paulo,	10 ml In sodium citrate	15	2	800rpm for 10'	1300 rpm for 10'	492000/ μ L (183%)		
				2	800rpm for 10'	1600 rpm for 10'	542583.3/ μ L (210%)		1) "the best technique for the preparation of the platelet rich plasma in dogs consists of the previous centrifugation of the blood at 800rpm for ten minutes, and then the plasma is separated and submitted to a second centrifugation of 1600rpm for 10 minutes, and the poor plasma is separated and discharged."

				2	800rpm for 10'	3200 rpm for 10'	615000/ μ L (222%)		2) "concentration obtained at 3200rpm, in relation to the plasma, but it should be noted that all the samples prepared by this technique showed medium to severe platelet damage, and Marx 5 inferred the process for the obtention of PRP should not cause harm to the platelets
Li et al. 2009	J Oral Maxillofac Surg	2 tubes (30ml) + 4,2 ml ACD	24	2	2000rpm for 15' a 4°C	4000rpm for 10' a 4 °C	1.1*10 ⁶ / μ L, (roughly 6 times greater than normal whole blood)	~ 1 ml	" The PRP and blood vessels in the latissimus dorsi muscle could cooperatively promote osteogenesis and vascularization in tissue-engineered bone"
Kim et al 2009.	Vet Dermatol	8 ml in citrate tube	1	2	629g for 10'	1233g for 15'	1.0-1.2*10 ⁶ / μ L (6 times normal whole blood)	~1 ml	"This case report suggests that autologous PRP may be beneficial in the management of large skin defects or in delayed wound healing."
Rabillard et al, 2009	Vet Comp Orthop Traumatol	-	6	2	350 g for 20' a 4°C	600 g for 15' a 4°C	a three- to six-fold increase of platelet concentration		"In this canine model employing a critical size ulnar gap, the combination of CaP granules and PRP did not effectively promote bone regeneration."
Shayesteh Y.S. et al 2010	Journal Compilation	40 ml	12	2	2400 rpm for 10' (PRP and PPP portion again centrifuged)	3600 rpm for 15'	2,070.78 x 10 ³ / μ L	Pellet resuspended in 1 ml of residual plasma	"The addition of PRP to palatal mucosal wound sites did not accelerate wound healing"

Sardari et al, 2011	Comp Clin Pathol	40 ml in ACD-A	5	2	120g for 5' (about 50% of plasma fraction adjacent to buffy coat subjected to 2° centrifugation)	280g for 5'	About 800.000/ μ L	1.5 ml	"The results of the present study demonstrated that PRP did not have significant effects to promote cutaneous regeneration and wound healing in dogs treated with dexamethasone at last 16 days after last injection. "
Zhu et al, 2012	J Endod	20 ml + 3 ml sodium citrate	4	2	200 g for 10' (no inclusion of white and red blood cells)	360g for 10'	> 1200000/ μ L.		"In the present study, we found that PRP alone or the combination of PRP and DPSCs did not enhance the regeneration of pulp-like tissues."
Silva et al. 2012	BMC Veterinary Research	8.5 ml + 1.5 ml di ACD-A	16	1	191 g for 6'		1072900 (84.01): lower PC fraction 652000 (56.47): upper PC fraction		"The methodology used in this study allows the concentration of a number of platelets and TGF- β 1 that might be acceptable for a biological effect for clinical or experimental use as a regenerative therapy in dogs."
Souza et al, 2012	Vet Comp Orthop Traumatol	8 ml + 2 ml di sodio citrato	21	2	160 g for 20' (with white blood cells and first 2 mm of red blood cells)	400 g for 15'	In all animals, the minimum increase in platelet count of 338% required for the PRP was obtained.	1 ml	"Platelet rich plasma can be used as an adjuvant therapy because it may promote better bone healing of a radial ostectomy treated with external skeletal fixation in dogs."

Table 2: Articles relating to PRP obtained from rabbits using a home made methods (Articles with positive outcome are highlighted in green, Articles with negative outcome are highlighted in red).

Authors	Journal	Subjects (n°)	Whole blood (ml) and anticoagulant	Centrifugation (n°)	Speed 1° centrifugation	Speed 2° centrifugation	Platelet concentration in PRP	Amount of PRP obtained (ml)	Conclusion
Efouglu et al., 2004	J Oral Maxillofac Surg	23	9 ml in EDTA	2	300g for 10' As suggested by Marx, all of the plasma and the top erythrocyte layer were transferred to an empty tube	5000g for 5'			
		Group 1: 13				Group 1: lower half is PRP	Mean : 1061000/ μ L (122.2037%)		
		Group 2: 10				Group 2: lower third is PRP.	Mean: 3134500/ μ L (429.6306 %)	It was not possible to repeat the cell-counting procedures because of the limited volume of PRP and PPP available	
Andrade, et al.,	Oral Surg Oral Med	50	9 ml + 1.5 ml di ACD	2	300 g for 10' a 22 °C	5000g for 5' within the	Mean 2324.08 x 10 ³ / μ L,		"The protocol evaluated

2008	Oral Pathol Oral Radiol Endod				rotor acceleration curve of 60'' and rotor braking curve of 55''	same parameters of temperature, acceleration, and braking curves.	DS 925.34, Median 2172.50 x 10 ³ /μL		produces a good PRP. Whole-blood parameters can predict PRP features. Whole blood Ht is an important variable for PRP preparation and PRP cytometry characterization . PRP platelet count is dependent upon whole- blood platelet count. "
									"It was experimentally shown that PRP with high platelet counts can be prepared using this modified method without the need for costly autotransfusion systems."
Nagata-	Eur J	10	5 ml	Group I:	160 G for 6'	-	781.875	1.2 ml	"double-

Messori et al., 2010	Dent			1 centrifugation	At room temperature		(±217.693)/μL		centrifugation protocol resulted in higher platelet concentrations than did the single-centrifugation protocol. However, the double-centrifugation protocol caused alterations in platelet morphology and was more sensitive to small processing errors."
			5 ml	Group II: 2 centrifugations	160 G for 20' At room temperature	400 G for 15' At room temperature	1986875 (± 685.020) /μl	0.5 ml	
Obata et al., 2012	Arthritis Research & Therapy	12	10 ml + 1 ml citrate dextrose-A solution	2	330 g for 15'	1000g for 10'	mean 57525000 ± 3937000 /μL, about 29 times greater than that of whole blood		"We have shown that the administration of autologous PRP-releasate was effective in restoring disc height and increasing chondrocytic

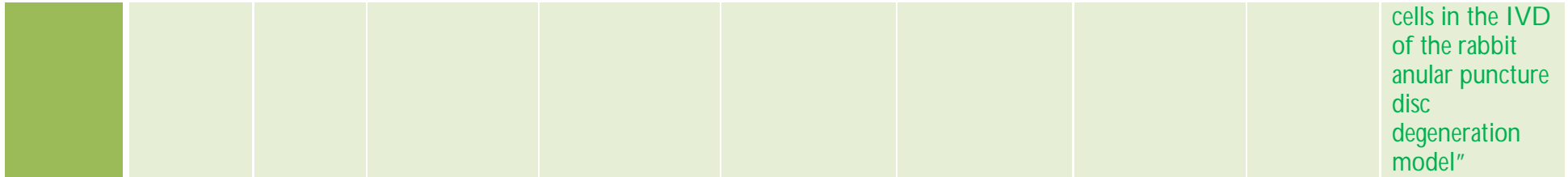


Table 3: Articles relating to **equine PRP** that use home made methods for PRP production (Articles with positive outcome are highlighted in green, Articles with negative outcome are highlighted in red)

Authors	Journal	Subjects (n°)	Whole blood (ml) and anticoagulant	Centrifugation (n°)	Speed 1° centrifugation	Speed 2° centrifugation	Platelet concentration in PRP	Amount of PRP obtained (ml)	Conclusion
Arguelle, Carmona et al., 2006	Research in Veterinary Science	26	125 ml divided in 25 tubes with sodium citrate 3.8%	I group: 1 centrifugation (5 ml in 8 tubes)	120 g for 5'		PC a platelet concentration 45%, 229000/ μ L (126000-364000) PC b platelet concentration 44% (228000/ μ L, 125000-361000)	PC a (50% fraction closet to the BC) PC b (50% fraction of the supernatant above PC a)	"The platelet collection efficiency and TGF- β 1 concentration efficiency were higher for PCs obtained by the single centrifugation tube method compared with PCs obtained by the double centrifugation tube method. "
				II group: 2 centrifugation	120 g for 5' (g and time used determined after preliminary studies (data not shown) done in the same laboratory)	240 g for 5'	PC c platelet concentration 71%, 272000 (138000-479000) PC d platelet concentration 21%, 191000 (112000-295000)	PC c (lowe 25% fraction of the centrifugated PC a sample) PC d (remaining upper 75% fraction)	"The results of this study let us think that PC b and PC c are maybe preferable for the clinical use in equine patients with musculoskeletal pathologies. "
Arguelles et al., 2008	Vet Rec	5	5ml * 8 tubes	1	120 G for 5'		229.000 / μ L		"All the horses had returned to their pre-injury level of

									performance by six months after the completion of the treatment, and none of them had suffered a recurrence after 20 months."
			5 ml *16 tubes	2	120 G for 5'	240g for 5'	272.000 / μ L		
De Rossi et al., 2009	Acta Cirurgica Brasileira	6	10 ml in 2 tubes with 10% sodium citrate	2	300g for 10'	640g for 10' at room temperature	1600000/ μ L		"Wounds treated with PRP gel exhibit more rapid epithelial differentiation and enhanced organization of dermal collagen compared to controls in equine
Torricelli et al., 2011	International Orthopaedics	13	Equine whole venous blood was collected with sodium citrate.	2	200 g for 5'	1000g for 15'	751 \times 10 ³ / μ L		the platelet concentration predicted the healing time: significantly faster recovery (p= 0.049) was observed in cases of PRP with more than 750 \times 103/ μ l platelets."

