UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

COLA DE FIBRINA CANINA PRODUZIDA COM FIBRINOGÊNIO OBTIDO POR CRIOPRECIPITAÇÃO E PRECIPITAÇÃO COM PROTAMINA A PARTIR DE DIFERENTES CATEGORIAS DE PLASMA POBRE EM PLAQUETAS

Autor: Monalyza Cadori Gonçalves
Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias na área de Clínica Cirúrgica Animal. Orientador: Prof. Dr. Carlos Afonso de Castro Beck

PORTO ALEGRE
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Monalyza Cadori Gonçalves

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Aprovada em 27 MAIO 2015

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Orientador e Presidente da Comissão

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Membro da Comissão

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Membro da Comissão

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Prof[a]. Dr[a]. Stella de Faria Valle
Membro da Comissão

DEDICATÓRIA

Dedico este trabalho a Deus, provedor da vida;

Aos professores e pesquisadores, que se sacrificam no oficio do ensino e promoção do crescimento intelectual e profissional da sociedade;

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“As dificuldades não são obstáculos, mas oportunidades de aperfeiçoamento.”
HS

RESUMO

A cola de fibrina tem sido utilizada em diferentes procedimentos cirúrgicos como agente hemostático, selante e de suporte adesivo. No entanto, seu emprego na Veterinária ainda é limitado devido à falta de formulações não dependentes dos componentes de origem humana e de validação baseada em necessidades e condições cirúrgicas de animais. Objetivou-se avaliar a viabilidade da produção de cola de fibrina canina com fibrinogênio obtido por crioprecipitação (crio) e precipitação por protamina a partir de fontes plasmáticas mais disponíveis em bancos de sangue e centros hospitalares. Quatro categorias de plasma pobre em plaquetas foram utilizadas: plasma fresco (*FR*), congelado dentro de oito horas da colheita e processado em menos de uma semana após congelamento; plasma fresco congelado (*FFP)*, com armazenamento inferior a um ano; plasma fresco congelado que ultrapassou um ano de armazenamento (*eFFP*), e plasma congelado com período entre colheita e congelamento maior que oito horas e de armazenamento superior a um ano (*FP*). No estudo *in vitro* de cada técnica, avaliou-se a concentração de fibrinogênio precipitado por meio do método de Clauss, as propriedades reológicas do gel por tromboelastografia (TEG) e as características estruturais do coágulo por microscopia eletrônica de varredura (SEM). O estudo *in vivo* consistiu da avaliação da praticidade de aplicação e das propriedades hemostáticas e adesivas das colas de fibrina resultantes em fígado e intestino de coelho (Oryctolagus cuniculus). Em avaliação prévia do protocolo de crio quanto ao uso de bolsas ou tubos, o aproveitamento do material inicial não diferiu, mas os tubos se mostraram mais simples, rápidos e homogêneos para o processamento, além de permitirem aumento da concentração final. O protocolo crio em comparação ao de protamina foi superior na precipitação de fibrinogênio coagulável nas avaliações de Clauss e TEG. Os coágulos formados se mostraram semelhantes entre os dois protocolos na SEM, no modelo de hemostasia hepática e na adesão à serosa intestinal. O uso de aprotinina com o protocolo de protamina não prejudicou a aplicação da cola sobre o intestino. Na crio, plasmas com maior tempo de armazenamento (eFFP, FP) se mostraram significativamente superiores aos mais frescos (FFP, FR) nas análises por Clauss e TEG. Não foi possível identificar diferenças estatísticas entre os tipos de plasma no protocolo protamina em nenhum dos parâmetros avaliados. Estudos adicionais e ajustes nos testes para avaliação de soluções concentradas são

necessários para determinação do efeito dos protocolos e tempo de armazenamento do plasma congelado sobre o fibrinogênio precipitado e demais componentes plasmáticos na cola de fibrina. Adequações e pesquisas ainda são necessárias para aproveitamento da precipitação de fibrinogênio por protamina e a partir de plasma fresco com a finalidade de obtenção rápida de cola de fibrina. Bolsas de plasma menos requisitadas em bancos de sangue veterinários representam uma fonte importante de fibrinogênio para a produção de cola de fibrina canina em centros hospitalares apropriadamente equipados, viabilizando seu uso em diferentes aplicações cirúrgicas e pesquisas relacionadas.

Palavras-chave: selante de fibrina, adesivo cirúrgico, sangue heterólogo, cão

ABSTRACT

Canine Fibrin Glue Produced with Fibrinogen Concentrated from Cryo- and Protamine Precipitation Using Different Platelet Poor Plasma Categories

Fibrin glue (FG) has been widely used in surgery for hemostatic, adhesive, sealant, and would healing support. In veterinary surgery, however, its use has been hindered by lack of specie-specific formulations and validation of its properties and biological characteristics. This study evaluated methods of fibrinogen precipitation from canine plasma envisioning autologous and allogeneic FG production for surgical use. The efficacy of cryo and protamine fibrinogen precipitation methods in producing canine FG was assessed by analysis on feasibility of each protocol with most available canine plasma sources, rheological and structural characteristics of the resultant FG clot and the hemostatic and adhesive properties of FG during in vivo application. The plasma categories studied included fresh plasma (FR), obtained and frozen within 8 hours from blood collection and processed within a week; fresh frozen plasma (FFP), frozen within 8 hours from blood collection and stored for up to a year; expired fresh frozen plasma (eFFP), plasma frozen within 8 hours from blood collection but stored for more than a year; and, frozen plasma (FP), which was frozen after 8 hours from collection and stored for more than a year. Comparison of cryoprecipitation among plasma types was previously performed in both 120-mL bags and 50-mL tubes and analyzed by Clauss. Total precipitation capacity did not differ significantly between bags and tubes. Nevertheless, the processing was more easily and homogeneously performed in tubes and allowed tailoring the final concentration. Cryoprecipitation generated better results in Clauss and TEG in comparison to protamine protocol. The resultant fibrin glue clots of cryo- and protamine-precipitation showed similar ultrastructure in scanning electron microscopy (SEM) and performance in the in vivo evaluations with the rabbit hepatic and intestinal incision models. The use of aprotinin in the protamine clot seemed beneficial in the intestinal evaluation. With cryoprecipitation, eFFP and FP were superior to FFP in the assessments performed by Clauss and TEG. Fresh plasma performed poorly with cryoprecipitation. Significant differences were not detected among plasma categories processed with protamine precipitation in any of the assays performed. While cryoprecipitation was more reliable regarding homogeneity and capacity to increase final

fibrinogen concentration, protamine protocol was faster and simpler considering the equipment required. Although, older plasma units generated significantly more cryo-precipitated and/or clottable fibrinogen, further studies are needed to validate the assays with such high concentrated solutions and to elucidate the effect of freezing storage on precipitation and clottability of fibrinogen intended for FG production. Adjustments on protamine protocol and improvements on fibrinogen precipitation from fresher plasma sources would support the use of autologous or allogeneic plasma for on-site production of canine FG. Veterinary hospitals, blood banks, and patients can benefit from usage of surplus plasma units for FG production aiming surgical and scientific needs.

Keywords: *fibrin sealant, surgical adhesive, heterologous blood, dog*

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE SIGLAS, ABREVIAÇÕES E SÍMBOLOS

FG	*fibrin glue*, cola de fibrina
FR	*fresh plasma,* plasma fresco
FFP	*fresh frozen plasma*, plasma fresco congelado
FP	*frozen plasma*, plasma congelado
eFFP	*expired fresh frozen plasma*, plasma fresco congelado vencido
IU	*international units*, unidades internacionais
ANOVA	*analysis of variance*, análise de variância
r^2	*Pearson's correlation coefficient*, coeficiente de correlação de Pearson
SD	*standard deviation*, desvio padrão
CV	*coefficient of variation,* coeficiente de variação
P	*significance level*, nível de significância estatística
pRBC	*packed red blood cells*, concentrado eritrocitário
AABB	*American Association of Blood Banking*, Associação Americana de Bancos de Sangue
TEG	*thromboelastography*, tromboelastografia
SEM	*scanning electronic microscopy*, microscopia eletrônica de varredura
R	*reaction time for clot formation*, tempo para início da formação do coágulo
K	*kinetics, time to achieve a certain clot firmness*, cinética do coágulo
MA	*maximum amplitude, maximum clot strength*, máxima amplitude
TMA	*time to maximum amplitude*, tempo para alcançar amplitude máxima
G	*shear elastic modulus strength (dynes/cm^2)*, resistência elástica
E	*elasticity constant (dynes/sec)*, constante de elasticidade
LY60%	*clot lyses at 60 minutes after maximum strength in percentage*, lise do coágulo
IM	*intramuscularly*, via intramuscular
PO	*per os, orally*, via oral
Bid	*bis in die, twice a day*, duas vezes ao dia
Tid	*ter in die, three times a day,* três vezes ao dia
IV	*intravenously*, via intravenosa
H&E	*hematoxylin and eosin stain*, coloração por hematoxilina e eosina

SUMÁRIO

1 INTRODUÇÃO

Em condições fisiológicas, a malha de fibrina formada como produto final da cascata de coagulação, serve como matriz para o crescimento de células de regeneração e formação de fibras de colágeno (BORIN *et al.*, 2008; GENTRY; BURGESS; WOOD, 2008; MOSESSON, 2005). Alguns estudos sugerem que durante a formação e lise do coágulo, os peptídeos liberados pela reações enzimáticas também favorecem o regeneração tecidual local. A capacidade da fibrina de polimerização e adesão a tecidos adjacentes, por meio de fatores associados, junto à sua biocompatibilidade e degradação pelo organismo, levaram ao desenvolvimento de colas de fibrina para uso em procedimentos cirúrgicos em diversos órgãos e com finalidades variadas (SPOTNITZ, 2014; SHARMA *et al.*, 2009; MACGILLIVRAY, 2003; REISS; OZ, 1996; SIERRA, 1993).

Tanto em procedimentos convencionais como minimamente invasivos, o uso da cola de fibrina tem reduzido o tempo operatório e a morbidade por meio do controle de hemorragias; impedimento de escape de ar por lesões pulmonares; diminuição do risco de extravasamento de conteúdo gastrintestinal, biliar ou urinário; atenuação de aderências; suporte a suturas e tecidos divulsionados, e sustentação do processo regenerativo e cicatricial (PORTILLA-DE BUEN *et al.*, 2014; SPONITZ; BURKS, 2012; LIPPERT *et al.*, 2011; PARK *et al.*, 2011; RICE; BLACKSTONE, 2010; BISHOFF *et al.*, 2003; DUNN; GOA, 1999; RUTGEERTS *et al.*, 1996). Em alguns centros hospitalares, este tipo de selante é rotineiramente utilizado como adjuvante na hemostasia de órgãos parenquimatosos, como selante de linhas de sutura em vísceras ocas e vasos, como suporte para rafias neurais e reconstruções ósseas, e adesivo tecidual para pele e córnea (KIM *et al.*, 2010; SUN *et al.*, 2010; HOVANESIAN; BEHESNILIAN, 2007; STOLTZE; HARMS, 2005; ZUCCHELLI; SILVESTRI, 2004; MINTZ *et al.*, 2001). Menos frequentemente, mas já clinicamente difundido, o selante de fibrina tem sido utilizado para rafias tendíneas, ligamentares e cartilaginosas (KIM *et al.*, 2014; HANKEMEIER *et al.*, 2009). Além do uso em procedimentos cirúrgicos, a característica de biodegradação gradativa, junto à ausência de toxicidade e ao poder adesivo, levou ao uso da cola de fibrina como arcabouço celular e meio de liberação local e lenta de substâncias, como antibióticos, hormônios e quimioterápicos, potencializando novos mecanismos terapêuticos (VEDAKUMARI; SASTRY, 2014;

FERRIS *et al.*, 2012; WU *et al.*, 2012; SPICER; MIKOS, 2010; ANITUA *et al.*, 2006; OPTIZ *et al.*, 2001).

As colas de fibrina, em geral, são produzidas a partir da extração do fibrinogênio plasmático. As comerciais são fabricadas priorizando o isolamento apenas do fibrinogênio e garantindo a esterilidade do produto. Isso permite que suas formulações sejam mais homogêneas, com maior concentração de proteína coagulável e biologicamente mais seguras. No entanto, esses processos elevam o custo do produto final. Além disso, por necessitarem de plasma humano para sua produção, a indisponibilidade temporária no fornecimento pela empresa fabricante, ou devido ao embargo por órgãos regulamentadores, dificulta sua aquisição e seu uso em cirurgias emergenciais (SPOTNITZ, 2014; PARK *et al.*, 2011; JACKSON, 2001; BRENNAN, 1991). O custo e o uso de componentes humanos também prejudica o emprego do produto comercial na veterinária, tanto em necessidades cirúrgicas como em pesquisas relacionadas (BLATTLER *et al.*, 2007; MARONE *et al.*, 1999; WHEATON *et al.*, 1994).

Entre os métodos descritos de precipitação de fibrinogênio plasmático, a crioprecipitação tem sido considerada a mais fisiologicamente favorável devido à concentração associada de fatores indutores de proliferação celular e à ausência de resíduos químicos. Características convenientes da crioprecipitação incluem a capacidade de produzir uma solução de fibrinogênio com concentrações suficientes para as principais necessidades cirúrgicas; possibilidade de mudança na diluição para aumentar o volume a uma concentração que não limite sua eficácia; segurança biológica, por permitir o uso de sangue autólogo ou alógeno de doadores previamente testados, e baixo custo de processamento. a Associação dessas características têm feito da crioprecipitação o método considerado padrão–ouro de obtenção de fibrinogênio para produção de cola de fibrina (SILVER; WANG; PINS, 1995b; BRENNAN, 1991; SPOTNITZ *et al.*, 1987).

No entanto, algumas aplicabilidades cirúrgicas recentes têm dependido de colas de fibrina com uma maior concentração de fibrinogênio que aquela produzida com a crioprecipitação, como o selamento de vísceras sob constante peristaltismo e a hemostasia sem manobras de garroteamento durante cirurgias minimamente invasivas (SPOTNITZ, 2014; LIPPERT *et al.*, 2011; KAETSU; TAKANORI; SHINYA, 2000). Na veterinária, a crioprecipitação ainda tem outros possíveis inconvenientes. O volume mínimo de plasma necessário para a crioprecipitação pode ser um fator limitante quando há escassez de bancos

de sangue ou porte insuficiente dos potenciais doadores, sendo estes o próprio paciente ou outro animal (BROOKS, 2010). Em casos que há doadores de porte apropriado, a produção de cola de fibrina ainda pode ser inviabilizada pelo tempo mínimo exigido para o processo de crioprecipitação que leva em média de 30 a 36 horas (SILVER; WANG; PINS, 1995a).

A precipitação de fibrinogênio por protamina para produção de cola de fibrina cirúrgica tem sido recentemente descrita como método de alto poder de concentração, rápido processamento, e menor necessidade de equipamentos exclusivos a bancos de sangue (ALSTON *et al.*, 2007). No entanto, poucos estudos tem sido publicados sobre as características do coágulo formado por este método e sobre as aplicações cirúrgicas possivelmente beneficiadas pelo seu uso (ALSTON *et al.*, 2008). Além disso, não há relatos da utilização deste protocolo na veterinária.

Em geral, a fonte de fibrinogênio utilizada para a produção de cola de fibrina é o plasma pobre em plaquetas. Os tipos mais disponíveis de plasma pobre em plaquetas em bancos de sangue veterinários são o plasma fresco congelado e o plasma congelado, que diferem entre si em relação ao tempo de processamento até o congelamento e/ou tempo de armazenamento após o congelamento. Se a colheita de sangue for feita no local de processamento, para a produção imediata da cola de fibrina, o plasma utilizado seria considerado como plasma fresco. Estes critérios de classificação são adotados referentes à perda relativa dos fatores lábeis de coagulação durante o armazenamento (ABRAMS-OGG; SCHNEIDER, 2010; GENOVEZ, 2008). Como a concentração de fibrinogênio tem se mostrado estável nestas categorias de plasma e os fatores lábeis não são necessários para a cola de fibrina, o efeito da categoria do plasma sobre a obtenção do fibrinogênio para a produção da cola de fibrina não tem sido estudado. Um outro aspecto relevante é que as características relacionadas aos componentes do sangue canino tem divergido daquelas observadas em sangue humano, podendo haver aspectos ainda não identificados em relação ao comportamento associado dos elementos plasmáticos caninos relevantes ao pretendido uso em questão (URBAN; COUTO; IAZBIK, 2013; GIBSON, 2007).

O desenvolvimento de cola de fibrina, a partir de componentes espécie-específicos, necessita de fontes plasmáticas e protocolos biologicamente seguros com comprovado custo-benefício (BUTCHA; SEMPLE, 2005; PENTELEZ; RUBIN, 2005). Objetivando a produção de cola de fibrina canina para uso em procedimentos cirúrgicos, estudou-se as soluções concentradas de fibrinogênio obtidas por crioprecipitação e precipitação por protamina a

partir de fontes plasmáticas mais disponíveis em bancos de sangue e centros hospitalares, avaliando a concentração de fibrinogênio no precipitado e as características reológicas, estruturais e biológicas dos coágulos resultantes.

Os resultados deste trabalho foram organizados na forma de três artigos. O estudo realizado com a precipitação de fibrinogênio por frio analisando o uso de bolsas e tubos, diferentes temperaturas de congelamento e distintas categorias de plasma está apresentado no artigo 1 (Capítulo 3). O artigo 2 compõe as avaliações *in vitro* realizadas com as soluções concentradas de fibrinogênio obtidas com os protocolos de precipitação por frio e pelo uso de protamina a partir de diferentes categorias de plasma (Capítulo 4). O estudo *in vivo* com a cola de fibrina produzida com fibrinogênio precipitado por frio e pelo uso de protamina a partir de plasma fresco congelado está descrito no artigo 3 (Capítulo 5).

2 REVISÃO DE LITERATURA

2.1 Fibrina

A fibrina é o produto final da cascata da coagulação após a ação enzimática da trombina, sobre o fibrinogênio e polimerização dos monômeros gerados, formando uma rede. Nesta reação, a glicoproteína fibrinogênio é identificada como fator I e a protease trombina como fator II. A estabilização da malha de fibrina pelo fator XIII, a torna insolúvel, servindo como base para a formação do coágulo. O coágulo é constituído pela interação do plasma sanguíneo, plaquetas e tecido lesionado (FERGUSON; NURNBERGER; REDL, 2010; MOSESSON; SIEBENLIST; MEH, 2001).

2.1.1 Formação do coágulo de fibrina

A molécula do fibrinogênio é composta por dois domínios D periféricos e um domínio central E. Cada domínio D tem três cadeias polipeptídicas: alfa, beta e gama, unidas no domínio central E. Na presença de trombina, os peptídeos das cadeias sofrem clivagem proteolítica e é iniciada a polimerização dos monômeros e filamentos de fibrina.

Com a clivagem de uma ligação peptídica de cada cadeia alfa e de cada cadeia beta e remoção dos peptídeos, o monômero de fibrina formado tem a carga eletrostática do seu domínio central E mudada. Essas mudanças nas forças eletrostáticas vão ser responsáveis pela polimerização dos monômeros de fibrina. No entanto, este polímero é instável. Na presença do fator estabilizador de fibrina (fator XIII), esses filamentos fazem ligações cruzadas covalentes entre si estabilizando a rede recém-formada (MOSESSON; SIEBENLIST; MEH, 2001).

Enquanto as cadeias gama sofrem ligações cruzadas dentro de alguns minutos, as cadeias alfa tem este processo completado entre 10 minutos até duas horas. Esta estabilização confere ao coágulo maior força mecânica e resistência à digestão proteolítica (NURNBERGER *et al.*, 2010).

2.1.2 Atuação fisiológica do coágulo

Em condições fisiológicas, a malha de fibrina serve como matriz para o crescimento de fibroblastos e formação de fibras de colágeno. Este efeito sobre a formação do tecido de granulação, incluindo um aumento na deposição de colágeno e presença de fibroblastos, tem sido amplamente relatado. A liberação dos fibrinopeptídeos do fibrinogênio e os produtos da degradação da fibrina participam da quimiotaxia leucocitária e macrofágica, contribuindo para limpeza da lesão e liberação de fatores de crescimento celular. Ainda, estudos têm demonstrado que coágulos de fibrina são invadidos por novos capilares e células de regeneração, essenciais para o crescimento tecidual (JACKSON, 2001; MOSESSON, 2005; SAHNI; FRANCIS, 2000; SIERRA, 1993).

Após a ativação do fibrinogênio e de fatores importantes no processo de coagulação sanguínea, como plaquetas, a trombina presa ao coágulo auxilia no processo de reparação tecidual. Em culturas celulares, a trombina também tem mostrado ter efeito mitogênico (FERGUSON; NURNBERGER; REDL, 2010; COX; COLE; TAWIL, 2004; BERING, 1944).

2.2 Cola de fibrina

A biocompatibilidade da fibrina e sua degradação pelo organismo levaram à busca e ao desenvolvimento de uma cola para uso em diferentes órgãos e com variados objetivos. A alta elasticidade deste material é uma característica útil no seu emprego em tecidos que precisam se manter flexíveis como pulmão, intestino, enxertos de pele e ainda junto a telas para hérnias. Embora não tão adesiva quanto as colas sintéticas como o cianoacrilato, a cola de fibrina tem a vantagem de ser um suporte adesivo de biocompatibilidade superior e possuir componentes fisiológicos indutores da reparação tecidual (FERGUSON; NURNBERGER; REDL, 2010; RICHTER *et al.*, 2010).

2.2.1 Usos

A cola de fibrina tem sido utilizada na hemostasia cirúrgica, principalmente de órgão parenquimatosos, no selamento de defeitos teciduais, evitando o vazamento de conteúdos

líquidos ou gasosos, e como agente adesivo para unir superfícies ou camadas teciduais em diferentes fases cirúrgicas. Em geral, seu uso reduz o tempo operatório e tem a vantagem de ser um material absorvível. Cirurgias de quase todos os órgãos, sob diferentes cenários e objetivos, têm sido beneficiadas com o uso da cola de fibrina. Seu uso é amplamente descrito em cirurgias cardiovasculares, pulmonares, gastrintestinais, urogenitais, neuronais, maxilo-faciais, oftálmicas, ortopédicas, oncológicas, plásticas e minimamente invasivas (JACKSON, 2001; MINTZ *et al.*, 2001; SALTZ *et al.*, 1991; SILVER; WANG; PINS, 1995a).

2.2.1.1 Hemostasia

Na hemostasia, a cola de fibrina tem sido utilizada com sucesso em órgãos parenquimatosos, como fígado e baço, em lesões superficiais e profundas, e em cirurgias cardíacas e renais. As situações de aplicação da cola nesses órgãos variam desde punções de biópsias, ressecções, anastomoses e lesões extensas por trauma (BISHOFF *et al.*, 2003; KRAM *et al.*, 1988; RADOSEVICH; GOUBRAN; BURNOUF, 1997).

A utilidade e eficiência da cola são observadas, em especial, em pacientes com distúrbios de coagulação ou que estejam recebendo terapia com antitrombóticos. Em pacientes com hepatopatias ou tumores, onde há reduzido número de plaquetas, proteínas e fatores de coagulação, o uso do selante de fibrina diminui grandemente o risco de hemorragias. Assim como em cirurgias hepáticas, evita o vazamento biliar pós-cirúrgico (FERGUSON; NURNBERGER; REDL, 2010; DUNN; GOA, 1999; KRAM *et al.*, 1988).

2.2.1.2 Selamento

É amplamente relatado o uso da cola de fibrina para selar defeitos, incisões, ressecções e anastomoses de vários órgãos dos sistemas respiratório, cardiovascular, gastrintestinal, geniturinário e ocular.

Selamento de suturas traqueo-bronquiais e pulmonares, incluindo ressecções parciais da traquéia e brônquios principais, têm sido realizados eficientemente com o uso da cola de fibrina. Em reconstruções traqueais, autores relatam estabilidade, selamento total ao vazamento de ar, suporte na cicatrização, diminuição significante no número de suturas na

anastomose e boa compatibilidade local e sistêmica do biopolímero (ORLOWSKI *et al.*, 1986; SILVER; WANG; PINS, 1995a).

Em cirurgia cardiovascular, além da hemostasia em sangramentos de baixa pressão, o selante tem sido utilizado em foco de hemorragias microvasculares e sobre linhas de sutura, anastomoses ou locais de punção. Diferentes procedimentos cirúrgicos têm sido favorecidos, incluindo implantação de válvulas, enxertos vasculares e dissecções aórticas e de aneurismas (KJAERGARD; FAIRBROTHER, 1996; RADOSEVICH *et al.*, 1997).

A cola tem sido usada eficientemente no fechamento de fístulas entero-cutâneas e selamento de anastomoses esofagogástricas e colo-retais, perfurações intestinais e rupturas peritoneais (ALABALA *et al.*, 2006). Em estudo com animais, a anastomose de cólon com o uso do selante foi comparado à sutura em dupla camada. O autor relata que na avaliação após sete dias, não houve diferença significativa na força de ruptura entre as anastomoses com e sem suturas (KJAERGAARD *et al.*, 1987). Sua aplicação no recobrimento de defeitos de órgãos abdominais de ratos diminuiu a força das aderências intraperitoniais em comparação com o grupo controle (LINDENBERG; LAURITSEN, 1986).

Em urologia e ginecologia, o selante tem permitido a rafia de aberturas e anastomoses com menor número de suturas e se mostrado útil no reparo de perfurações vesicais, anastomoses uretero-ureterais, reconstruções uretrais e ressecções transuretrais de próstata (MINTZ *et al.*, 2001; SALTZ *et al.*, 1991).

Em procedimentos oftálmicos, o adesivo de fibrina tem sido empregado no fechamento de fístulas esclerais, tratamento de descolamento de retina, extração de catarata extracapsular e restauração de lesões corneanas, conjuntivais e do canal lacrimal. Em estudo retrospectivo, relatou-se menor taxa de casos de deiscência em fechamento de córnea com o uso da cola de fibrina e menor desconforto relatado por pacientes em relação à sensação de corpo estranho, em comparação ao uso de suturas oculares (HOVANESIAN; BEHESNILIAN, 2007; SHARMA *et al.*, 2003; ZAUBERMAN; HEMO, 1988).

Ainda em procedimentos no crânio, tem sido utilizada para tratar rinorréia de fluido cérebro-espinhal após cirurgia de hipófise e empregada no reparo de perfurações da membrana timpânica, sob visualização microscópica (ALBALA *et al.*, 2006; DUNN; GOA, 1999).

2.2.1.3 Suporte adesivo

A cola de fibrina tem sido também utilizada para união de bordas teciduais, diminuição de espaço morto e fechamento de aberturas ou fístulas em diferentes áreas *cirúrgicas* (SALTZ *et al.*, 2001).

Para fechamento de hérnias, a cola de fibrina tem sido aplicada junto a telas com ou sem suturas, em procedimentos convencionais e laparoscópicos. É relatado que com o número menor de suturas, os pacientes tendem a sofrer menos dor pós-operatória. (MINTZ *et al.*, 2001; REISS; OZ, 1996).