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CHAPTER 6

Effectiveness of manual double centrifugation method for preparation of Canine Platelet-Rich Plasma

6. Effectiveness of manual double centrifugation method for preparation of Canine Platelet-Rich Plasma

6.1 Abstract

The platelet-rich plasma (PRP) is a product derived from whole blood with a platelet concentration higher than normal range in a small amount of plasma. Regenerative capacities of PRP deriving from platelet growth factors. For this reason PRP is used in human and veterinary medicine for its capacity to stimulate cell proliferation, angiogenesis, wound healing, production of fibroblasts, collagen, osteoblasts and to accelerate the healing process. In veterinary medicine the methods use to produce PRP are not standardized and extremely numerous. The aim of this study was to describe and evaluate a manual double centrifugation method to produce canine PRP. 28 blood samples (5-10 ml with 1 ml of sodium citrate) from 28 healthy dogs were analyzed. The first centrifugation (2500 rpm for 10') resulted in two components, blood cell component in the bottom and serum component (SEC) in the upper fraction of the tube. All SEC, the buffy coat and the first 2 mm of red blood cell was submitted to a second centrifugation (4000 rpm for 15') and resulted in two components, platelet poor plasma (PPP) and platelet pellet in the bottom. The amount of PPP used to resuspend platelet pellet was calculated considering that all platelets previously present in the SEC was in platelet pellet. Then 50% of the calculated PPP was used to resuspend platelet pellet with the aim to obtain the final platelet concentration of about 1 million / μ l.

The method proposed in this study permitted in 14/28 samples of PRP (50%) to reach the human target of 1 million platelets/ μ l \pm 20% and/or the target of three to six fold platelet concentration in whole blood. According to a part of human literature the concentration of platelet in PRP does not seem to be necessarily linked to its effectiveness. For this reason to complete the evaluation of the proposed method will be necessary assess the platelet viability and after apply the PRP in vivo.

6.2 Introduction

The platelet-rich plasma (PRP) is a product derived from whole blood, characterized by the presence of a platelet (PLT) concentration higher than the normal range in a small volume of plasma (Marx et al., 2001).

The numerous growth factors present in the platelet alpha granules (Derived Platelet Growth Factor, Epidermal Growth Factor, Insulin Growth Factor, Vascular Endothelial Growth Factor etc.) are capable of stimulating cell

proliferation, angiogenesis, wound healing, production of fibroblasts, collagen, osteoblasts and to accelerate the healing process (Anitua et al., 2004).

In the human medicine the regenerative capacity of PRP are mainly applied in orthopedic surgery, maxillofacial surgery, dentistry, medicine, cosmetic surgery (Martinez-Zapata et al., 2009, Eppley B et al., 2006) and in dermatology such as, the healing of chronic skin ulcers (Driver et al., 2006).

In veterinary medicine studies about the production and application of PRP are very scarce. In the most studies animals were used as models for human medicine (Jensen et al., 2004, Casati et al., 2007, Li et al., 2009, Rai et al., 2007, Nagata et al., 2010). The use of PRP for therapeutic purpose in animals has been documented in musculoskeletal, tendal and soft tissue injuries in horses (Arguelles et al., 2008, Waselau et al., 2008, Bosch et al., 2011, Torricelli et al., 2011) and in a single dog with a large cutaneous lesion (Kim et al., 2009).

In veterinary medicine only few studies were conducted and there is a lack of suitable standardization and definition of the different PRP preparations (Arguelles et al., 2006, Textor et al., 2011, Nagata et al., 2010). In particular in canine species there are few studies that described and evaluated manual method (Souza et al., 2012, Ferraz et al., 2007, Silva et al., 2012) or semi-automated method (Stief et al., 2011) to produce canine PRP.

In dog the potential use of autologous thrombocytic growth factor to accelerate tissues regeneration requires improved methods of isolating platelet-rich plasma (PRP) (Weibrich et al., 2003). The manual protocols described to prepare PRP differ widely and do not give detailed information about the amount of starting whole blood, the type of anticoagulant used, the times and speed of centrifugation used, whether or not buffy coat was included in the final PRP and the final platelet concentration obtained in PRP (Rabillard et al., 2009, Silva et al., 2012, Souza et al., 2012, Kim et al., 2009). To date there are no exhaustive studies carefully describing the manual method to obtain PRP in the dog and there is not yet been identified the most effective concentration of PLT in PRP in the canine species. The arbitrary target use in human medicine of platelet concentration in PRP of 800000-1000000 plt/ μ L, that is three to six fold platelet concentration in whole blood has been proven as clinically effective by several studies. Nonetheless the relationship among platelet concentration, growth factor concentration and clinical effectiveness is far from being clear (Mazzucco et al., 2009) both in human that in veterinary medicine. Thus the aim of this study was to clearly describe a manual double centrifugation method to obtain small amount of autologous canine PRP starting to a relative small amount of whole blood.

6.3 Material and methods

Twenty eight adult healthy dogs weighing from 20 to 45 Kg, both sexes, between 1 and 7 years were use. With owners consent the blood samples were extracted from each dog fasted from 12 hours during routine heartworm test screening control for blood donor dogs.

Five to ten milliliter of blood were collected from the cephalic vein using large gauge needle (21 gauge) in order to minimize either animal trauma and platelet activation.

The blood collected was immediately placed into test tube with a conical bottom contains 1 ml of sodium citrate 3.8% (Tube A) (Photo 1a) and gently sloped several time to mix the blood with the anticoagulant. All samples were maintained at room temperature (18-25 ° C), processed and analyzed within 24 hours of sampling.

On all whole blood (WB) samples complete blood count was performed using an automatic analyzer (CELL-DYN 3500 Plus, Abbott, Germany). All samples were accurately maintaining on laboratory blood rocker for minimum 5 minutes before the platelet count.

6.3.1 Protocol of PRP preparation

All samples were subject to the same double centrifugation protocol; the first in order to divide blood cells elements to plasma, the second one to concentrate platelets into a single pellet.

First centrifugation: The blood samples were centrifuged at 610 g (2500 rpm) for 10 minutes at room temperature using a laboratory centrifuge (EBA 20 – Hettich, Germany) resulting in two components, blood cell component (BCC) in the bottom of the tube and serum component (SEC) in the upper fraction of the tube. In all cases was possible to see buffy coat between the two components (Photo 1b).

To be sure to include the buffy coat (rich of young giant platelets) on the SEC fraction, a mark was made 2 mm below the line between BCC and SEC. Then all contents above this mark was pipetted and transferred to another 10 ml graduated conical tube without anticoagulant (Tube B). In particular firstly 80% of the top SEC was pipetted and transferred in tube B with a 1000µL pipette (adjustable manual air cushion pipette 100-1000 µL, Eppendorf, North America) while remaining SEC (included the buffy coat) was pipetted with a 200 µL pipette (adjustable manual air cushion pipette 10-200 µL, Eppendorf, North America) to be more precise.

Before the second centrifugation, the quantity of SEC was recorded and the count of PLT on the SEC was performed using an automatic analyzer (CELL-DYN 3500 Plus, Abbott, Germany).

Second centrifugation: Tube B was then centrifuged again at 1600 g (4000 rpm) for 15 minutes resulting in two components: platelet poor plasma (PPP) in the

upper fraction of the tube and platelet pellet in the lower fraction (visible as a red button on the bottom of the tube) (Photo 1c).

The PPP of each tube was pipetted and transferred to another 10 ml test tube without anticoagulant.

The amount of ml of PPP used to resuspend the platelet pellet was calculated considering that all platelets previously present in the SEC were collected inside the platelet pellet. Then the amount of PPP used to suspend the platelet pellet was calculated with the aim to obtain the final concentration of PLT of about 1 million platelets / μ L (PRP) (Marx et al., 1998, Marx et al., 2001).

We decided to resuspend the pellet in 50% of the amount of PPP indicated by the calculation to compensate the platelets lost due to a possible non-total precipitation of platelets in the pellet and to the operator.

The solution thus obtained (PRP) was pipetted sometimes gently in order to disrupt and resuspend any platelet aggregates.

The platelet and leukocyte count and the haemoglobin concentration were automatically determined using CELL-DYN 3500 Plus (Abbott, Wiesbaden, Germany) in whole blood, SEC and PRP.

All samples were accurately maintained on laboratory blood rocker for minimum 5 minutes before the platelet count.