Na neurocirurgia, o selante tem sido utilizado para reparar nervos periféricos, incluindo plexos braquiais e tronco principal, com indicação de que não há impedimento de crescimento axonal através da anastomose (SUN *et al.*, 2010). A aplicação é normalmente feita por meio do recobrimento do local lesionado. No entanto, em estudo com ratos, a aplicação entre segmentos do nervo facial intratemporal, observou-se que, embora possa ter havido obstrução mecânica da cola entre os seguimentos, esta não demonstrou efeito proibitivo significante na regeneração do nervo HYATT; WANG; KWOK, 2010).

Em procedimentos ortopédicos, seu emprego tem sido auxiliado no tratamento de diferente lesões musculoesqueléticas, fortalecendo adesão entre segmentos de tendões, cartilagem e osso e fragmentos ósseos. No entanto, nestes casos, reforços estruturais são geralmente promovidos por outros materiais. O selante tem demonstrado promover uma superfície mais lisa e com menos adesões em cirurgias de tendão, maior indução de crescimento ósseo com o uso de enxertos e a formação de camada protetora contra deslocamento de fragmentos ósseos a estruturas nobres (HASHIMOTO *et al.*, 1992; REISS; OZ, 1996; SCHWARZ *et al.*, 1989; SILVER; WANG; PINS, 1995a; YOU *et al.*, 2007).

Em cirurgias reconstrutivas, especialmente em áreas de maior tensão, tem permitido diminuir o número de suturas, atenuar a tensão em retalhos, prevenir edema e hematomas, melhorar a cicatrização e minimizar a formação de cicatriz. Tem demonstrado também ser eficiente na eliminação do espaço morto e na promoção de maior pega de enxertos de pele por aumentar a probabilidade de vascularização. (ROSSI *et al.* 2009; HERMETO *et al.*, 2012; SALTZ *et al.*, 1991).

2.2.1.4 Matriz biológica e sistema de liberação lenta de substâncias

A fibrina é considerada uma opção ideal para matriz biológica e arcabouço celular. Sua estrutura e comportamento biológico permitem que fatores de crescimento ou medicações sejam liberados lentamente e que células se fixem e proliferem na área lesionada. Ainda, com a sua degradação gradativa, espaço é liberado para crescimento de novo tecido (SIERRA, 1993; SPICER; MIKOS, 2010).

Antibióticos são liberados da fibrina por difusão simples, o que depende bastante do gradiente de concentração entre o coágulo e o ambiente em que se encontra. Incorporados à fibrina, os antibióticos são retidos por maiores períodos que quando instilados diretamente em cavidades corpóreas. Ainda assim, são retidos por períodos menores que em cimentos ósseos, não permanecendo mais que o tempo indicado. Alguns antibióticos, contudo, podem interferir na coagulação da fibrina, no entanto, a suplementação com fator XIII pode diminuir esse efeito em alguns casos (REDL *et al.*, 1982; FERGUSON; NURNBERGER; REDL, 2010).

Alguns fatores de crescimento, como fator básico de crescimento de fibroblastos (bFGF) e fator de crescimento endotelial vascular (VEGF), e outras moléculas bioativas têm sido incorporados a matrizes de fibrina. Assim, esses peptídeos acabam sendo liberados de acordo com a degradação do arcabouço, sincronizando a degradação da cola e remodelação da lesão com a regeneração tecidual (HASHIMOTO *et al.*, 1992; MOSESSON; SIEBENLIST; MEH, 2001). Já se tem demonstrado que arcabouços de fibrina com esses fatores têm melhorado a angiogênese e revascularização (SAHNI; FRANCIS, 2000). Fatores de crescimento neuronal (NGF, NT-3) e quimioterápicos, como o 5-fluorourazil, o taxol e a carboplatina, também têm sido incorporados à fibrina como sistema de liberação em diferentes estudos (SALTZ *et al.*, 1991; NURNBERGER *et al.*, 2010; SPICER; MINKOS, 2010).

2.2.2 Componentes da cola

A cola é obtida essencialmente pela mistura de uma solução de fibrinogênio, a proteína coagulável, e uma solução de trombina, a enzima ativadora da formação do coágulo. Além desses, para a formação do coágulo, precisam estar presente o cálcio, que é adicionado

à fração de trombina, e o fator XIII, que é geralmente precipitado com o processamento do fibrinogênio. Certos aditivos, como antifibrinolíticos, são algumas vezes adicionados à formulação para retardar a fibrinólise (SIERRA, 1993; WOSNIAK, 2003).

2.2.2.1 Fibrinogênio

O fibrinogênio é o componente base para o início da reação que formará a cola de fibrina. É solúvel no sangue e corresponde em 0,2% do volume sanguíneo total. Sua concentração final na cola influencia a elasticidade e aderência aos tecidos (FERGUSON; NURNBERGER; REDL, 2010). Para a obtenção do fibrinogênio, distintas formas de precipitação a partir do sangue determinam concentrações finais diferentes, variando desde 10 até 115mg/mL na solução (REISS; OZ, 1996).

A crioprecipitação é considerada o método "padrão-ouro" por resultar em maiores concentrações de proteína coagulável. No entanto, é o que exige mais tempo e quantidade de sangue para o processamento. Considerando que este método, que é também o mais utilizado na fabricação das colas comercialmente disponíveis, necessite de um ou mais doadores, o risco de transmissão de doenças infecciosas precisa ser minimizado por intensa triagem da inocuidade microbiológica do sangue colhido e técnicas de inativação de possíveis agentes. Isso acarreta complexidade e custo ao processamento (SPOTNITZ, 2014; SILVER; WANG; PINS, 1995b; SPOTNITZ *et al.*, 1987).

Quando a disponibilidade de sangue é limitada ou a necessidade imediata, podem ser utilizados outros processos de precipitação de fibrinogênio a partir de sangue autólogo, ou de um doador apenas. Dentre as alternativas estão os métodos de precipitação química ou proteica e formulações com plasma rico em plaquetas. Contudo, esses protocolos fornecem, em diferentes graus, menores concentrações finais de fibrinogênio em comparação com o método de crioprecipitação e colas comerciais. Alguns dos processos químicos ainda possuem limitações por risco de toxicidade (ALSTON *et al.*, 2007; RADOSEVICH *et al.*, 1997; YOU *et al.*, 2007).

Na Medicina, as colas produzidas em centros hospitalares apropriadamente equipados são mais baratas e prontamente disponíveis que as comerciais. No entanto, possuem a desvantagem da variável, e comumente menor, qualidade do produto e consequente inconsistência do desempenho clínico (MINTZ *et al.*, 2001; SPOTNITZ; BURKS, 2012).

2.2.2.2 Trombina

A trombina atua sobre o fibrinogênio para formar os monômeros de fibrina e indiretamente na estabilização da malha resultante. Ativando também o fator XIII, este catalisará a formação de ligações cruzadas, ou covalentes, entre aminoácidos, estabilizando o polímero (MOSESSON; SIEBENLIST; MEH, 2001).

Uma cola com menor concentração de trombina (4–6 IU/mL) promove polimerização mais lenta, permitindo uma melhor pré-mixagem e manipulação. Isso permite que o cirurgião tenha mais tempo para adaptar e posicionar tecidos, como um enxerto de pele, por exemplo. Já a pulverização da cola com altas concentrações de trombina (500 IU/mL) confere um rápido e eficiente estabelecimento da hemostasia (FERGUSON; NURNBERGER; REDL, 2010).

2.2.2.3 Cálcio

O Fator XIII, que é ativado pela trombina, depende de cálcio para a catálise da reação que formará as ligações covalentes responsáveis pela estabilidade do polímero formado. Desta forma, a presença de cálcio é importante para o desempenho biomecânico da cola (WOSNIAK, 2003).

Para um nível adequado de ligações cruzadas, é necessário a adição de $CaCl_2$ em concentrações mínimas de 5mM, sendo comumente utilizadas concentrações de 10 a 50mM. A adição de cloreto de cálcio aumenta a velocidade de formação do coágulo, mas retarda em até 15 minutos o alcance máximo de sua adesão tecidual (ALSTON *et al.*, 2007; MOSESSON; SIEBENLIST; MEH, 2001).

2.2.2.4 Fator XIII

Além da atuação na estabilização da rede de fibrina, conferindo resistência elástica à cola, há estudos indicando que o fator XIII promove ligações cruzadas da fibrina com o colágeno, proporcionando maior força adesiva aos tecidos. O fator XIII também promove ligações cruzadas da fibrina com a fibronectina, um glicoproteína plasmática importante na adesão e crescimento celular (ALSTON *et al.*, 2007; REDL; SCHLAG, 1984).

Embora recuperado com o fibrinogênio, em até aproximadamente 50% de sua concentração plasmática (10μg/mL), o fator XIII pode ser adicionado à solução concentrada de fibrinogênio no intuito de aumentar a elasticidade (ALSTON *et al.*, 2007). Há relatos da adição do fator XIII buscando retardar a lise do coágulo, especialmente em locais onde as enzimas fibrinolíticas estão presentes em altas concentrações, como órgãos parenquimatosos e vasos sanguíneos (SIERRA, 1993).

2.2.2.5 Antifibrinolíticos

A fibrinólise ocorre por mecanismos de degradação enzimática ou atividade fagocítica. Enzimas proteolíticas estão presentes no próprio coágulo, em tecidos corporais e podem ainda ser liberadas por células inflamatórias (MOSESSON; SIEBENLIST; MEH, 2001).

A velocidade de degradação da cola depende do órgão ao qual foi aplicada, espessura da camada colocada e quantidade de plasminogênio precipitado durante sua produção. A atividade fibrinolítica é mais alta em órgãos parenquimatosos, vasos sanguíneos e espaços pleurais. Uma camada mais espessa de cola tende a demorar mais para ser degradada, mas dependendo da configuração tecidual à qual foi aplicada, pode retardar o processo de reparação tecidual (FERGUSON; NURNBERGER; REDL, 2010; SIERRA, 1993).

Graus de inibição da fibrinólise podem ser alcançados com a adição de substâncias como a aprotinina e os ácidos tranexâmico e epsilonaminocapróico. A aprotinina, uma inibidora natural de proteases, tem se mostrado mais eficiente que outros agentes sintéticos. Na produção de selante com protamina, a adição de aprotinina ou ácido tranexâmico não alterou as propriedades mecânicas do produto (ALSTON *et al.*, 2007). O ácido tranexâmico em aplicação subdural provocou convulsões em ratos, sendo contraindicado em cirurgias neurológicas. Como a atividade proteolítica se dá também por proteases leucocitárias, inibidores de elastase ou catepsina G também podem prevenir a lise prematura do coágulo (FERGUSON; NURNBERGER; REDL, 2010; REISS; OZ, 1996).

As variações dos protocolos relatados na literatura indicam que a escolha por adição das diversas substâncias na produção da cola, bem como suas concentrações, vai depender do órgão ao qual vai ser aplicada e do objetivo esperado com seu uso.

2.2.3 Formas de aplicação

A cola pode ser utilizada em diferentes formas, sendo as mais comuns a líquida, como aerossol e em telas de colágeno. No tratamento de lesões superficiais, a cola é utilizada em forma de spray, com uma fina camada sobre o defeito, ou mesmo com injeção dentro ou sobre a lesão. Em lacerações profundas, a cola é injetada distendendo levemente a região para promover um efeito hemostático e tamponante. Para lesões de espessura completa, a cola é injetada dento e sobre o defeito antes do fechamento de suturas previamente colocadas para aproximar os bordos da ferida (FERGUSON; NURNBERGER; REDL, 2010; SILVER, 1995a).

Ainda que a cola de fibrina possua muitas vantagens no selamento de defeitos e controle de hemorragias não possui rigidez suficiente para atuar como reforço em locais onde carga mecânica é solicitada, pois mesmo forças pequenas e movimento tecidual pode resultar em falência. Além disso, os efeitos selantes da fibrina são considerados mais importantes nos primeiros dias após o trauma, durante as fases de proliferação e granulação da cicatrização (SALTZ *et al.*, 1991; SIERRA, 1993).

2.3 Uso de cola de fibrina na veterinária

O uso da cola de fibrina em animais tem servido principalmente como modelo para estudo da cola de origem humana (PORTILLA-DE BUEN *et al.*, 2014; PARK *et al.*, 2011; ALSTON *et al.* 2008; FALSTROM *et al.*, 1999; HASHIMOTO *et al.*, 1992). Aplicações em equinos têm sido descritas com produtos comerciais e a partir de sangue autólogo buscando a adesão de enxertos de pele e córnea e em procedimentos ortopédicos, incluindo uso como veículo de transporte para células-tronco (TEXTOR *et al.*, 2014; HALE *et al.*, 2012; ROSSI *et al.*, 2009). Embora a cola de fibrina esteja descrita em livros de cirurgia de pequenos animais (MACPHAIL, 2013; ANDERSON, 2012; FAHIE, 2012), poucos são os estudos com crioprecipitado a partir de plasma canino (WHEATON *et al.*, 1994) ou uso da cola comercial nesta espécie (HERMETO *et al.*, 2012; BLATTLER *et al.*, 2007).

Várias aéreas da cirurgia veterinária poderiam se beneficiar com o uso da cola de fibrina, incluindo áreas de pesquisa terapêutica (SPOTNITZ, 2014; VEDAKUMARI; SASTRY, 2014; NURNBERGER *et al.*, 2010; SPICER; MIKOS, 2010). No entanto, o custo

e o uso de componentes humanos dificulta o emprego e estudo do produto comercial. O aproveitamento dos benefícios com a utilização da cola de fibrina na cirurgia veterinária depende de pesquisas direcionadas às necessidades relevantes dos animais e de protocolos exequíveis e que resultem em soluções cuja composição possa ser adaptada ao uso pretendido. Este estudo buscou avaliar dois métodos de obtenção de fibrinogênio a partir das fontes de plasma canino mais disponíveis em bancos de sangue realizando análises *in vitro* e *in vivo* do coágulo resultante.