To establish the linearity of Cell-Dyn 3500 to count canine platelet over the canine PLT normal value a pooled PRP sample with high platelet concentration (1 ml volume; 863000 $\text{plt}/\mu\text{L}$) obtained from the same unit of canine fresh whole from Department Veterinary Blood Bank, was assessed at different dilutions.

To establish the inter-assay variation of the procedure 10 blood samples of 8 ml from one unit of canine fresh whole blood obtained from Department Veterinary Blood Bank were subject to the protocol of PRP preparation on the same day. The coefficient of variation (CV) was calculated as $(\text{SD}/\text{mean}) \times 100$.

6.4 Statistical analysis and Platelet counting

All quantitative measurements were described using summary statistics (mean, standard deviation, median, minimum, maximum). Mean, standard deviation and median of whole blood (ml) used to produce PRP, platelet concentration in WB, SEC and PRP, ml of SEC obtained after the first centrifugation, PPP aliquot used for the resuspension of platelet pellet were calculated after calculating normal distribution of parametric data using the D'Agostino-Pearson test.

Mean, standard deviation, median of hematocrit (Ht), leukocyte (WBC) and platelet count (PLT) from WB and PRP were calculated after calculating normal distribution of parametric data using the D'Agostino-Pearson test. The difference between Ht, WBC and PLT between WB and PRP were tested by carried out based on Mann-Whitney test for independent samples.

The influence of these variables on PRP value and the correlation among each parameter in whole blood and PRP were calculated using Spearman's coefficient of rank correlation (ρ). Also the correlation between ml of WB use to produce PRP, platelet concentration in SEC and platelet concentration in PRP were calculated using Spearman's coefficient of rank correlation (ρ).

To assess the platelet increase over whole blood baseline value was used the following equation:

Platelet concentration over whole blood baseline = (Platelet Count in PRP/ Platelet count in whole blood) x 100 (Marx et al., 2004).

To establish the inter-assay variation of the procedure 10 blood samples of 8 ml from one unit of canine fresh whole blood were subject to the protocol of PRP preparation on the same day. The coefficient of variation (CV) was calculated as (SD/mean)x100.

For all test significance was set at $P < .05$. Statistical analyses were performed using commercial software (MedCalc Software, Mariakerke, Belgium).

6.5 Results

PRP was prepared in approximately 30 minutes. Twenty-eight samples were subjected to double centrifugation methods. The time and speed of centrifugation was chose based on the previous centrifugation that failed to achieve any significant platelet enrichment. After various assays optimal platelet enrichment was achieved with double centrifugations at the speed of 2500rpm for 10 minutes and 4000 rpm for 15 minutes respectively.

Number of platelets, mean, standard deviation and median of platelet concentration in WB, SEC and PRP, ml of SEC obtained after the first centrifugation, PPP aliquot used for resuspension of platelet pellet, and amount of platelet concentration over whole blood baseline (%) are reported in Table 1.

Platelet count of 1 million/uL \pm 20% was achieved by 12/28 samples (42.9%) while platelet concentration (%) of minimum 300% was achieved by 11/28 samples (39,3%). Taken together 14/28 (50%) samples has reached the target (platelet concentration higher than 800000 plt/uL or a minimum of three fold platelet concentration in whole blood) (Table 1) (Photo 1).

Mean, standard deviation, median of hematocrit (Ht), leukocyte (WBC) and platelet count (PLT) in WB and PRP and statistical analyses of difference were reported in Table 2.

Significant and positive correlations between platelet count in WB and in PRP ($\rho = 0.490$, $p = 0.0081$) and between WBC in WB and in PRP ($\rho = 0.384$, $p = 0.0435$) were observed. No correlation between Ht in WB and Ht in PRP ($\rho = -0.157$, $p = 0.4447$) was presented.

No correlation was presented between ml of WB use to produce PRP and platelet concentration in SEC ($\rho = 0,1665$, $p = 0,5167$) and between ml of WB and platelet concentration in PRP ($\rho = 0,04838$, $p = 0,8069$).

The CV of platelet count performed on 10 samples of PRP obtained from one fresh whole blood unit was 8.43 (Table 3)

The linear correlation value between the full strength and the diluted platelet counts on pooled PRP sample was found to be $r = 0.98$. Mean of recovery was 16,4% (Table 4).

Figure 1: a - Tube A containing whole blood, b -Tube A after first centrifugation with visible buffy coat, c - Tube B after second centrifugation with visible platelet pellet on the bottom of the tube

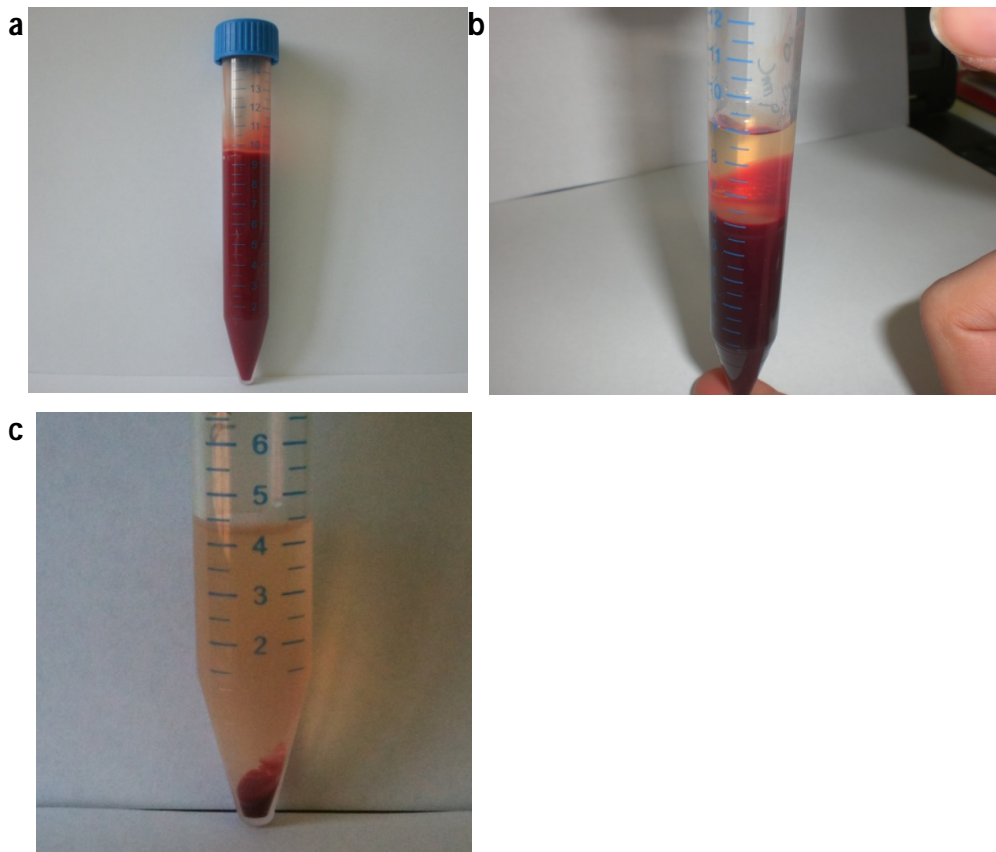


Table 1: Data obtain from 28 samples submitted to manual double centrifugation for produce canine PRP

Sampl es	MI of whole blood	PLT in WB (/uL)	PLT in SEC (/uL)	ml SEC	ml PPP for resuspension	PLT in PRP	PLT concentration (%)
1	7	204000	250000	3,5	0,4	818000	401
2	9	313000	160000	4,4	0,4	1388000	443
3	8	252000	151000	3,2	0,2	427000	169
4	6	194000	48800	3	0,1	658000	339
5	5	193000	60700	3,2	0,1	1348000	698
6	5	98900	57400	2,5	0,1	170000	172
7	5	98100	93000	4,1	0,2	569000	580
8	7,2	442000	612000	3	0,9	825000	187
9	7,5	629000	942000	3,5	1,7	997000	159
10	7	395000	344000	2,3	0,4	997000	242
11	6,5	321000	320000	2,5	0,4	740000	231
12	7	355000	277000	3	0,4	559000	157
13	7	340000	277000	3	0,4	500000	147
14	8,5	237000	268000	3	0,4	620000	262
15	8,5	357000	425000	4	0,9	1135000	318
16	9	314000	375000	3,5	0,7	785000	250
17	9	156000	234000	4	0,5	457000	293
18	10	140000	151000	4	0,3	260000	186
19	9,5	106000	147000	4	0,3	211000	199
20	9	163000	134000	4	0,3	172000	106
21	9	375000	504000	4,4	1,1	1411000	376
22	9	241000	104000	3,5	0,2	1121000	465
23	8,5	270000	317000	2,5	0,4	540000	200
24	8,5	275000	360000	2,5	0,5	752000	273
25	10	167000	167000	4	0,3	873000	523
26	9	174000	223000	3	0,3	1490000	856
27	9	346000	390000	3,8	0,7	1390000	402
28	10	247000	387000	2	0,4	559000	226
Mean	8,03	264392,9	277817,9	3,3357 14	0,464286	777571.43	316,4286
SD	1,5238 09	118922,7	191340,2	0,6717 66	0,344496	394454.37	178,8048
Media n	8,5	264392,9	259000	3,35	0,4	746000	256

Figure 1: Platelet count in WB and PRP of each sample.