3 ARTIGO 1 – CRYOPRECIPITATION OF FIBRINOGEN FOR CANINE FIBRIN GLUE PRODUCTION WITH DIFFERENT TYPES OF PLATELET POOR PLASMA

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3.1 Abstract

Fibrin glue (FG) is considered a valuable tool in surgical settings for its hemostatic, sealing and adhesive properties. Cryoprecipitation has been considered the most suitable method for producing autologous and allogeneic human FG. However, different techniques with varying results have been published. This study evaluated the process of fibrinogen cryoprecipitation from different types of platelet poor plasma using 120mL-bag units and 50mL-tubes aiming canine FG production. Different aspects of the process were analyzed, including plasma sources, freezing temperatures and concentration capacities of the methods employed. Precipitation of fibrinogen was assessed by Clauss assay. Effect of freezing temperatures of -20°C, -80°C, or -196°C, prior to cryoprecipitation was carried out using 50mL-tubes. Results considering containers, plasma sources, and freezing temperatures were submitted to statistical analyzes. Fibrinogen measurements were used to estimate the efficiency of the cryoprecipitation process in different containers and after second cryoprecipitation cycles. Cryoprecipitation performed with older fresh frozen plasma units yielded significantly more fibrinogen than fresher ones. No difference was detected among freezing temperatures prior to cryoprecipitation. The ability to tailor final fibrinogen concentration, aiming FG production for surgical applications or research purposes, was considered an advantage of processing with 50-mL tubes. The cryoprecipitation methods performed to increase fibrinogen yield per initial plasma volume and in the final fibrinogen concentrated solution may improve efficiency of usage of the available resources. More than 6-month old blood bank plasma units, processed singly or fractioned, stand as good resources for obtaining

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fibrinogen concentrates to be used in canine FG.

Keywords: fibrin sealant, fresh frozen plasma, hemostasis, dog.

3.2 Introduction

Fibrin glue (FG), also referred as fibrin sealant, is a plasma-derived product widely used in surgery for hemostatic, adhesive, sealant, and wound healing support. FG facilitates clot formation independently of patient's coagulation factors content and enzymatic reactions and provides a local environment where cells can proliferate and differentiate efficiently (BORIN *et al.*, 2008; ANITUA *et al.*, 2006; ABIMARAN *et al.*, 2002). The utilization of FG has lately increased in several surgical specialties including cardiovascular, thoracic, minimally invasive, gastrointestinal, urologic, ophthalmic, neurosurgery, orthopedics, oncology, reconstructive, and plastic surgery (SPOTNITZ, 2014; KIN *et al.*, 2010; LIPPERT *et al.*, 2010; RICE; BLACKSTONE, 2010; ALBALA; LAWSON, 2006; COTHREN *et al.*, 2004; SHARMA *et al.*, 2003; BRADY *et al.*, 1993). Fibrin glue production and usage in hospitals and research settings has been widely reviewed and studied through retrospective, preclinical and clinical trials (SPOTNITZ, 2010; ALBALA; LAWSON, 2006; MACGILLIVRAY, 2003; JACKSON, 2001; REISS; OZ, 1996; SILVER; WANG; PINS, 1995b; SIERRA *et al.*, 1993). At hospitals, the usage of FG has been reported to reduce the need for re-operation due to bleeding or wound dehiscence and to decrease hematoma formation. In these studies, reduction in hospitalization time, morbidity, and even mortality rate due to decreased postoperative hemorrhagic events were also associated with use of FG (ZUCCHELLI; SILVESTRI, 2004; MINTZ *et al.*, 2001). In minimally invasive surgery, among other usages, hemostasis with FG has also been valuable for controlling nonsuturable and noncauterizable bleeding (PARK *et al.*, 2011; KLINGER *et al.*, 2006). Multi-center studies have reported that, by reducing blood loss, the employment of FG minimized the need for perioperative allogeneic blood transfusions and associated risks and morbidities (SPOTNITZ, 2014; DUARTE *et al.*, 2012; DHILLON, 2011; FATTAHI; MOHAN; CALDWELL, 2004; JACKSON, 2001; RADOSEVICH; GOUBRAN; BURNOUF, 1997). Moreover, due to its similarities to the extracellular matrix, and by acting as a cell carrier and source of growth factors, FG has been used and studied as cellular scaffolds and drug-delivery system for different clinical and experimental applications (VEDAKUMARI;

SASTRY, 2014; FERRIS *et al.*, 2012; KIM *et al.*, 2010; SPICER; MIKOS, 2010; MARONE *et al.*, 1999).

Fibrin glue is acquired by mixing a solution of thrombin and calcium with another solution of fibrinogen, as the last step of coagulation cascade pathway when a clot is formed (NURNBERGER *et al.*, 2010; BERING, 1944). Fibrinogen content dictates the gel consistency and is the major component affecting FG effectiveness for most surgical applications (KIM *et al.*, 2014; LACAZE *et al.*, 2012; KAETSU; TAKANORI; SHINYA, 2000). Although human commercial products are available, their inconsistent availability and high cost impair their use in veterinary surgery and research (KUNIO; SCHREIBER, 2013; SPOTNITZ; BURKS, 2012; MINTZ *et al.*, 2001).

Cryoprecipitation is considered the 'gold standard' method for producing FG from either pooled or donor blood (FERGUSON; NURNBERGER; REDL, 2010; SPOTNITZ, 1987). Nevertheless, different cryoprecipitation protocols may yield diverse and variable fibrinogen concentration results. This fact is often associated with the cryoprecipitation techniques, mostly affected by initial plasma volume, centrifugation regimens, and final concentrated solution volumes (ABRAMS-OGG; SCHNEIDER, 2010; GIBSON, 2007; BRENNAN, 1991). Additionally, temperatures of freezing and plasma sources have varied among studies and may have accounted for some differences seen with the resultant FG (EHRENFEST; RASMUSSON; ALBREKTSSON, 2008; REISS; OZ, 1996; SILVER; WANG; PINS, 1995b). During separation of cellular components from blood, the platelet poor plasma can also be subjected to different processing and environmental variations. The influence of all these supposedly subtle modifications on the final FG product is poorly defined (NG *et al.*, 2013; KUNIO; SCHREIBER, 2013; VALBONESI, 2006; RADOSEVICH; GOUBRAN; BURNOUF, 1997).

Moreover, the shortage of veterinary blood banks and adequate blood donor population limits the access to plasma for production of canine FG (BROOKS, 2010; YAXLEY *et al.*, 2010). The unavailability of the specific equipment needed, which is usually found only in blood banks facilities, may also restrict cryoprecipitation processing. Increased knowledge on efficient methods of fibrinogen precipitation for FG production may facilitate studies aimed at improving FG performance and clinical applications. This study evaluated the feasibility of the production of concentrated fibrinogen solutions by cryoprecipitation using distinct plasma categories and different processing techniques. Further maneuvers for

increasing total fibrinogen yield per mL of fibrinogen solution were also explored. Identified difficulties during the cryo process and implemented adjustments are additionally discussed.

3.3 Materials and Methods

3.3.1 Canine Plasma Resources

All canine plasma units were from licensed Veterinary Blood Banks[3, 4] and obtained from registered and appropriately screened donor dogs meeting the standard guidelines for collection and blood processing techniques (ABRAMS-OGG; SCHNEIDER, 2010). The study was submitted and approved by an ethics commission of research[5] according to national and international regulations (COBEA, Brazilian Law 11,794/November 8th, 2008).

All plasma categories used in direct comparisons of fibrinogen yield were from the same blood bank and processed through the same protocol for separation of blood cellular components. In brief, separation of plasma from whole blood was performed by two sequential centrifugation series using a four-bag closed system (Terumo Medical®). The first centrifugation (2,700 *x g*, for 3 minutes, at 22°C) was carried out to isolate the erythrocytes lineage and the second one (4,000 *x g*, for 5 minutes, at 22°C) to separate the platelet concentrate. The supernatant of the second centrifugation consisted of platelet poor plasma and was transferred to one or two satellite bags.

The platelet poor plasma unit was either frozen in bags at the blood bank (single unit of 100 to 220mL[2] or two subdivisions of 120-mL unit[1]) or further divided into 50-mL Falcon tubes in the laboratory where they were subsequently frozen. While all plasma bags were frozen at temperatures equal to or lower than -30°C, 50-mL tubes were frozen at -80°C, unless otherwise stated.

3.3.2 Plasma Categorization

For scientific purposes, the standard classification of fresh frozen plasma and frozen

[3] Blue Ridge Veterinary Blood Bank, Purcellville, VA, USA (Privately owned veterinary blood bank)
[4] Blut's, Porto Alegre, RS, Brazil (Privately owned veterinary blood bank)
[5] Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil – registration number 24362, PROPESQ approval: March 14th, 2013; CEUA approval: April 11th, 2013

plasma, were further categorized in this study. The plasma categories studied included fresh plasma (FR), consisting of plasma obtained within 8 hours from blood collection; fresh frozen plasma (FFP), which was frozen within 8 hours from blood collection and stored for less than a year; expired fresh frozen plasma (eFFP), which consisted of plasma frozen within 8 hours from blood collection but stored for more than a year; and, frozen plasma (FP), which was frozen after 8 hours from collection and was also stored for more than a year.

For comparing cryoprecipitation among plasma types most often available in veterinary blood banks, cryo-processing was performed in 120-mL bags with FFP (6-8 month-old, n=9), eFFP (12-20 month-old; n=9), and FP (18-22 month-old; n=9). Cryoprecipitation of plasmas categories that would be available to veterinary hospitals either as autologous or allogeneic unit collected on-site, or as a bag-unit purchased from a veterinary blood bank, was carried out in 50-mL tubes with FR (less than a week-old; n=4) and FFP (6-10 month-old; n=8).

3.3.3 Cryoprecipitation with Bags and Tubes

In both bags[6] and tubes[7], cryoprecipitate was produced by slowly thawing the plasma at 2°C and, when only flocculation could be seen in almost completely absence of ice crystals, the plasma was centrifuged at 4,000 *x g*[8, 9] for 10 minutes, at temperatures set at 2°C, oscillating between 1 and 3°C (SILVER; WANG; PINS, 1995a; BRENNAN, 1991). For centrifugation, bags were held in a straightened and up-right position, within the bucket, by being tied around a second water-filled bag, previously refrigerated. After centrifugation, as the precipitated content remained at the bottom of the bag, the resultant supernatant plasma was transferred to a satellite bag through squeezing the unit in a blood extractor. With 50-mL tubes, the supernatant was poured out until 1.6mL of supernatant was left with the cryoprecipitate.

After dilution of the cryoprecipitate, the cryo final volume was determined and

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[6] Cornell University Hospital for Animals and Animal Health Diagnostic Center, Cornell University, Ithaca, NY, USA

[7] REPROLAB, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

[8] refrigerated centrifuge CR412, M4 swing-out rotor (USA)[1]

[9] refrigerated centrifuge Thermo® ALC-PK 121R, AM-10 rotor (BRA)[2]

samplings were collected for analysis. Duplicate aliquots containing 0.5 to 1mL of initial plasma, supernatant, and cryo-precipitated solutions were stored at -20°C for Clauss assay. The remaining cryo-precipitated and supernatant solutions were refrozen in 50-mL tubes for further processing techniques to increase final fibrinogen yield. Randomized cryoprecipitate samples that were processed in tubes (n=4) were collected for standard microbiological culture.

3.3.4 Freezing Temperatures prior to Cryoprecipitation

The effect of freezing temperature on cryoprecipitation was evaluated by transferring plasma from FFP bags to 50-mL tubes by a quick thawing-freezing cycle (37°C, in water bath, for 10-15 minutes) prior to cryo-processing (YAXLEY *et al.*, 2010). Samples were refrozen either at -20°C for 24h (n=3), -80°C for 18h (n=3), or -196°C for 5 minutes by immersion in liquid nitrogen followed by a 2-hour period at -20°C (n=4) (SILVER; WANG; PINS, 1995b). The FFP units were about 8 to 10-month old and had at least one 50-mL sample subjected to every freezing temperature studied.

3.3.5 Extra Concentration Procedures

A second cryoprecipitation cycle was performed with some cryoprecipitate and supernatant from bags. These approaches were intended to increase the fibrinogen concentration in the final solution and the productivity with the available material (SILVER; WANG; PINS, 1995a). These additional cycles were performed in 50mL-tubes as described above for different plasma categories. The condensed material from these second cryo cycles was redissolved in 0.8mL of the supernatant from each process, to achieve a minimum final volume of 1mL.

3.3.6 Fibrinogen Concentration Measurement

Clauss method[10, 11] was used for assessment of fibrinogen content in plasma prior to

[10] automated, photo-optical density system, Stago® (USA)
[11] semi-automated, mechanical-magnetic system, Stago® (BRA)

cryoprecipitation process, cryo-precipitated solution, supernatant, cryo of cryo-precipitated solution, and cryo of supernatant.

Prior to Clauss, aliquots were thawed in water-bath at 37^{o}C for 3 minutes. When needed, the concentrated fibrinogen solutions were diluted prior to the assay (1/2 to 1/3^{8} or 1:20 to 1:40^{9}). The assays were performed in batches of 20 to 40 samples at each time, with the calibration curve being reestablished at every day of measurement.