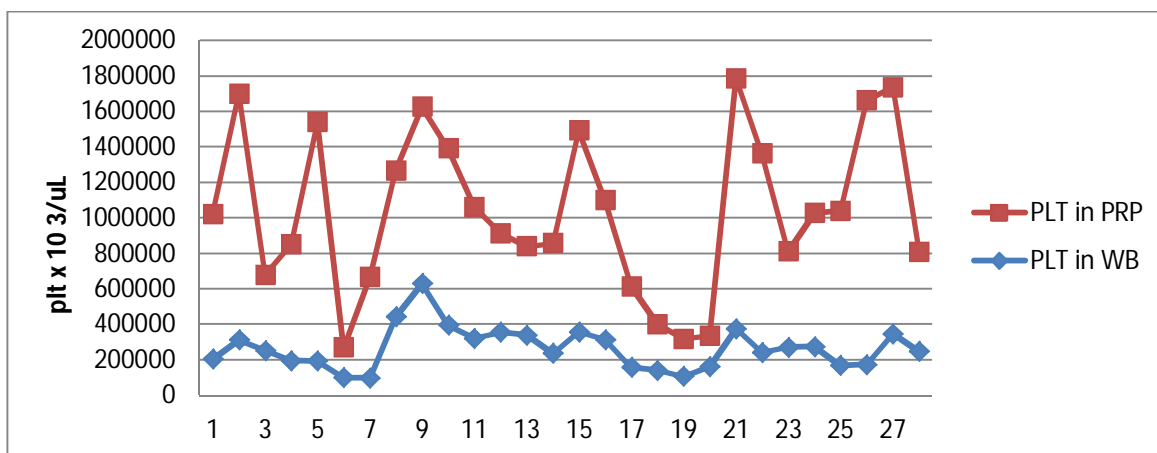


Table 2: Mean, standard deviation and median values of Hematocrit (Ht), leukocytes (WBC) and PLT on WB and PRP and Spearman's coefficient of rank correlation (rho) results on 28 samples subjected to home made double centrifugation

Variable	Sample	Mean	SD	Median	Distribution	P Mann-Whitney	Spearman's coefficient (rho)
Ht (%)	Whole blood	40.9	4.939	40.25	Normal (p = 0.9166)	< 0.0001	rho = -0.157, p = 0.4447)
	PRP	4.6988	3.6668	3.8250	Not normal (p < 0.0001)		
WBC(/μL)	Whole blood	9537,9	2.9540	9035	Normal (p = 0.2838)	< 0.0001	rho = 0.384, p = 0.0435
	PRP	28588,2	24.6352	19950	Not normal (p = 0.0004)		
Platelet count (PLT/μL)	Whole blood	264392.86	118922.71	249500	Not normal (p = 0.0226)	< 0.0001	rho = 0.490 p= 0.0081
	PRP	777571.43	394454.37	746000	Normal (p = 0.4327)		

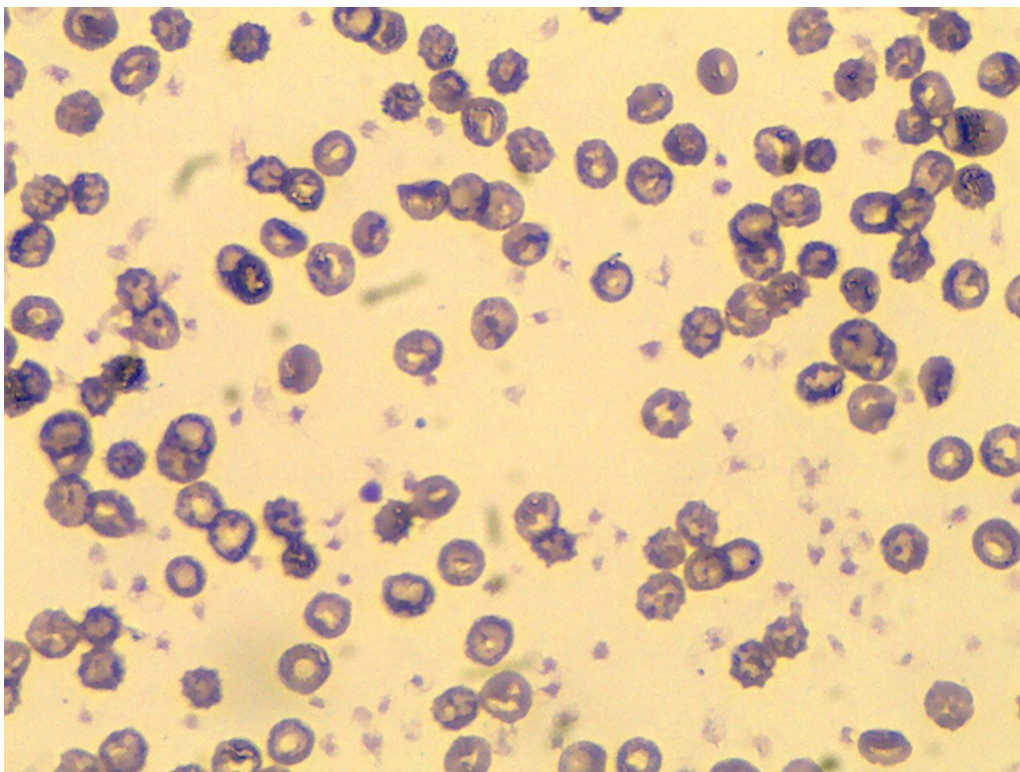
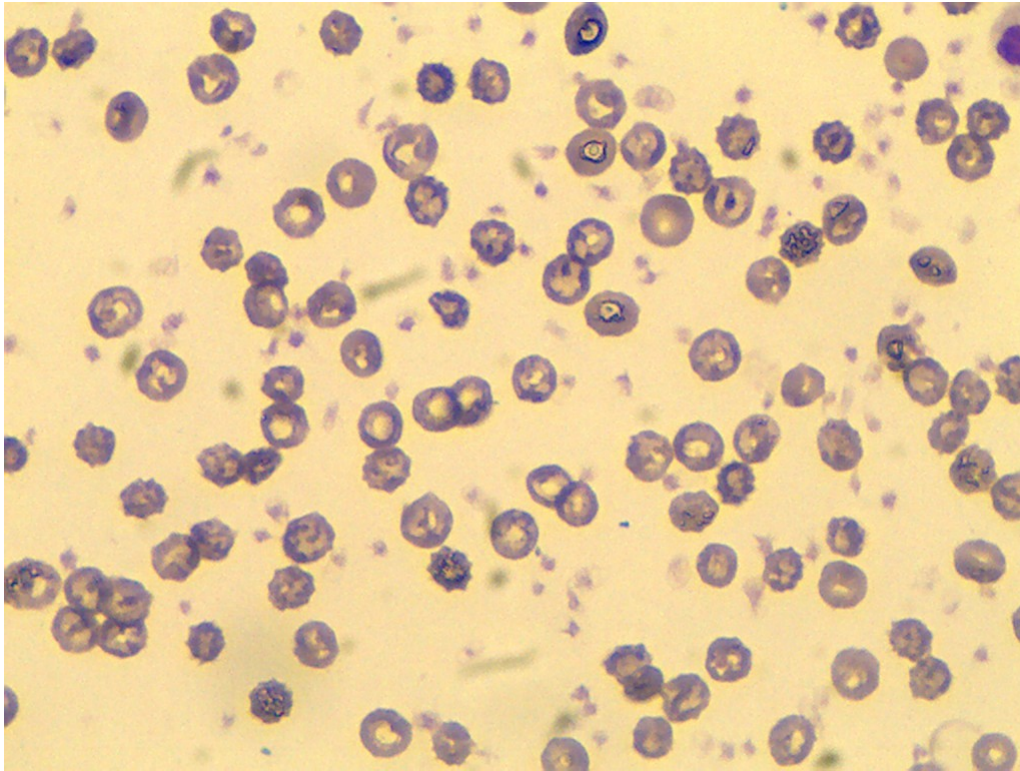
Table 3: Data about repeatability of manual double centrifugation method to obtain canine PRP on 10 samples obtained from one unit of fresh whole blood

Samples	PLT in Whole Blood	PLT in PRP (/μL)	%
1	455000	956000	210,11
2	431000	815000	189,1
3	408000	1033000	253,19
4	417000	903000	216,55
5	406000	1029000	253,45
6	387000	910000	235,14
7	395000	901000	228,1
8	389000	1008000	259,12
9	376000	1062000	282,45
10	377000	1044000	276,92
Mean	404100	966100	240,413
SD	25004,22	81481,35	29,96885

Table 4: Platelet concentration and percent recovery of platelet using Cell-Dynn 3500 Analyzer on a diluted PRP pooled sample.

Dilution	Expected (x 1000/μL)	Observed (x 1000/μL)	Recovery
1:1	858	858	100
1: 0.75	643.5	753	117
1: 0.5	429	500	116.5
1: 0.25	214.5	248	115.6

Photo 1: PRP smear stained with a rapid method for cytological preparations (MGG STAIN QUICK BIO-OPTICA) (magnification x1000). This sample has reached both targets (PLT count 1388000/ μ L, platelet concentration 443%). All components are easily recognizable).



6.6 Discussion

The aim of this study was evaluated a manual double centrifugation protocol to obtain canine PRP. Our method not requiring special equipment and permit to obtain the final product in 30 minutes. In our study the arbitrary target of 1000×10^3 platelet/ $\mu\text{L} \pm 20\%$ used in the majority of human medicine studies has been reached in 12/28 samples (42.9%) while the target of three to six folds platelet concentration in whole blood was achieved by 11/28 samples (39,3%). A total of 14/28 samples (50%) reached one or both the human target for PRP.

A mean of 0,5 ml of PRP starting from a mean 8 ml of whole blood was achieved in our study similar to the result of Jensen et al. (2004) that isolated 0,6 ml of PRP from each test tube containing 9 ml of whole blood.

The mean of platelet concentration in our PRP is 777571.43 / μL . This value is lower respect other studies for producing canine PRP with home made method (Kim et al., 2002, Choi et al., 2004, You et al., 2007, Ning et al., 2009, Zhu et al., 2012) but higher respect other studies (Casati et al., 2006, Ferraz et al., 2007).

The speed of the centrifugation may influenced the final PRP concentration (Casati et al., 2006, Ferraz et al., 2007, Silva et al. 2012). We have varied the force and time of centrifugation in order to obtain the optimal platelet enrichment on the basis of the previous studies conducted on the dogs (Choi et al., 2004, You et al., 2007, Li et al., 2009, Kim et al., 2009). In particular our speed and time of centrifugation are similar to other three studies (You et al, 2007, Choi et al, 2004, Kim et al, 2009) but in these studies the target of 1million of platelets/ μL was reached. Also the anticoagulant may have influence on the PRP platelet concentration, Landsberg et al. (2000) reports that EDTA gave higher yields than the citrate but seems that the platelets appeared to be more damaged. Although EDTA gave greater yields of platelets they appeared damage by the presence of the EDTA while the citrated-derived plasma had sufficient platelets to produce good clots (Landsberg et al., 2000)

Even if the target has been reached in 50% of the samples the significant difference between PLT in WB (median 249500/ μL) and PLT in PRP (median 746000/ μL) ($P < 0.0001$) and the significant and positive correlations between platelet count in WB and platelet count in PRP ($\rho = 0.490$, $p = 0.0081$) are indices of the capacity of the method to concentrate the platelets in a small amount of plasma. Furthermore the significant difference between WBC in WB (median 9035/ μL) and WBC in PRP (median 19950/ μL), ($p < 0.0001$) and the significant correlation between WBC in WB and WBC in PRP ($\rho = 0.384$, $p = 0.0435$) with WBC in PRP higher that WBC in WB are indices of good buffy coat inclusion during the preparation method. In buffy coat are in fact present giant and young platelets.

The variables to be assessed during the PRP production are numerous and all can influence the final result. Also for this reason, the literature is extremely

varied with regard to method of production, the final platelet concentration and clinical efficacy of PRP. Platelets are extremely delicate blood components that may undergo changes in number or quality at each step of the production of PRP. Platelets are easily stressed by withdrawal of blood, by subsequent manipulations (amount, rate, duration of centrifugation, the centrifuge and quality settings for acceleration and breaking), the operator's manual, the anticoagulant used. Furthermore platelets are labile particles that quickly lose their properties during storage. All these variables can lead to changes in both the quantity of platelets found in the final product that their quality as a possible activation of the product during any of the stages of production leads to the release of growth factors, most of which will be lost with the removal the plasma.

Although the biological rationale for the use of PRP is theoretically sound, both in human that veterinary medicine, the precise methodology that will result in the optimal benefits from this therapy is yet to be elucidated. In particular the precise PRP concentrations that would be ideal are yet to be determined. The majority of human studies take the definition of PRP from 2 studies from Marx (Marx et al, 1999, Marx et al, 2001) which report that a good PRP could have 1 mln platelets / μL in 5-ml volume of plasma or a minimum 338% increased in platelet compared to whole blood. At the same way also in veterinary medicine it is used the same target of "good PRP" even if there are differences between human platelets and platelets of veterinary species regarding platelet size, optimal centrifugation for their isolation and platelet reactivity. In studies conducted on animals the different home made protocols proposed have obtained platelet concentration in PRP between 400 and 2000 x 10^3 plt/ μL in dog, between 1000 and 5700 x 10^3 plt/ μL in rabbit and between 200 and 1600 x 10^3 plt/ μL in horse with different in vivo effects. A canine platelet concentration of 460.000 plt/ μL (320% of whole blood) seem to be not sufficient to promote bone formation (Casati et al., 2007) while a platelet concentration of 1 milion/ μL or more seem to be effective in some studies (Ning et al., 2009, You et al., 2007) and not effective in others (Rabillard et al., 2009, Zhu et al., 2012) to promote osteogenesis and vascularization. In rabbit (Weibrich et al., 2004) advantageous biological effects for bone regeneration seem to occur when platelet concentration in PRP was approximately 1 mln / μL with lower effects at suboptimal concentration and paradoxically inhibitory effect at higher concentration. The similar inhibitory effect is reported in a study on canine PRP (Choi et al., 2004).

A recent study (Torricelli et al., 2011) carried on the horse has already shown that platelet concentrations greater than 750000/ μL significantly accelerate the healing time of wounds.

So it would seem that the activity of the PRP is more related to the concentration of growth factors and their activation rather than to the concentration of platelets. Growth factors present in PRP are likely to depend on the particular technique used to obtain the PRP (Weibrich et al., 2003)

It must be noted that numerous controversial results exist for the platelet concentration request to have therapeutic effects associated with PRP alone and in combination with bone substitutes, in animal studies and in particular in the dogs (Rai et al., 2007, Marx et al., 2000). For example in a study conducted by Jansen et al. (2004) a canine PRP with 1 mln platelet/ μ L did not show significant effect in bone formation while in a study conducted by Rai et al. (2007) a canine PRP of 450000 platelet/ μ L demonstrated activity in the treatment of critical-sized defects of the mandible.

In light of the currently reported in bibliography our method allows to obtain a "good PRP" in 50% of cases. The amount of PRP (mean 0.5 ml) we obtained starting from a minimum quantity of blood (5-9 ml) allow the application in the field of dermatology and ophthalmology. This method could be used in clinic, if only limited volumes of PRP are needed.

In addition the inter-assay variation of our method evaluated on 10 samples taken from the same fresh whole blood units gave a value of 8.43. To our knowledge there are no studies that have evaluated the inter-assay variation of method for the production of PRP. For this reason, we can only compare our data with the value of inter-assay reported for platelet counts in human medicine (Westgard, 2012). In human medicine, a within-subject biologic variation for platelet count of 9.1 is considered acceptable. Therefore on this basis we can assume that our method performed by an experienced operator gives an acceptable value of inter-assay.

However, we believe that it is necessary to improve the protocol proposed by us. The points to be evaluated will be the use of a different anticoagulant (such as ACD), the production of PRP within 6 hours of collection of whole blood, the use of a centrifuge which allows to set acceleration and deceleration and the assessment of new speed and centrifugation time in order to allow a better division of the various blood components and to minimize the resuspension of platelets in the plasma portion during braking. Additionally we would like to confirm the automatic platelet counts by performing counts with 2 different machines (for example Cell Dyn and ADVIA 120) to minimize the possibility of an erroneous platelet count.

Unfortunately, in our study, we evaluated only the quantity of platelet in PRP samples and not the platelet integrity (BIAS). In particular qualitative alterations in the platelets may affect the regenerative potential of PRP for the loss of bioactive growth factors.

To complete the evaluation of this method to produce canine PRP will be necessary

assess the platelet viability (for example using tests like pH, hypotonic stress, P-Selectin and platelet aggregation levels) and PRP application in vivo.

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ATTACHMENT 1

**Linea guida relativa all'esercizio
delle attività sanitarie riguardanti
la medicina trasfusionale in campo
veterinario.**

Ministero della Salute

DIPARTIMENTO PER LA SANITÀ PUBBLICA VETERINARIA, LA NUTRIZIONE
E LA SICUREZZA DEGLI ALIMENTI
DIREZIONE GENERALE DELLA SANITÀ ANIMALE E DEL FARMACO
VETERINARIO

UFFICIO IV

Linea guida relativa all'esercizio delle attività sanitarie riguardanti la medicina trasfusionale in campo veterinario.

Campo di applicazione

La presente linea guida si applica al sangue intero di origine animale prelevato da animali di proprietà di persone giuridiche e/o fisiche per lo scopo trasfusionale. Essa non si applica ai prodotti derivati dal sangue, regolati dal decreto legislativo del 6 aprile 2006, n.193.

Prelievo di sangue intero

Si definisce "sangue intero" il sangue prelevato, per lo scopo trasfusionale, dal donatore riconosciuto idoneo, utilizzando materiale sterile e sacche regolarmente autorizzate dal Ministero della salute, contenenti una soluzione anticoagulante-conservante.