3.3.7 Feasibility of Cryoprecipitation with High and Low Volumes of Plasma

Cryo processing for FG production in each of the containers used (120mL-bags and 50-mL tubes) was evaluated considering the concentration capacity (mg/mL of fibrinogen), the possible amount of aliquots attained with a minimum concentration of 10mg/mL of fibrinogen from a given initial plasma volume (considering the average of 240mL of platelet poor plasma from one unit of 450mL of blood), and the total fibrinogen precipitation (%; total final fibrinogen content/total initial fibrinogen content).

3.3.8 Statistical Analyses

Clauss results were tested for normality using Komogorov-Smirnov Test, considering the different configurations analyzed. A General Linear Model of ANOVA was used to detect differences among groups. When required, Tukey test was used as post hoc analysis of differing groups.

Pearson correlation test (r^2) was performed to determine whether a significant linear relationship existed between initial and final fibrinogen concentration, volume of cryoprecipitate from bags and final fibrinogen concentration, and initial volume in tubes and final fibrinogen concentration.

The intra-assay coefficient of variation of Clauss method for measuring cryo-precipitated solutions was based on analyses of duplicates performed in the same day, in different days, and in different dilutions (*SD/mean;* n=4).

Minitab and SAS Platforms were used for both descriptive and statistical analyses. Differences with a *P* value <0.05 were considered significant.

3.4 Results

3.4.1 Canine Plasma Category

Plasma types processed in bags were compared separately to those processed in tubes. Fibrinogen yield obtained with cryoprecipitation of different categories of canine plasma with 120mL-bags are summarized in table 1. Plasmatic fibrinogen concentrations prior to cryoprecipitation process and fibrinogen content in the supernatant solutions are also shown. Differences among plasma categories processed in 120-mL bags were assessed within specific ranges of final cryo volumes (Figure 1).

Table 1 – Fibrinogen yield obtained with cryoprecipitation of different categories of canine plasma processed in 120mL-bags.

Plasma Source	*Mean Concentration (mg/mL)*	*± SD*	*n*	*Concentration Range (mg/mL)*	*Quantity of Cryo with 10mg/mL**	*Total Precipitation*
120-mL Bag						
FFP						
Initial plasma	1.93	0.70	8	1.05 - 3.12	-	-
Supernatant	1.57	0.27	3	1.30 - 1.84	-	-
Cryo - 6 to 16mL	**7.11** [b]	**2.22**	**7**	**5.42 - 11.22**	12-30mL/unit	22 to 37%
eFFP						
Initial plasma	1.95	0.73	5	0.90 - 2.75	-	-
Supernatant	-	-	0	-	-	-
Cryo - 6 to 16mL	**11.04** [a]	**2.35**	**6**	**7.14 - 13.92**	12-30mL/unit	27 to 51%
FP						
Initial plasma	1.95	0.53	4	1.37 - 2.09	-	-
Supernatant	1.88	0.55	4	1.41 - 2.67	-	-
Cryo - 6 to 16mL	**10.27** [ab]	**2.43**	**6**	**6.48 - 12.96**	12-30mL/unit	23 to 31%

Fibrinogen measurements performed by Clauss method. Plasmatic fibrinogen concentrations prior to cryoprecipitation process and fibrinogen content in cryo-supernatant solutions are also presented. Solutions of different plasma types were compared. Superscripts (a, b) indicate statistical difference among cryoprecipitate from the plasma types processed in 120-mL bags within the range of 6 to 16mL of final volume (FFP *versus* eFFP: *P*=0.020; FFP *versus* FP: *P*=0.065; eFFP *versus* FP: *P*=0.836). **FFP:** fresh frozen plasma (6-8 month-old); **eFFP:** expired fresh frozen plasma (12-20 month-old); **FP:** frozen plasma (18-22 month-old); *SD*: standard deviation; *n*: number of samples.
***** Estimation considering a standard unit with 450mL of blood, yielding 240-mL bag of platelet poor plasma.

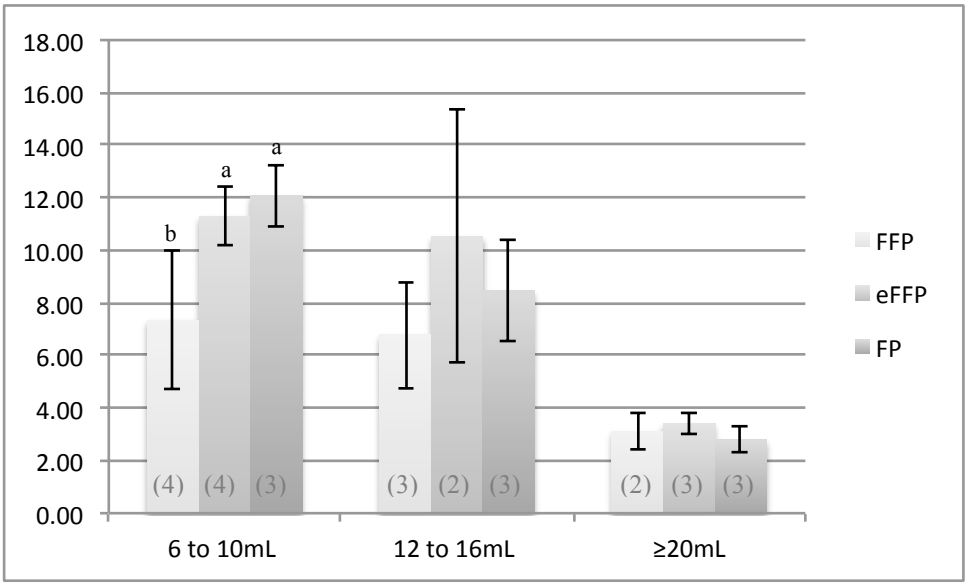

Figure 1 – Fibrinogen concentration in distinct ranges of cryoprecipitate final volumes after processing of 120mL-bags with different categories of canine plasma.

Method of measuremnt: Clauss. Statistical evaluation was performed to compare cryoprecipitate of different plasma types processed in 120-mL bags within a same final volume range. Letters **a** and **b** indicate statistical difference among plasma types (P<0.05). Numbers in parenthesis indicate amount of samples. **FFP**: fresh frozen plasma (6-8 month-old); **eFFP**: expired fresh frozen plasma (12-20 month-old); **FP**: frozen plasma (18-22 month-old).

In volumes of 6 to 16mL, eFFP yielded more fibrinogen in cryoprecipitate than FFP (P=0.02). Within the range of 6 to 10mL of final volumes, FFP cryo-precipitated solutions had less fibrinogen than those obtained with eFFP and FP (P=0.03 and P=0.02, respectively). Between eFFP and FP there was no statistical difference regarding fibrinogen yield in any final volume. In ranges above 15mL of final volume, no difference could be detected among any type of plasma.

The fibrinogen content in initial plasma and in the supernatant of cryo did not differ statistically between themselves in each plasma category, and neither among plasma types. Supernatants of eFFP were not measured. With cryoprecipitation performed in bags, fibrinogen concentration in the cryoprecipitate had a certain level of positive correlation with the initial plasmatic concentration of fibrinogen (Pearson correlation, r^2=0.57, P=0.03). As expected, there was a more significant negative correlation between fibrinogen concentration in the cryoprecipitate and the final volume of cryo in bags r^2=-0.56, P=0.01).

Fibrinogen yield obtained with cryoprecipitation of different categories of canine plasma processed in 50mL-tubes are presented in table 2. Cryoprecipitation performed in 50-mL tubes with FFP yielded significantly more fibrinogen than FR (P<0.001). With cryoprecipitation of FR and FFP in tubes, fibrinogen concentration in the cryoprecipitate did not correlate with the initial plasmatic concentration of fibrinogen.

Table 2 – Fibrinogen yield obtained with cryoprecipitation of different categories of canine plasma processed in 50mL-tubes.

Plasma Source	*Mean Concentration (mg/mL)*	*± SD*	*n*	*Concentration Range (mg/mL)*	*Quantity of Cryo with 10mg/mL**	*Total Precipitation*
50-mL Tube						
FR						
Initial plasma	1.42	0.49	2	1.07 - 2.31	-	-
Supernatant	1.46	0.27	2	1.27 - 2.47	-	-
Cryo - 2mL	**4.67** [x]	**0.41**	**4**	**4.30 - 5.50**	5mL/unit	9 to 16%
FFP						
Initial plasma	1.93	0.45	5	1.19 - 2.20	-	-
Supernatant	1.13	0.87	3	0.14 - 2.02	-	-
Cryo - 2mL	**15.14** [y]	**3.10**	**8**	**12.10 - 19.40**	10-20mL/unit	23 to 53%
Supernatant of FFP						
Initial supernatant	1.51	0.40	4	1.08 - 1.87	-	-
Cryo of supernatant - 1mL	**8.97**	**2.48**	**7**	**6.03 - 12.89**	2-4mL/unit	10 to 20%

Fibrinogen measurements performed by Clauss. Plasmatic fibrinogen concentrations prior to cryoprecipitation process, fibrinogen content in cryo-supernatant solutions, and results of second cryoprecipitation cycle of supernatant are also presented. Superscripts (x, y) indicate statistical difference among plasma types processed with 50-mL tubes. Differences were considered significant when P value was <0.05. **FFP:** fresh frozen plasma (6-10 month-old); **FR:** fresh plasma (<1 week-old); *SD*: standard deviation; *n*: number of samples.

* Estimation considering a standard unit with 450mL of blood, yielding 240mL of platelet poor plasma.

3.4.2 Effect of Freezing Temperatures prior to Cryoprecipitation

No significant differences in fibrinogen yield were observed among plasma counterparts that were frozen at -20°C, -80°C, or -196°C/20°C for periods of 24h, 18h, or 2h, prior to cryoprecipitation (Table 3).

Table 3 – Effect of temperature of freezing on cryoprecipitation of thawed canine fresh frozen plasma

Freezing Temperature	Freezing Time prior to Cryoprecipitation	*Mean (mg/mL)**	± *SD*	*n*	*Range (mg/mL)*
-20°C	**24h**	14.99	4.01	3	12.50 - 18.12
-80°C	**18h**	15.30	2.81	3	11.47 - 19.35
-196°C	**5' (+2h at -20°C)**	15.83	4.48	4	10.96 - 21.68

Fibrinogen measurements by Clauss from cryo-precipitated solutions obtained with different freezing temperatures and periods prior to concentration process. The plasma category used was 8 to10-month old fresh frozen plasma (FFP[2]) stored and thawed in bags and then refrozen and processed in 50-mL tubes. Ressuspention was performed in 1.6mL of supernatant. Mean, standard deviations (± *SD*), minimum and maximum values, and number of samples (*n*) are presented.
*No statistical difference was detected among different freezing temperatures (P>0.05).

3.4.3 Efficiency of Cryoprecipitation

The main parameters considered for estimation of the cryoprecipitation efficiency, of each processing and volumes studied, are presented in table 1 and table 2 according to the container used.

Considering the final volumes in the range 6 to 16mL, which yielded the highest fibrinogen contents, the highest concentration measurement obtained with 120-mL bags was about 14mg/mL (eFFP). The mean fibrinogen concentration of all plasma types in final volumes of 6 to 16mL was 9.34mg/mL (*SD*=2.83). Volumes in the ranges of 6 to 10mL/bag and 12 to 16mL/bag did not differ significantly among their fibrinogen content in either plasma category or with all types together. Final volumes above 20mL yielded statistically less fibrinogen per mL of cryo than volumes equal or lower than 16mL ($P<0.001$). With 50-mL tubes, in final dilutions of 2mL per tube, final concentrations varied from 4mg/mL to 22mg/mL, considering FR, FFP, and thawed-refrozen FFP.

Following strict criteria to consider a centrifugation process as adequate, the capacity of fibrinogen cryoprecipitation with bags ranged from 22% to 51% in final volumes of 6 to 16mL, considering all plasma types. In this range of final volumes, FFP had a mean of fibrinogen precipitation of 30.63% (*SD*=7.46), eFFP showed a mean of 41.33% (*SD*=10.17),

and FP a mean of 17% (*SD*= 5.66). With 50-mL tubes, in final volumes of 2mL, the mean precipitation capacity of FR was 14% (*SD*=4.6) and of FFP was 32% (*SD*=12.09).

Cryo-precipitation of cryo-supernatant, performed in 50-mL tubes and dilution into 1mL, produced final concentrations with a mean of 9mg/mL. Fibrinogen yield of cryoprecipitation of supernatants did not correlate with initial fibrinogen concentration. Second cryoprecipitation cycle in 50-mL tubes of cryo solutions obtained from bags (10 to 25mL), generated fibrinogen concentrations up to 31mg/mL, being able to concentrate, at the most, three times the initial fibrinogen content (*n*=6; data not shown).

3.5 Discussion

Cryoprecipitation is considered the safest and most cost-effective method for producing FG with plasma sources from blood banks or single donors (BRENNAN, 1991; SPOTNITZ *et al.*, 1987). With this method, large amounts of concentrated fibrinogen solutions can be prepared and additional freezing-thawing cycles can further increase the final fibrinogen content (REISS; OZ, 1996; SILVER; WANG; PINS, 1995a). Blood bank plasma products, after separation of packet red blood cells (pRBC), are an important resource for fibrinogen cryoprecipitation. Hospitals and laboratory settings may also be able to alternatively process cryo with adapted techniques using simpler equipment and smaller volumes (SILVER; WANG; PINS, 1995a). This can be beneficial in circumstances of blood donors or blood bank-produced plasma scarcity (BROOKS, 2010; ISAACSON; HERMAN, 1996). Thus, such adaptations could allow on-site autologous or allogeneic production for a planned surgery, even from a small sized dog (ABRAMS-OGG; SCHNEIDER, 2010). Fibrinogen content produced through different methods of cryoprecipitation intended for FG production from canine plasma was evaluated through a quantitative, clot-based, functional assay.