Il prelievo di sangue intero deve essere effettuato da un medico veterinario, attuando una metodica che garantisca asepsi, con un sistema a circuito chiuso, compatibilmente con la specie animale, e con dispositivi non riutilizzabili. Il direttore sanitario della struttura trasfusionale definisce un protocollo dettagliato delle procedure di prelievo, con particolare riguardo alla detersione e disinfezione della cute prima della venipuntura, e vigila sulla sua applicazione. Nel caso in cui si rendesse necessaria più di una venipuntura deve essere utilizzato un nuovo dispositivo di prelievo. Preliminarmente al prelievo è necessario ispezionare le sacche per verificare l'assenza di eventuali difetti, la scadenza, la corretta quantità di anticoagulante in esse contenuta ed il suo aspetto.

Dopo ciascun prelievo, i contenitori e le sacche debbono essere accuratamente ispezionati per verificare l'assenza di qualsiasi difetto. Debbono essere inoltre adottate misure volte ad evitare ogni possibilità di errore nell'etichettatura della sacca e delle corrispondenti provette.

Nell'allegato 2 alla presente linea guida sono illustrate le fasi della procedura da seguire per la raccolta del sangue.

Idoneità alla donazione e benessere animale

Occorre anzitutto valutare le condizioni generali di salute dell'animale donatore, con particolare attenzione agli stati di debilitazione, iponutrizione, edemi, anemia, ittero, cianosi, dispnea e lesioni cutanee.

Ad ogni donazione l'animale donatore deve essere sottoposto obbligatoriamente agli esami di laboratorio di cui all'allegato 1 alla presente linea guida, volti ad escluderne gli stati patologici e la positività degli indicatori delle malattie trasmissibili, nonché ad individuarne le principali caratteristiche immunoematologiche.

Le modalità previste per l'attuazione della procedura di donazione di unità di sangue intero non devono provocare sofferenza, angoscia o danni durevoli in capo ai donatori.

Preparazione, conservazione ed etichettatura del sangue intero

Il sangue intero, prelevato utilizzando materiale sterile e sacche autorizzate dal Ministero della salute, deve essere conservato in frigoemoteca ad una temperatura di 4°C (+/-) 2°C, per un periodo di tempo adeguato al tipo di anticoagulante-conservante impiegato, che deve essere in ogni caso definito sulla base della sopravvivenza post-trasfusionale delle emazie uguale o superiore al 75% a 24 ore.

I frigoriferi utilizzati per la conservazione del sangue devono assicurare un'adeguata ed uniforme temperatura al suo interno, devono essere provvisti di termoregistratore e di gruppo di continuità dedicato esclusivamente a questo scopo.

La data di scadenza del sangue si identifica con l'ultimo giorno in cui il predetto può essere considerato utile agli effetti della trasfusione; la data di scadenza deve essere indicata in etichetta ed è consentito l'utilizzo massimo entro 30 giorni dal momento del prelievo.

Sui contenitori di unità di sangue devono essere apposte etichette conformi a quanto indicato nell'allegato 6 alla presente linea guida.

Trasporto, distribuzione e somministrazione del sangue .

Il sangue intero deve essere trasportato in contenitori termoisolanti dotati di appositi sistemi di controllo della temperatura interna, ed i contenitori per il trasporto di unità di sangue devono essere pre-raffreddati a + 4°C. Il sangue deve essere trasportato ad una temperatura compresa tra +1° C e +10°C (sangue intero fresco o conservato) .

Prima della somministrazione di sangue intero ai riceventi devono essere eseguite delle indagini volte ad accertare la compatibilità fra il donatore ed il ricevente.

Presso ogni struttura dedita alla medicina trasfusionale veterinaria deve essere adottato, per ciascuna unità di sangue, un sistema di sicuro riconoscimento dell'animale ricevente cui la stessa unità è stata assegnata.

Tracciabilità e registrazione dati

Presso ogni struttura trasfusionale deve essere predisposto un sistema di registrazione e di archiviazione dei dati che consenta di ricostruire il percorso di ogni unità di sangue, dal momento del prelievo fino alla sua destinazione finale.

I dati inerenti il donatore devono essere registrati e aggiornati in uno schedario donatori (cartaceo o informatico) a cura del direttore sanitario di ogni struttura trasfusionale. Le operazioni di registrazione vanno effettuate dopo che sia stata ultimata ogni singola fase di lavoro. Il registro deve essere conservato per tre anni presso ogni struttura trasfusionale, e per le strutture destinate alla commercializzazione del sangue intero deve essere vidimato dall'ASL competente per territorio. Il suddetto schedario deve essere tenuto in modo da:

- a) contenere i dati del proprietario dell'animale;
- b) contenere i dati clinici dell'animale donatore;

c) attuare la normativa vigente in materia di identificazione animale, facilitando la tracciabilità della donazione;

d) consentire l'introduzione di informazioni riguardanti eventuali reazioni avverse.

Nel caso di reazioni avverse del ricevente, correlate alla trasfusione, deve essere possibile, attraverso il precitato schedario, risalire al donatore e verificare i risultati di tutte le indagini compiute.

Allegato n. 1

ESAMI OBBLIGATORI AD OGNI DONAZIONE DI SANGUE: CANE, GATTO E CAVALLO

A seconda della tipologia d'impiego del sangue si possono distinguere due possibili opzioni nel pannello di esami di laboratorio da eseguire sul donatore:

- sangue intero di pronto impiego o d'emergenza preparato all'interno della struttura veterinaria e da utilizzare all'interno della medesima senza possibilità di cessione ad altre strutture;
- sangue intero reperibile in commercio.

Lo stato di salute del donatore deve essere verificato ad ogni donazione, oltre che con una visita clinica accurata, anche con un pannello di esami standard che, all'occorrenza ed in particolari situazioni epidemiologiche, può essere ampliato. Queste informazioni devono essere trascritte sulla cartella clinica dell'animale donatore, che deve essere conservata per tutta la durata dell'impiego dello stesso come donatore e per un anno dopo la sua esclusione o conclusione dell'attività come soggetto donatore.

Nel caso di sangue intero di pronto impiego o d'emergenza, le analisi di laboratorio da eseguire sono quelle elencate nella tabella 1 per il cane, nella tabella 2 per il gatto e nella tabella 3 per il cavallo. Nel caso di sangue intero reperibile in commercio le analisi di laboratorio da eseguire sono quelle riportate nella tabella 4 per il cane, nella tabella 5 per il gatto e nella tabella 6 per il cavallo.

Tabella 1 – Cane: Elenco degli esami di laboratorio da eseguire sul sangue intero di pronto impiego o d'emergenza preparati all'interno della struttura veterinaria, da utilizzare all'interno della medesima e non cedibile ad altre strutture.

Esame	Analiti
Gruppo sanguigno	DEA 1.1°
Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofilo, eosinofilo, basofilo, linfociti, monociti) morfologia WBC n° PLT, MPV (morfologia PLT) Proteine plasmatiche totali ricerca microscopica per Babesia spp. nel buffy coat*
Sierologico	Leishmania Infantum ^a , Ehrlichia Canis ^a , Babesia Canis ^{b*} , Dirofilaria Immitis ^{a**}

Legenda: * alternativo all'indagine IFI per Babesia canis; ** se in trattamento profilattico regolare si può omettere l'esame; a= è possibile l'impiego di test rapidi di tipo ambulatoriale; b- IFI=Immunofluorescenza Indiretta.

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio;

Tabella 2 – Gatto: Elenco degli esami di laboratorio da eseguire sul sangue intero di pronto impiego o d'emergenza preparati all'interno della struttura veterinaria, da utilizzare all'interno della medesima e non cedibile ad altre strutture.

Esame	Analiti
Gruppo sanguigno	A e B ^a

Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofili, eosinofili, basofili, linfociti, monociti) morfologia WBC n° PLT, MPV (morfologia PLT) Proteine plasmatiche totali Ricerca microscopica per Mycoplasma haemofelis nello striscio di sangue
Sierologico	FIV ^a , FeLV ^a

Legenda: a= è possibile l'impiego di test rapidi di tipo ambulatoriale.

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio; FIV=virus immunodeficienza felina; FeLV=virus leucemia felina.

Tabella 3 – Cavallo - Elenco degli esami di laboratorio da eseguire sul sangue intero di pronto impiego o d'emergenza preparati all'interno della struttura veterinaria, da utilizzare all'interno della medesima e non cedibile ad altre strutture

Esame	Analiti
Gruppo sanguigno	Fattore Aa e Qa
Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofili, eosinofili, basofili, linfociti, monociti) morfologia WBC n° PLT, MPV (morfologia PLT) Proteine plasmatiche totali ricerca microscopica per Babesia caballi Theileria equi. nel buffy coat
Urine	Chimico, fisico e sedimento
Parassitologico	Feci

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio. Note: Si suggerisce di utilizzare solo come donatori in emergenza coloro che hanno effettuato di recente (15 gg) gli esami sierologici indicati nella successiva tabella 6

Tabella 4 – Cane: Elenco degli esami di laboratorio da eseguire sul sangue intero reperibile in commercio.