3.5.1 Effect of Canine Plasma Categories

Older plasma sources showed comparable or better fibrinogen precipitation to that of fresher plasmas with cryo performed with bags and tubes. Veterinary blood banks in many countries follow the human guidelines for processing and storing blood products (AABB;

GENOVEZ, 2008; BRECHER, 2005). According to these standards, after blood collection and separation of cellular components, platelet poor plasma can receive different classifications according to storage procedures. The main categorizations of frozen platelet poor plasma consider the time elapsed from whole blood collection to plasma freezing, the period the unit has been stored frozen, and whether it has been thawed and refrozen. Platelet poor plasma is only considered FFP if it was frozen within 8 hours from collection, has been stored at -18ºC or lower temperatures for less than a year from collection, and no planned or incidental thawing has occurred. If any of these requisites is unfulfilled, the FFP is downgraded to FP category (ABRAMS-OGG; SCHNEIDER, 2010; GIBSON, 2007). These classifications take into account the concerns with factor VII's undesired activation and, mainly, the stability of labile coagulation factors V and VIII when the blood plasma remains for more than 8 hours in room temperature or stays frozen for over a year (BROOKS, 2010). These events are considered more detrimental to situations when the plasma is intended for transfusion therapy of congenital or acquired deficiencies of, or related to, factor V and VIII (HOGAN; BROOKS, 2010). Similarly to most of the factors in the coagulation cascade, these factors are important in the thrombin generation pathway (SMITH, 2010; GENTRY; BURGESS; WOOD, 2008). For FG production, however, activated thrombin is provided, so, these factors are not considered of utmost importance. Fibrinogen, however, is crucial for effectiveness of FG in all the intended surgical applications (DUARTE *et al*., 2012; FERGUSON; NURNBERGER; REDL, 2010; ZUCCHELLI; SILVESTRI, 2004). Different studies have shown that fibrinogen levels are not significantly diminished after storage periods of up to 14 days under refrigeration (GROCHOWOSKY *et al*., 2014; WANG *et al*., 2014; ATHANASIOU *et al*., 2013) and up to 5 years in temperatures under -18ºC (URBAN; COUTO; IAZBIK, 2013; BRECHER, 2005). Studies addressing the effect of freezing storage time on cryoprecipitation for FG production, however, were not found. Indeed, the plasmatic fibrinogen concentrations prior to cryo were not statistically different in the older plasma compared to the fresher categories studied, which is in accordance to the reports on conservation of fibrinogen levels under prolonged freezing storage of canine plasma (YAXLEY *et al*., 2010). Unexpectedly, however, the final fibrinogen content was significantly higher in cryoprecipitates obtained with older plasma units.

The experimental set performed with bags showed increased fibrinogen yield with cryoprecipitation of eFFP and FP, in comparison to FFP. Both eFFP and FP acquired for this

study were stored frozen for more than a year. Still, with 120-mL bags, statistical differences among plasma categories were not always detectable. This could be due to the great variability of final fibrinogen concentration related to the hindrances associated with greater volume yields and separation of supernatant from bags. Supernatant of cryo was separated by a plasma extractor in which inopportune folding of the bag, during heavy centrifugation, impaired proper squeezing and obstructed the cryo-supernatant flow during its removal. Additionally, the squeezing of a folded bag induced detachment and consequently expulsion of the precipitated content, which could occur unnoticeably. These uncontrolled events during cryo-processing of bags, combined with the small number of samplings within some final volume ranges, could have masked the possible changes in fibrinogen precipitation from different plasma types and impaired proper statistical analysis. Cryoprecipitation performed in 50-mL tubes with fresh frozen plasma aliquots that were stored frozen from 6 to 10 months (FFP) yielded a significant higher fibrinogen content than fresh frozen plasma collected and processed within a week (FR). Some of these aliquots were from same plasma unit that were processed 6 months apart. So far, information to corroborate these findings was only obtained by informal statements from transfusion medicine specialists (personal communication: Dr. Eliane Bandinelli, Biosciences' Institute/UFRGS; MSc. Magnus L. Dalmolin, LacVet/UFRGS).

Second thawing-freezing cycle of canine fresh frozen plasma did not show decrease in fibrinogen levels either. These findings were in accordance with the study of Yaxley *et al*., (2010). When compared to the FFP samples that were frozen directly into tubes after blood collection (FFP; Table 2), the thawed-refrozen plasma (FFP-80ºC; Table 3) generated similar fibrinogen contents. This observation may motivate apportioning of old plasma units accordingly to the equipment available with no need for long freezing periods prior to cryoprecipitation. Moreover, these studies regarding fibrinogen stability are important considering the adverse conditions to which fresh frozen plasma may be subjected at blood banks or hospital settings (GROCHOWOSKY *et al*., 2014; YAXLEY *et al*., 2010). While these plasma units may be relegated regarding transfusion purposes, or even discarded, they may become a valuable source of fibrinogen for FG production. Additionally, many designated usages of FFP in veterinary practice can still be treated with cryo-supernatant (BROOKS, 2010; BROOKS; LAFOCARDE, 2010; HOGAN; BROOKS, 2010).

3.5.2 Effect of Freezing Temperature

Since thawing and refreezing of FFP has shown maintenance of fibrinogen concentrations of original plasma (YAXLEY *et al.*, 2010), this approach was chosen to evaluate the effect of freezing temperature on cryoprecipitation performed in 50-mL tubes.

Freezing thawed plasma (FFP) in 50-mL tubes at temperatures of -20°C, -80°C, and -196°C did not show to influence fibrinogen yield in cryoprecipitation of canine plasma. Freezing with a minimum of five-minute immersion in liquid nitrogen followed by 2h in -20°C was sufficient to yield a fibrinogen content comparable to that attained with 18h of freezing at -80°C. Very low freezing temperatures are preconized to decrease the period of exposure of blood plasma through temperatures that can induce factor VII activation (HOGAN; BROOKS, 2010; GENTRY; BURGESS; WOOD, 2008). Although preservation of fibrinogen is the main concern for FG production, activation of factor VII could induce some thrombin generation and, with the consequent fibrin monomers formation, less fibrinogen would be available for precipitation. In this study, freezing 50-mL tubes at -20°C did not induce detectable changes in the final fibrinogen concentration after cryoprecipitation. Different freezing temperatures and volumes used for cryoprecipitation aiming production of human FG have been reviewed, but no significant differences in fibrinogen yield have been mentioned (FERGUSON; NURNBERGER; REDL, 2010; SILVER; WANG; PINS, 1995a). Although -20°C and -80°C freezing temperatures for cryoprecipitation have been reported (SILVER; WANG; PINS, 1995b; WHEATON *et al.*, 1994), no study using -196°C was found. The possibility of using temperatures such as -20°C and liquid nitrogen expands the circumstances in which FG can be produced when blood banks facilities are not available. Moreover, the use of tubes with the evaluated temperatures may be valuable in reducing the time needed for cryoprecipitation, what can be beneficial to enable FG production in emergency needs and diminish the wait on a planned or high-risk bleeding surgery.

3.5.3 Efficiency of Cryoprecipitation

Clauss measurements were used to estimate the efficiency of the cryoprecipitation process in tubes and bags considering initial and final volumes.

Evaluation of the efficiency of the cryoprecipitation with bags showed that final volumes within 6 to 16mL would be the most efficient ones considering higher fibrinogen yields and precipitation capacity. Consensually, higher final volumes of cryo would be disadvantageous because it usually increases the dilution of the precipitate and lower fibrinogen concentration. However, there was an inability to correlate fibrinogen concentration with cryo volume in more strict ranges of final volume in bags. In part, this could be due to the high variation of fibrinogen concentrations within a specific volume, most likely associated with the separation hindrances of wrinkled bags, and to the small number of observations for each set. In 50-mL tubes, precipitated contents from plasma volumes of 50mL were diluted in 1.6mL for cryo and in 0.8mL for supernatant. An advantage seen with the polypropylene tube was that, due to the way the cryoprecipitate adheres to the container wall after centrifugation, small volumes of supernatant such as 0.5mL were able to dissolve the precipitated material, increasing the final concentration (APPENDIX A). Thus, volumes and concentrations seemed more easily tailored in 50mL-tubes cryoprecipitation. The starting volume in tubes to produce one mL with a minimum fibrinogen concentration of 10mg/ml, from FFP, was 40mL (GONCALVES, 2015; APPENDIX A).

Overall, the cryoprecipitation performed in bags with final volumes of 6 to 12mL showed similar precipitation capacity to 50-mL tubes. Fibrinogen precipitation of more than 50% was achieved with bags, but such results had much variability. Although the tubes seemed to have lower precipitation capacity, they had lower variation, especially when considered within more specified plasma categories. The overall capacities of precipitation seen in this study are in accordance with the ranges reported in literature with cryoprecipitation (BROOKS, 2010; GENOVEZ, 2008; WHEATON *et al.*, 1995; SPOTNITZ *et al.*, 1987).

The relevance of choosing either bags or tubes for fibrinogen cryoprecipitation has many facets. While bags decrease contamination risk during handling and processing, tubes allowed more homogeneous and reproducible results with less equipment-demanding and faster process (APPENDIX 2). Different from 50-mL tubes, many difficulties occurred during processing and centrifugation of plasma bags. Intercurrences with bags included bag rupture, ice formation during centrifugation, visible precipitate dislocation with supernatant, and loss of cryo by solubilization of fibrinogen during supernatant extraction. Moreover, the lack of homogeneity on final volume and final concentration of fibrinogen in the

cryoprecipitate in bags may yield unpredictable results during FG preparation. The use of 50-mL tubes for cryoprecipitation and mixing of cryo batches seemed to be advantageous. Yet, all steps of the process require strict sterility guidelines (BRECHER, 2005; BRENNAN, 1991). The use of such open systems increases the risk of contamination and has not been advised if aseptic processing cannot be ensured (KUNIO; SCHREIBER, 2013). Measures for avoiding environmental contamination of solutions during handling were carefully complied and no bacterial growth was observed in samples incubated for a week. Under the adopted conditions, very low risk of contamination has been considered (BUTCHA; SEMPLE, 2005; BRENNAN, 1991). Still, the plasma can also be a source of infectious agents (GIBSON, 2007). Thus, culture of samples prior to clinical usage is considered an unavoidable approach to ensure biological safety (BUTCHA; SEMPLE, 2005).

Timing for total defrosting of bags and tubes varied from 10 to 22 hours. At homogeneous 2^{o}C, the average time for thawing was 11 to 13 hours when two bags or three tubes, at the most, were place to defrost together, in volumes from 30 to 50mL in the tubes, and 100 to 200mL, in the bags. The most suitable temperature for thawing and the best moment for centrifugation of FFP in 50ml tubes in the presented laboratory conditions were determined previously by thawing tubes with same plasma source at 2^{o}C and 4^{o}C until some or none ice-crystals were seen, followed by Clauss evaluation (preliminary studies). More flocculation remained suspended even after an hour from absence of ice-crystals, with more fibrinogen being precipitated with thawing performed at 2^{o}C. These findings agree with other results relating slower speed of thawing, higher volumes of plasma, and higher fibrinogen concentration in cryoprecipitate (SILVER; WANG; PINS, 1995a). Higher volumes of plasma in bags, lower temperature of storage, simultaneous thawing of containers and more homogeneous refrigerator temperature were associated with increased thawing time. Especially when working with bags, the time of thawing can differ significantly, so spare time should be considered if FG production is intended for a planned surgery.

Second cryoprecipitation cycles were warranted to increase productivity of the available plasma resource. Clauss measurements of supernatant showed no statistical difference from that of starting plasma. This motivated the second freeze-thawing cycle of the supernatant to precipitate more fibrinogen, expecting to increase the production of FG from the scarce material. The second cryoprecipitation has been performed in other studies with apparently rewarding results, yet the initial fibrinogen concentration before and after the

second cycle was not stated (SILVER; WANG; PINS, 1995b; WHEATON *et al.*, 1994). Besides avoiding the cost of an extra bag, second cryoprecipitation cycles performed in 50-mL tubes demonstrated to be feasible and increase cryo content. Although some studies mentioned a third cryoprecipitation cycle, this procedure has consensually shown no worth (MINTZ *et al.*, 2001; WHEATON *et al.*, 1994). Second freeze-thawing cycle of cryo did not consistently increase the final fibrinogen content in samples (up to 4x). Possibly, greater concentration capacities were not observed due to the initial volumes being 30mL or less. This could be related to the nature of thawing in polypropylene tubes. Small and thinner columns of frozen material (<35mL) seemed to thaw faster and not promote endurance of fibrinogen flakes into suspension in the liquid plasma. This was evident in thawing amounts of 35mL and under in 50-mL tubes. Indeed, a correlation between the ability to concentrate more fibrinogen with higher volumes in tubes was detected (r^2=0.933, P=0.007). Yet, the second cryo cycle of cryo from bags could potentially be performed in 50mL tubes as a pool of small cryo volumes. Thus, a cryoprecipitate with increased fibrinogen concentration could be strategic for surgical needs that require higher FG performance.

3.5.4 Final Considerations

Replicates were performed with the Clauss methodology to observe its consistency for evaluation of fibrinogen concentrates, which can be 5 to 10 times more concentrated than normal plasma usually tested by the assay. It was observed a higher coefficient variation from that of the laboratory and the ones reported for standard plasma evaluations (STANG; MITCHELL, 2013; MACKIE *et al.*, 2003). This may have also influenced the lack of statistical difference in some comparisons among plasma types or processing adaptations.

The effect of storage time of canine platelet poor plasma on fibrinogen cryoprecipitation and clottability needs further evaluations. Information that could explain the higher fibrinogen yield with cryoprecipitation of older frozen plasmas was not found. Additionally, Clauss is a quantitative and functional assay, so whether the measured fibrinogen was higher in older plasmas due to increased precipitation capability and/or clottability of the protein is not known. More homogeneous studies are warranted to evaluate fibrinogen precipitation methods from plasma categories after different periods of storage at low temperatures.

Insufficient fibrinogen levels for achieving a needed fibrin clot property may hamper the efficacy of the derived FG in specifically intended applications. A wide range of fibrinogen yield (10 to 40mg/mL) has been presented in studies with cryoprecipitation (SHARMA *et al.*, 2003; WHEATON *et al.*, 1994; SPOTNITZ *et al.*, 1987; REISS; OZ, 1996). Also, most descriptions of cryoprecipitation for FG do not discuss strategies to overcome the lack of uniformity with bag processing. Indeed, this may be a source of discouragement and lack of interest in such simple and inexpensive technique for large-scale fibrinogen precipitation and FG production (SPOTNITZ, 2010). The different approaches here described aimed to circumvent some of the difficulties seen with performing cryoprecipitation. Advantages seen with 50mL-tubes included faster freezing, easier handling for storage and centrifugation, simpler and more efficient supernatant separation, and more homogeneity on final fibrinogen concentration. Moreover, the ability to tailor final fibrinogen concentration, aiming FG production for surgical applications or research purposes, was considered a positive aspect of the 50-mL tubes. Yet, rigorous measures of sterile handling must be adopted to ensure microbiological safety of the final product.

3.6 Conclusions

Different plasma categories produced divergent fibrinogen concentrations in cryoprecipitate, with higher yields detected in plasmas with longer freezing storage periods. Cryoprecipitation of canine plasma sources from veterinary blood banks was feasible and more easily performed in tubes than in bags.

Improvement of fibrinogen precipitation methods could help FG production for surgical applications and research in veterinary medicine.

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3.8 Acronyms and Abbreviations

FFP fresh frozen plasma
FG fibrin glue
FP frozen plasma
FR fresh plasma
eFFP expired fresh frozen plasma
P significance level
pRBC packed red blood cells
r^2 Pearson's product-moment correlation coefficient
SD standard deviation

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4 ARTIGO 2 – VISCOELASTIC PROPERTIES AND ULTRAESTRUCTURE OF CANINE FIBRIN GLUE PRODUCED WITH CRYO- AND PROTAMINE-PRECIPITATED FIBRINOGEN FROM DIFFERENT PLASMA CATEGORIES

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4.1 Abstract

Fibrin glue (FG) usage in veterinary surgery is still dependent on suitable production methods capable of yielding adequate fibrinogen concentrations from the available material. This study evaluated two methods of fibrinogen precipitation for canine FG production from platelet-poor plasma categories most available at veterinary blood banks. Fibrinogen concentrate produced through cryoprecipitation and precipitation with protamine from fresh frozen plasma (FFP), expired fresh frozen plasma (eFFP), and frozen plasma (FP) were analyzed considering fibrinogen yield, rheological properties during FG polymerization and ultrastructure of the resultant clot. The precipitation processes were performed with volumes of 40mL in 50-mL Falcon tubes and each cryo-precipitated fibrinogen sample had a protamine-precipitated counterpart from the same bag. Clauss assay was performed for determination of the functional fibrinogen obtained with both protocols, from each specific plasma source. The resultant FG clots from all samples were further evaluated by thromboelastography (TEG) for assessment of rheological characteristics and some were analyzed through scanning electronic microscopy (SEM) for ultrastructure characterization. Cryoprecipitation generated better results in Clauss and TEG in comparison to protamine protocol. In cryoprecipitation, eFFP and FP yielded more fibrinogen and stronger viscoelastic properties than FFP. Significant differences were not detected among plasma categories

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processed with protamine precipitation in Clauss and TEG assessments. The resultant FG clots of cryo- and protamine-precipitation showed similar fiber arrangement in scanning electron microscopy (SEM). Cryoprecipitation seemed more consistent regarding homogeneity and capacity to increase final fibrinogen concentration. Protamine protocol showed to be faster and simpler considering the equipment required. Additional studies are needed to improve protamine precipitation and better elucidate the effect of freezing storage time on precipitation and/or clottability of fibrinogen intended for canine FG production. Veterinary hospitals, blood banks, and patients can benefit from usage of surplus plasma units for FG production aiming surgical and scientific applications.
Keywords*:* fibrin sealant, surgical adhesive, fresh frozen plasma, frozen plasma, dog

4.2 Introduction

Fibrin glue (FG) is considered a valuable tool in human surgery and in regenerative and therapeutic research for cellular-scaffolds and drug-delivery (SPOTNITZ, 2014, FERGUSON; NURNBERGER; REDL, 2010; MACGILLIVRAY, 2003). Hospitals worldwide have used FG for hemostasis, tissue bonding, and sealing support in basically all surgical specialties (ALBALA; LAWSON, 2006; ZUCCHELLI; SILVESTRY, 2004; MINTZ *et al.*, 2001). FG is composed by a solution of thrombin and calcium with a solution containing fibrinogen, factor XIII, and variable adhesive proteins such as fibronectin and vonWillebrand factor (NURNBERGER *et al.*, 2010; BERING, 1944). For mostly all applications, fibrinogen is the major component affecting FG effectiveness and desired biological properties (KIM *et al.*, 2014; KAETSU; TAKANORI; SHINYA, 2000).

In veterinary medicine, FG production is still dependent on cost-effective fibrinogen precipitation methods and proper validation of its properties. Cryoprecipitation has been considered gold-standard method for precipitation of fibrinogen and associated factors (SILVER; WANG; PINS, 1995a). Still, the lower-end of unpredictable fibrinogen concentrations generated by cryoprecipitation with plasma bags have not been considered to be sufficient for some intended FG applications. Moreover, standard cryoprecipitation takes more than a day to be completed and demands refrigerated centrifuges and high volumes of plasma (SPOTNITZ, 2010; BRENNAN, 1991). Protamine protocol has been advocated as being capable of precipitating more than 90% of total plasmatic fibrinogen and feasible with

small amounts of plasma. Also, its process should take less than an hour and does not require refrigerated centrifuges (ALSTON *et al.*, 2007). Protamine started being used for fibrinogen precipitation aiming the diagnosis of dysfibrinogenemia in the early 40's (MYLON; WINTERNITZ; SUTO-NAGY, 1942) and, later, disseminated intravascular coagulation ("paracoagulation test"; HORN; HAWIGER; COLLINS, 1969; DENPFLE and HEENE, 1987). Currently, protamine is widely used to neutralize the anticoagulant effect of heparin therapy (BAILEY; KOENIGSHOF, 2014; TANEJA *et al.*, 2014). While protamine is considered one of the most positively charged biologic molecules, fibrinogen is the most negatively charged molecule in plasma (AINLE *et al.*, 2009; HALL; SLAYTER, 1958). Indeed, some authors have mentioned that protamine has more affinity to fibrinogen than to any other molecule in blood (DENPFLE and HEENE, 1987; MYLON; WINTERNITZ; SUTO-NAGY, 1942). Other chemical methods of fibrinogen precipitation have been described and utilized (BRENNAN, 1991), but its efficiency has been controversial and its chemical safety questioned (ALSTON *et al.*, 2007; SILVER; WANG; PINS, 1995b).

The source of fibrinogen can also be a limitation even for production of commercial human FGs (SPOTNITZ; BURKS, 2012). Autologous plasma is not always an option, considering volume and/or quality required. Even though allogeneic platelet poor plasma from blood banks is the most accessible supply, it may experience periods of scarceness, especially the fresh frozen plasma units. Other categories of platelet poor plasma, like frozen plasma that have less transfusion usefulness due to possible reduction of thrombin generation factors, can become a more available material (SPOTNITZ *et al.*, 1987).

Although most fibrinogen precipitation methods may yield adequate clottable protein content for FG production, major differences can be found between the individual protocols and plasma sources regarding functional fibrinogen and other components and factors (SIERRA, 1993; NAIR; DHALL, 1991). Such differences may influence biological properties, biomechanical features, and *in vivo* efficacy (DICKNEITE *et al.*, 2003). Knowledge of coagulation kinetics and viscoelastic properties of FGs are important for adjustments on components' concentration, changes in ratio between fibrinogen and thrombin solutions, and addition of substances for a defined purpose (HICKERSON; NUR; MEIDLER, 2001; GLIDDEN; MALASKA; HERRING, 2000; KJAERGARD *et al.*, 2000). Moreover, this information can help the estimation of efficacy of a given FG formulation for an intended use and on the tissue where it will be applied (CARLSON *et al.*, 2014;

WOSNIAK, 2003). The ultrastructure of the resultant fibrin clots is also of interest considering its influence on clot stiffness and elasticity, fibrinolysis, and cellular migration (NURNBERGER *et al.*, 2010; LAURENS; KOOLWIJK; DE MAAT, 2006; WEISEL, 2004; COLLET *et al.*, 1996). For some applications, FG structure may be particularly relevant. When FG is applied for cell scaffolding, cellular matrix, or in a tissue where support of healing process is expected, the final arrangement of fibrin fibers has shown to either promote or limit cellular movement (CECCARELLI; PUTMAN, 2014; HO *et al.*, 2006; COX; COLE; TAWIL, 2004).

This study analyzed cryo and protamine protocols for fibrinogen precipitation from different category of canine plasma usually available from blood banks and accessed their resulting FGs' rheological characteristics and structure.

4.3 Material and Methods

4.3.1 Plasma Sources

All canine plasma was obtained from the same commercial veterinary blood bank[14]. The separation of plasma from the cellular components of whole blood was achieved through standardized techniques for all plasma types, followed by -30^{o}C freezing storage (ABRAMS-OGG; SCHNEIDER, 2010). Twelve 120-mL bags were shipped over-night in dry ice. Upon arrival, the units were thawed in water bath (37^{o}C) for approximately 20 minutes and the plasma was transferred to 50mL-polypropylene centrifuge tubes into final volumes of 40mL. For standardization purposes, all 40-mL aliquots were refrozen at -80°C for 48h preceding the fibrinogen precipitation processes (BRENNAN, 1991). From each bag, one 40mL-sample was directed to the cryo protocol and another was used for the protamine precipitation process.

The platelet-poor plasma categories studied included fresh frozen plasma (FFP; 4-month old; n=4), consisting of fresh plasma that was frozen within eight hours from blood collection and stored for less than a year; expired fresh frozen plasma (eFFP; 22 to 24-month-old; n=4), which consisted of plasma frozen within eight hours from blood collection, but

[14] Blue Ridge Veterinary Blood Bank, Purcellville, VA, USA (Privately owned veterinary blood bank).

with storage time exceeding a year; and, frozen plasma (FP; 28-month old; n=4), which was frozen after 8 hours from collection and was also stored for more than a year.

4.3.2 Fibrinogen Precipitation Methods

For each precipitation technique, the 50-mL tubes were processed as group of three samples at a time, one tube from each plasma kind.

4.3.2.1 Cryoprecipitation Protocol

Cryoprecipitation was performed accordingly to a methodology previously described (SILVER; WANG; PINS, 1995a). Briefly, cryoprecipitate was obtained by slowly thawing the plasma at 2°C for 9 to 13 hours. When only very small ice crystals could be seen among fibrinogen flakes, the plasma was centrifuged at 4,200 *x g* for 10 minutes, at temperatures between 1°C and 3°C, to form a sediment at the bottom of the tube. Before decantation of the supernatant plasma, 1mL of supernatant was retrieved for fibrinogen analysis and 0.8mL was recovered for dilution of the cryo-precipitated sediment. The cryo aliquots were apportioned and stored at -20^{o}C. Prior to use or assaying, the cryo solutions were thawed at 37^{o}C in water-bath for three minutes.

4.3.2.2 Protamine Precipitation Protocol

The protamine precipitation of fibrinogen was accomplished with a protocol published for human plasma FG (ALSTON *et al.*, 2007). A protamine[15] stock solution of 40mg/mL was prepared in advance, under aseptic conditions, and stored at 4^{o}C. The 40-mL samples were thawed at 37^{o}C for about 10 minutes, until all fibrinogen was solubilized, and were then left at room temperature of 22^{o}C for another 10 minutes. The amount of 13.4 mL of the protamine stock solution was added to the plasma to reach a final concentration of 10mg/mL. After mixing the content by lightly rotating the tube five times up side down, the solution was centrifuged at 1,000 *x g* for five minutes to sediment the precipitate (20^{o}C). One milliliter of supernatant was retained for evaluation before the plasma was discarded. The

[15] Sigma Chemical Co., St. Luis, MO; P4020.

remaining sediment was redissolved in 0.8ml of 0.2M sodium citrate (pH 7.4) on a vortex. The protamine-precipitated fibrinogen solution was stored at -20°C until later use. Aliquots were thawed at 37°C in water-bath for three minutes prior to evaluations.

4.3.3 Fibrinogen Measurement by Clauss Assay

A Clauss automated system[16] was used to measure fibrinogen concentrations in the protamine- and cryo-precipitated fibrinogen solutions, initial plasma, and supernatant of all plasma categories processed (FFP, eFFP, and FP). When necessary for proper assay function, the concentrated fibrinogen solution was diluted in the manufacturer's buffer solution prior to the test. For analysis of protamine-precipitated samples, calcium chloride ($CaCl_2$) was added to the buffer solution (40mM of final concentration) prior to dilution and loading of the aliquot into the Clauss machine. The calibration curve was reestablished for each day of measurement and assays were performed in batches of 20 to 40 samples.