Esame	Analiti
Gruppo sanguigno	DEA 1.1 ^a [DEA 1.2 (Aa), DEA 7 (Tr)]
Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofili, eosinofili, basofili, linfociti, monociti) morfologia WBC n° PLT, MPV (morfologia PLT) ricerca microscopica per Babesia spp. nel buffy coat*
Biochimico	Proteine plasmatiche totali, Albumina, Urea, ALP, ALT
Coagulazione	PT, aPTT, Fibrinogeno
Sierologico	Leishmania Infantum ^a , Ehrlichia Canis ^a , Rickettsia rickettsii ^b , Babesia Canis ^{b*} , Dirofilaria Immitis ^{a**} [Borrelia Burgdorferi ^b Brucella Canis ^c]
Urine	Chimico, fisico e sedimento
Parassitologico	Feci, Ricerca microfilaria nel sangue periferico

Legenda: * alternativo all'indagine IFI per Babesia canis; ** se in trattamento profilattico regolare si può omettere l'esame; a= è possibile l'impiego di test rapidi di tipo ambulatoriale; b- IFI=Immunofluorescenza Indiretta; c- AGID= Immunodiffusione in Gel di Agar.

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di

distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio; ALP=Fosfatasi Alcalina; ALT=Alanina Aminotransferasi; PT=Tempo di Protrombina; aPTT=Tempo di Tromboplastina Parziale attivato.

Note: Gli esami indicati tra parentesi quadra non sono obbligatori [DEA 1.2 e 7] [Borrelia Burgdorferi^b Brucella Canis^c]

Tabella 5 – Gatto: Elenco degli esami di laboratorio da eseguire sul sangue intero reperibile in commercio distribuito da aziende e/o da centri di raccolta.

Esame	Analiti
Gruppo sanguigno	A e B ^a
Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofilo, eosinofilo, basofilo, linfocito, monocito) morfologia WBC n° PLT, MPV (morfologia PLT) Proteine plasmatiche totali Ricerca microscopica per Mycoplasma haemofelis nello striscio di sangue
Biochimico	Proteine plasmatiche totali, Albumina, Urea, ALP, ALT
Coagulazione	PT, aPTT, Fibrinogeno
Sierologico	FIV ^a , FeLV ^a , FIP ^a
Urine	Chimico, fisico e sedimento
Parassitologico	Feci

Legenda: a= è possibile l'impiego di test rapidi di tipo ambulatoriale.

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio; ALP=Fosfatasi Alcalina; ALT=Alanina Aminotransferasi; PT=Tempo di Protrombina; aPTT=Tempo di Tromboplastina Parziale attivato; FIV=virus immunodeficienza felina; FeLV=virus leucemia felina; FIP=virus peritonite infettiva.

Tabella 6 – Cavallo: Elenco degli esami di laboratorio da eseguire sul sangue intero reperibile in commercio.

Esame	Analiti
Gruppo sanguigno	Fattore Aa, Qa, Ca, Db, Dc
Emocromo	n° RBC, Hgb, Hct, MCV, MCH, MCHC, RDW (morfologia RBC) n° WBC, Formula leucocitaria (neutrofilo, eosinofilo, basofilo, linfocito, monocito) morfologia WBC n° PLT, MPV (morfologia PLT) ricerca microscopica per Babesia caballi Theileria equi. nel buffy coat*
Biochimico	Proteine plasmatiche totali, Albumina, Urea,
Coagulazione	PT, aPTT, Fibrinogeno
Sierologico	Anemia infettiva, Morva, Ehrlichia Equi, Anaplasma phagocitophila, Babesia caballi Theileria equi.* Morbo Coitale Maligno
Urine	Chimico, fisico e sedimento
Parassitologico	Feci

Legenda: * alternativo all'indagine IFI per Babesia spp.

Codifica sigle: n°RBC=numero eritrociti; Hgb=emoglobina; Hct=ematocrito; MCV=volume corpuscolare medio; MCH=emoglobina corpuscolare media; MCHC=concentrazione emoglobinica corpuscolare media; RDW=ampiezza di distribuzione eritrocitaria; n°WBC=numero leucociti; n°PLT=numero piastrine; MPV=volume piastrinico medio; PT=Tempo di Protrombina; aPTT=Tempo di Tromboplastina Parziale attivato.

Allegato n.2

IDONEITA' ALLA DONAZIONE DI SANGUE E PROCEDURA DI RACCOLTA DEL SANGUE.

Cane

Nel cane il sangue per la donazione viene di norma prelevato dalla vena giugulare previa tricotomia della zona con il soggetto in stazione quadrupedale o in decubito laterale. Devono essere impiegate sacche autorizzate dal Ministero della Salute e la raccolta deve avvenire per gravità.

Elementi per l' idoneità alla donazione:

Peso corporeo	> 25 KG
Età	2 – 8 anni
Regolarmente vaccinati per:	Cimurro, leptosirosi, epatite, parvovirosi, rabbia
Carattere	Docile
Quantità da prelevare	1,5-2% del volume ematico corporeo ogni 9 settimane non superando i 18 ml /Kg
Profilassi routinarie	Filariasi cardio-polmonare
Identificazione	Anagrafe di specie

Gatto

Nel gatto il sangue per la donazione viene di norma, prelevato dalla vena giugulare previa tricotomia della zona.

Vi sono 2 opzioni per la raccolta di sangue:

- l'impiego di sacche di ridotte dimensioni con appropriata quantità di anticoagulante in rapporto al sangue introdotto.
- L'uso di una siringa con aspirazione lenta. Se il sangue prelevato con la siringa deve essere conservato in una sacca per l'impiego a distanza di tempo (oltre 12 ore) all'ago cannula munita di tubicino va collegato un rubinetto a tre vie prima dell'innesto sul cono della siringa. Questo dispositivo a tre vie consente, una volta raccolto il sangue nella siringa, di poterlo trasferire nella sacca di conservazione senza aprire le vie di collegamento, garantendo quindi una discreta sterilità del prodotto emotrasfusionale.

Elementi per l' idoneità alla donazione:

Peso corporeo	5-7 Kg
età	2 – 8 anni
Regolarmente vaccinati per:	Calicivirosi, Herpesvirosi, Panleucopenia infettiva, Clamidiosi, Leucemia virale.
Carattere	Docile
Quantità da prelevare	1,5-2% del volume ematico corporeo ogni 9 settimane non superando i 10 ml /Kg
Identificazione	Microchip registrato nella banca dati della struttura preposta al prelievo di sangue

Cavallo.

Nel cavallo il sangue per la donazione viene di norma prelevato dalla vena giugulare previa anestesia locale sopra il sito di prelievo e tricotomia della zona. Il sangue può essere raccolto

impiegando un dispositivo che produce una pressione negativa esternamente alla sacca al fine di aumentare la velocità di raccolta.

Elementi per l' idoneità alla donazione:

Peso corporeo	> 400 KG
età	3 – 10 anni
Regolarmente vaccinati per:	Tetano, Influenza e Rinopolmonite
Carattere	Docile
Quantità da prelevare	1,5-2% del volume ematico corporeo ogni 6 settimane non superando i 20 ml /Kg
Identificazione	Anagrafe di specie

Allegato n.3

CRITERI DI ESCLUSIONE PERMANENTE E TEMPORANEA DELL' ANIMALE CANDIDATO DONATORE AI FINI DELLA PROTEZIONE DELLA SUA SALUTE

Al fine della tutela della sua salute, deve essere giudicato permanentemente non idoneo alla donazione di sangue l'animale candidato donatore affetto o precedentemente affetto da una delle seguenti patologie:

malattie autoimmuni e immunomediate; malattie cardiovascolari; malattie del sistema nervoso centrale; neoplasie o malattie maligne; tendenza anomala all'emorragia; crisi convulsive.

Nel caso in cui l'animale candidato donatore sia o sia stato affetto in modo grave o cronico da malattia gastrointestinale, ematologica, respiratoria o renale, non compresa nelle categorie di cui sopra, il medico veterinario responsabile della selezione può avvalersi della consulenza specialistica prima della definizione del giudizio di idoneità o di non idoneità temporanea o permanente alla donazione.

Possono sussistere motivi per i quali è necessario, ai fini della protezione della salute dell'animale candidato donatore, rinviare la donazione; la decisione relativa alla durata del periodo di rinvio spetta al medico veterinario responsabile della selezione.

La gravidanza in atto costituisce motivo di inidoneità temporanea.

Allegato n.4

CRITERI DI ESCLUSIONE PERMANENTE E TEMPORANEA DELL' ANIMALE CANDIDATO DONATORE AI FINI DELLA PROTEZIONE DELLA SALUTE DELL' ANIMALE RICEVENTE

Inidoneità permanente.

Ai fini della protezione della salute dell'animale ricevente deve essere dichiarato permanentemente non idoneo alla donazione di sangue l'animale candidato donatore affetto o in precedenza affetto da una delle seguenti patologie o condizioni:

malattie autoimmuni e immunomediate, neoplasie maligne, diabete insulino-dipendente, epilessia, malattie cardiovascolari, glomerulonefrite cronica e pielonefrite, policitemia vera.

Inoltre le seguenti specie animali sono inidonee alla donazione del sangue se hanno contratto: nel cane la babesiosi; nel gatto la immunodeficienza felina (FIV), la leucemia virale felina (FeLV), la peritonite infettiva (FIP); nel cavallo la babesiosi, l'infezione da *Anaplasma phagocitophila* e da *Ehrlichia equi*, l'infezione che determina l'anemia infettiva (da lentivirus della famiglia dei retrovirus) e la morva (da *Pseudomonas mallei*).

Esclusione temporanea

In presenza di una delle sottoelencate patologie o condizioni l'animale candidato donatore deve essere dichiarato temporaneamente non idoneo alla donazione di sangue per un periodo di tempo di durata variabile in funzione della patologia o condizione rilevata:

nel gatto la Toxoplasmosi (dopo la guarigione clinica ed in assenza di anticorpi IgM) e l'infezione da *Mycoplasma haemofelis* (dopo guarigione clinica, scomparsa del parassita dal sangue periferico e indagine con PCR negativa); nel cane la Leishmaniosi (in presenza di titoli sierologici bassi e indagine PCR negativa sul linfonodo o sul midollo osseo), l'Ehrlichiosi (dopo la guarigione clinica in presenza di titoli sierologici negativi e indagine PCR sul sangue periferico negativa) e la Borreliosi (dopo la guarigione clinica in presenza di titoli sierologici negativi).

Rinvio di sei mesi

Trasfusione di sangue o trattamento con farmaci emoderivati, intervento chirurgico di rilievo, Allergia ai farmaci.

Rinvio di tre mesi

Parto o interruzione di gravidanza, Somministrazione di sieri di origine animale.

Rinvio di tre settimane

Somministrazione di vaccini costituiti da virus o batteri vivi attenuati.

Rinvio per 48 ore

Somministrazione di vaccini costituiti da virus o batteri uccisi o inattivati o da tossoidi.

Assunzione di farmaci.

Possono sussistere ulteriori ragioni per il rinvio temporaneo di un donatore ai fini della protezione dei riceventi la donazione: la decisione relativa alla durata del periodo di rinvio spetta al medico veterinario responsabile della selezione e comunque in accordo alla normativa vigente sui medicinali veterinari e rispettando i tempi di sospensione più lunghi.

Allegato n.5

PREPARAZIONE E CONSERVAZIONE DEL SANGUE

Il centro di prelievo del sangue intero destinato alla commercializzazione è costituito da locali in grado di garantire un'adeguata igiene delle procedure, equiparati ad una struttura veterinaria secondo la definizione dell'accordo Stato regione (Atti n. 1868 del 26 novembre 2003) e deve avere la disponibilità di particolari attrezzature di seguito elencate:

1. pinza multifunzione e anellini di alluminio o pinza saldatrice;
2. emofrigoteca a temperatura costante di 4-6 °C con registratore di temperatura, dotato di gruppo di continuità;
3. agitatore meccanico per la raccolta del sangue intero;
4. bilancia.

I locali di raccolta di sangue intero devono essere autorizzati dalle ASL competenti per territorio e devono adottare tutte le misure idonee a valutare e prevenire la diffusione delle malattie post-trasfusionali, principalmente quelle infettive.

Per le operazioni di preparazione del sangue intero devono essere utilizzate sacche autorizzate dal Ministero della salute.

Tipologie dei prodotti emotrasfusionali:

1. Sangue intero fresco: sangue prelevato da 6-8 ore;
2. Sangue intero conservato: sangue conservato oltre 6-8 ore;
3. Sangue intero in predeposito per autotrasfusioni: consiste in un'unità di sangue intero prelevata al paziente cui è destinata per corrispondere a proprie esigenze terapeutiche.

Allegato n.6

ETICHETTATURA DEL SANGUE INTERO

Su ciascuna sacca contenente sangue intero dovrà essere indicato tramite apposita etichetta:

1. nome ed indirizzo della struttura di prelievo del sangue intero;
2. numero identificativo della donazione ;
3. tipo del preparato;
4. peso netto del preparato;
5. data di prelievo e preparazione;
6. data di scadenza del prodotto;
7. composizione e volume della soluzione anticoagulante conservante e delle eventuali soluzioni aggiunti;
8. gruppo sanguigno dell'animale donatore;
9. modalità e temperatura di conservazione;
10. indicazione della specie animale;
11. dati anagrafici del donatore.

Nei sottoelencati preparati trasfusionali devono inoltre essere inclusi le seguenti diciture:

1. Sangue intero fresco, sangue intero conservato, Dicitura: “ *esclusivamente per uso veterinario – specie di destinazione:..... non utilizzabile a scopo trasfusionale se presenta emolisi o altre anomalie evidenti*” “*per la trasfusione utilizzare un adatto dispositivo munito di appropriato filtro*”

2. Sangue intero da predeposito per autotrasfusioni. L'etichetta di colore diverso dalle omologhe deve indicare la dicitura: **AUTODONAZIONE – STRETTAMENTE RISERVATA A:** . Generalità del tutore dell'animale; firma del medico responsabile del salasso; tipo di preparato; la dicitura: “ Non utilizzare a scopo trasfusionale se presenta emolisi o altre anomalie evidenti”; la dicitura: “ Per la trasfusione utilizzare un adatto dispositivo munito di appropriato filtro” ; la dicitura “ Esclusivamente per uso autologo – prove di compatibilità ed esami pre-trasfusionali **NON ESEGUITI**”.

ATTACHMENT 2

Science Publications on National Journals and Proceedings of National Conferences

Scientific Publication on National Journals

PEREGO R., BAGNAGATTI DE GIORGI G., SPADA E., DOMENICHINI G, PROVERBIO D. (2010) Dermatofitosi nella specie felina. Rassegna di Medicina Felina Anno 14, n° 1, pp. 7-16

SPADA E., PEREGO R., BAGNAGATTI DE GIORGI G., DELLA PEPA A., BAGGIANI L., PROVERBIO D. (2011) Gruppi sanguigni nel gatto e medicina trasfusionale felina. Rassegna di Medicina Felina, Anno 15, N° 3, pp. 7-13

SPADA E., PEREGO R., BAGNAGATTI DE GIORGI G., DELLA PEPA A., PROVERBIO D. (2011) Sindrome acquisita da fragilità cutanea felina. Rassegna di Medicina Felina, Anno 15, N° 4, pp 7-10.

DOMENICHINI G., SPADA E., DELLA PEPA A., BAGNAGATTI DE GIORGI G., DRAGONETTI V. , PEREGO R., PROVERBIO D.(2012) Caratteristiche di una popolazione di gatti di colonia della città di Milano. Rassegna di Medicina Felina, N° 2, pp 8-15.

Scientific Publications on Proceedings of National Conferences

PROVERBIO D., BAGGIANI L., PEREGO R., MILICI A., DOMENICHINI G., DELLA PEPA A., **BAGNAGATTI DE GIORGI G.**, FERRO E. (2010) Distribuzione dei gruppi sanguigni A, B E AB in gatti di razza ragdoll, Atti del LXIV Convegno Nazionale SISVet, 7-10 settembre 2010, Asti (AL)

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ATTACHMENT 3

Science Publications on Peer Review International Journals with Impact Factor

Scientific Publications on Peer Review International Journal with Impact Factor

PROVERBIO D., **BAGNAGATTI DE GIORGI G.**, DELLA PEPA A., PEREGO R., BAGGIANI L., SPADA E., BELLOLI A. (2011) Preliminary evaluation of total protein concentration and serum protein fraction obtained by electrophoresis of fresh and frozen wild horned viper serum (*Vipera ammodytes ammodytes*). Accepted for publication on Veterinary Clinical Pathology on December 20, 2011

SPADA E., PROVERBIO D., DELLA PEPA A., PEREGO R., BAGGIANI L., **BAGNAGATTI DE GIORGI G.**, DOMENICHINI G., FERRO E., CREMONESI F (2012) Seroprevalence of feline immunodeficiency virus, feline leukemia virus and Toxoplasma Gondii in stray cat colonies in northern Italy and correlation with clinical and laboratory data. Journal of Feline Medicine and Surgery, 2012 Jun;14(6), pp 369-77

PROVERBIO D., SPADA E., PEREGO R., DELLA PEPA A., **BAGNAGATTI DE GIORGI G.**, BAGGIANI L. (2012) Assessment of blood types of ragdoll cats for transfusion purposes. Accepted for publication on Veterinary Clinical Pathology on 19 July 2012.

SPADA E., PROVERBIO D., DELLA PEPA A., DOMENICHINI G., **BAGNAGATTI DE GIORGI G.**, TRALDI G., FERRO E. (2012) Prevalence of fecal-borne parasites in colony stray cats in northern Italy. Accepted for publication on Journal of Feline Medicine and Surgery on 11 December 2012

ATTACHMENT 4

Science Publications on Proceedings of International Conferences

Scientific Publications on Proceedings of International Conferences

SPADA E., DELLA PEPA A., MIGLIAZZO A., BAGNAGATTI DE GIORGI G., PEREGO R. PROVERBIO D. (2013). "Serological and molecular evaluation of *Leishmania infantum* infection in stray cats in a non-endemic area in Northern Italy". INTERNATIONAL SCIVAC CONGRESS – CANINE LEISHMANIOSIS AND OTHER VECTOR-BORNE DISEASE: OUR CURRENT STATE OF KNOWLEDGE. MARCH 8th – 10th, PISA, ITALY