4.3.4 Thromboelastography (TEG) of FG Clots

All samples previously assayed for fibrinogen content were submitted to TEG for assessment of kinetic characteristics of the resultant FG clots. Clots consisted of a mixture of fibrinogen and thrombin solutions. The concentration of components in each solution was prepared accordingly to that intended for *in vivo* application of canine FG (WHEATON *et al.*, 1994). The volume and ratio of fibrinogen and thrombin solutions followed TEG's methodology as described for fibrin sealants evaluation (GLIDDEN; MALASKA; HERRING, 2000). Cryo- and protamine- fibrinogen samples were thawed at 37°C for 3 minutes. The thrombin solution was prepared to contain 15UI/mL of thrombin and 40μmol/mL of $CaCl_2$. With protamine formulation, $CaCl_2$ concentration in the thrombin solution was 80μmol/mL.

Briefly, one hundred-eighty microliters from each aliquot were mixed in the TEG cup with three fast and sequential pipette mixing, just before the event marker was depressed to start data collection of clot formation. Three TEG workstations[17] were used concurrently to

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16 STACompact®, Diagnostica Stago, Parsippany NJ; manufacturer's thrombin reagent contained a mixture of calcium titrated human thrombin (approximately 80 NIH U/mL thrombin).

17 TEG 5000 Thrombelastograph®, Haemonetics Corp, Braintree, MA, USA.

evaluate the viscoelastic properties of cryo-FG and protamine-FG clots. TEG instruments were calibrated each day of use and all samples were assayed in duplicates. TEG data from each sample was recorded for 90 minutes, at 37°C, and processed simultaneously by TEG Analytical Software.

From the parameters recorded, the ones used for FG clot analysis were time to start clot formation (R), time from beginning of clot formation to a fixed level of clot firmness (K), rate of clot development (α-angle), maximum clot strength (MA), time to maximum clot strength (TMA), clot elasticity (G, E), and percentage of clot lyses at 60 minutes after reaching maximum strength (LY60%).

4.3.5 Scanning Electronic Microscopy (SEM) of FG Clots

Fibrinogen concentrated solutions obtained by cryo- and protamine- precipitation methods from the canine eFFP and FFP were clotted in microtubes and prepared for ultrastructure evaluation through SEM imaging. A clot with higher ratio (3:1) between fibrinogen to thrombin solutions with cryo (eFFP) was also evaluated.

In brief, cryo- and protamine-FG clots were prepared with a 1:1 ratio of fibrinogen to thrombin solution. In a 2-mL microtube, 100μL of fibrinogen solution were mixed with 100μL of thrombin (15UI/mL) containing 40μmol/mL of $CaCl_2$ (cryo) or 80μmol/mL (protamine). After 10 minutes from clotting, the clots were placed into a 0.05M Cacodylate buffer and incubated for fixation in 2% glutaraldehyde for two hours on ice. Then, after repeated rinsing in 0.05M Cacodylate buffer (three times, 5 minutes each time), they were placed in 1% Osmium Tetroxide in buffer, for one hour, and were rinsed again (three times, 10 minutes each time) in Cacodylate buffer; all these steps were performed on ice. For dehydration, the samples were immersed in graded series of increasing ethanol concentration (25%-50%-70%-95%-100%-100%) for 10 minutes each. The clots were then critical point dried, mounted, and sputter-coated with gold and palladium (AuPd).

Under a field emission electron microscope[18], a minimum of three transverse pictures, with at least two perpendicularly to each other, was taken per sample in doubled amplified pixel sizes (mainly 12.5 and 25nm, few 50 and 100nm; aperture size 20.00um, EHT 5kV).

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[18] LEO® Gemini Zeiss 1550FV FESEM, Germany.

Evaluation of ultrastructure and arrangement of the fibrin fibers was evaluated considering fiber diameter, pore size, and presence of debris.

4.3.6 Statistical Analyses

Normal distribution of Clauss and TEG results was confirmed using Komogorov-Smirnov Test. A General Linear Model of ANOVA was used to analyze differences between protocols and among plasma types. All subsets were considered as a single factorial group. For thorough evaluation, each factor (precipitation protocols and plasma sources) was analyzed separately and together accordingly to configurations of clinical relevance. Tukey Test was used for identification of differing groups.

Pearson correlation test (r^2) was used to evaluate the relationship between initial and final fibrinogen content, and between TEG variables K, MA, and G, with sample's final fibrinogen concentration. Correlation of TEG parameters was performed using the mean of replicates from each sample and assessing every protocol and plasma type separately and combined.

Differences were considered significant when $P<0.05$. Minitab and SAS Platforms were used for both descriptive and statistical analyses.

4.4 Results

4.4.1 Fibrinogen Measurements

The mean concentration and statistical differences of fibrinogen measurements by Clauss in the precipitate obtained by cryo and protamine protocols, from different sources of canine plasma, are summarized in table 4.

Considering all plasma sources together, cryoprecipitation yielded more clottable fibrinogen than protamine precipitation protocol ($P=0.004$). When comparing precipitation protocols specifying each type of plasma, only eFFP showed significantly higher fibrinogen yield with cryo than with protamine precipitation ($P=0.001$).

Table 4 – Fibrinogen concentration of canine initial plasma and precipitated solutions obtained from cryo and protamine protocols with different categories of plasma.

	Initial Plasma			Cryo			Protamine		
Plasma Type	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*
FFP	1.39	0.19	4	15.54 ^{b}	4.82	4	10.11	2.70	2
eFFP	1.58	0.47	5	25.50 ^{aA}	1.84	4	12.55 ^{B}	4.44	5
FP	2.48	0.46	3	25.20 ^{ab}	7.05	4	18.89	14.86	2
All together	**1.74**	**0.58**	**12**	**22.08** ^{x}	**6.64**	**12**	**13.42** ^{y}	**7.00**	**9**

Fibrinogen measurements performed by Clauss. Superscripts indicate statistical difference (x, y: between protocols without plasma source distinction; A, B: between protocols with eFFP; a, b: among plasma types in cryo). Differences were considered significant when *P* value was <0.05. **FFP:** fresh frozen plasma (4-month old); **eFFP:** expired fresh frozen plasma (22 to 24-month old); **FP:** frozen plasma (28-month old); *SD*: standard deviation; *n*: number of samples.

With cryoprecipitation, comparing plasma categories, eFFP yielded significantly more fibrinogen than FFP (P=0.008). FP also showed a tendency to generate more fibrinogen than FFP (P=0.056). Statistical difference was not detected between eFFP and FP. FFP samples yielded concentrates containing 9.95 to 19.83mg/mL of fibrinogen, eFFP ranged from 23.32 to 27.80mg/mL, and FP had concentrations from 17.28 to 32.94mg/mL.

In the protamine precipitation, no difference among plasma types could be detected. Yet, there was an inability to generate Clauss assay readings with all protamine samples of FFP and FP. Fibrinogen measurements were between 7.02 to 29.40mg/mL, considering all plasma categories.

Since every unit of canine plasma was submitted to cryo and protamine protocols, the initial fibrinogen concentration from a plasma sample was the same in both precipitation processes. The initial fibrinogen content varied from of 1.13mg/mL to 2.93mg/mL and did not differ statistically among plasma sources. Cryo-supernatant had a mean of 1.13mg/mL of fibrinogen (±0.7; n=4), considering all plasma types; while in the supernatant of protamine, fibrinogen was undetectable (<0.1mg/mL, n=3).

No statistical correlation was detected between the precipitated fibrinogen content and the initial plasmatic concentration in either cryo (r^2=0.29, P=0.35) or protamine precipitation (r^2=0.33, P=0.37).

The observed inter-assay coefficient of variation of the method using a canine plasma control ranged from 4.5 to 7.5%. Coefficient of variation (CV) of Clauss within replicates and dilutions of concentrate fibrinogen essays of cryo varied from 2% to 19%.

4.4.2 Thromboelastography of FG Clots

TEG results are presented in table 5. TEG plotted curves obtained from clot development of cryo-FG and protamine-FG are exemplified in figure 2.

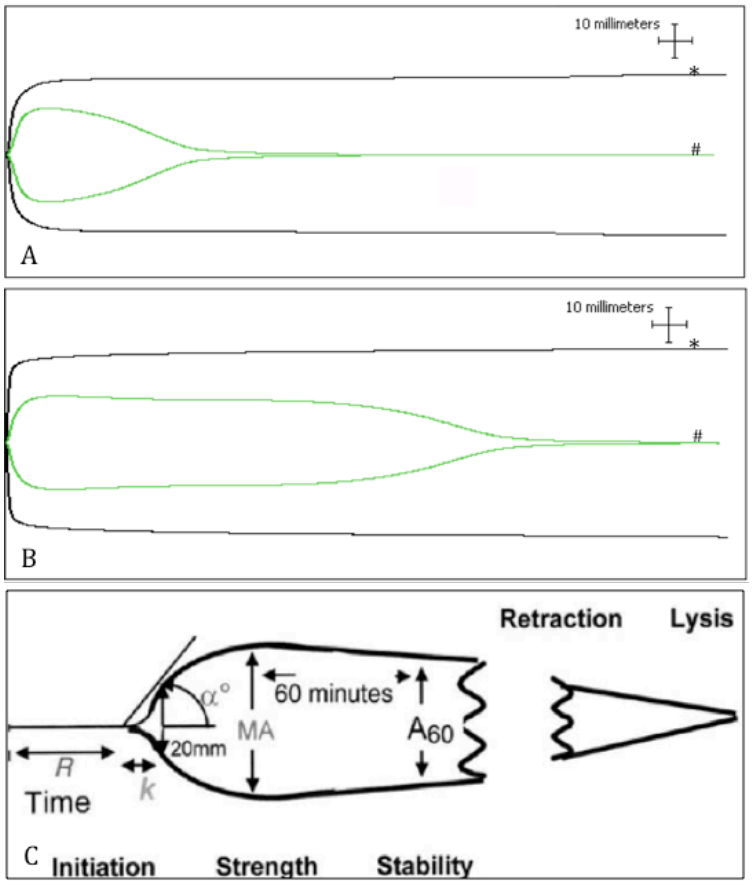

Figure 2 – Thromboelastograph (TEG) tracings obtained from clot development of cryo-FG, protamine-FG derived from different canine plasma categories.

Rheological properties of cryo- (*) and protamine-FG (#) from different canine fresh frozen plasma (FFP; A) and expired fresh frozen plasma (eFFP; B) are presented. The wider waves (black lines*) show higher amplitude with cryo samples, denoting greater clot strength (MA). The decrease in the width of the curve ("leaf shape") with time in protamine samples (green lines#) indicates spontaneous fibrinolysis. **A**: TEG response of fibrin glue clot derived from FFP (Clauss measurements: cryo: 19.28mg/mL; protamine: 12.02mg/mL). **B**: TEG response of fibrin glue clot derived from eFFP (Clauss measurements: cryo=25.62mg/mL; protamine=17.24mg/mL). **C**: Representative clot development pattern and fibrinolysis in TEG. Kinetics variables are illustrated. R = reaction time, from start of test to initial fibrin formation, when amplitude reaches

2mm; K = kinetics; time taken from beginning of clot detection (2mm of amplitude) to achieve a certain level of clot strength (20 mm of amplitude), considered the amplification phase; α^{o} = alpha angle, slope between R and K; assesses the rate of clot formation; MA = maximum amplitude, represents overall stability of the fibrin clot, maximum stiffness; A60= amplitude at 60 minutes with or without fibrinolysis. (Adapted from WHITING; DINARDO, 2014).

When compared to cryo, without plasma type distinction (n=12; Table 5), protamine-FG showed statistically (P<0.01) longer time to start clot formation (R), slower propagation (K, α-angle, TMA), decreased clot strength (MA, G, E), and spontaneous clot lysis (LY60).

Table 5 – Thromboelastography (TEG) of canine fibrin glue (FG) clots obtained from cryo- and protamine protocols without distinction between categories of canine plasma

	R		**K**		**a-angle**		**TMA**		**MA**		**G**		**E**		**LY60**	
All plasma	(min)		(min)		(degree)		(min)		(mm)		(d/cm^2)		(d/sc)		(%)	
Samples	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Cryo	**0.23** ^{x}	**0.06**	**1.23** ^{x}	**1.05**	**81.14** ^{x}	**5.22**	**8.38** ^{x}	**2.75**	**41.36** ^{x}	**12.77**	**3932.51** ^{x}	**1980.05**	**78.65** ^{x}	**39.60**	**0.00** ^{x}	**0.00**
Protamin	**0.64** ^{y}	**0.36**	**2.71** ^{y}	**2.05**	**63.00** ^{y}	**15.52**	**7.84** ^{x}	**2.85**	**29.53** ^{y}	**10.46**	**2242.58** ^{y}	**1050.07**	**44.85** ^{y}	**21.01**	**41.23** ^{y}	**23.96**

Results are presented as means and standard deviation (*SD)*, considering all replicates obtained with TEG of clots derived from canine fresh frozen plasma (FFP), expired fresh frozen plasma (eFFP), and frozen plasma (FP). Differences were considered significant when *P* value was <0.05. Superscripts indicate statistical difference between protocols without plasma source distinction (x, y). **R** = reaction time, in minutes (first evidence of clot formation); **K** = kinetics, in minutes (time taken from beginning of clot detection to achieve a certain level of clot strength); **α-angle** = rate of clot formation, in degrees (speed at which fibrin build up and cross linking takes place); **TMA** = time to maximum amplitude, in minutes; **MA** = maximum amplitude, in millimeters (maximum stiffness of the clot); **G** = shear modulus strength, in dynes/cm^2 (clot elasticity); **E** = extensibility, in dynes/second (clot elasticity constant); **LY60** = lysis at 60 minutes, in percentage (clot disintegration).

When plasma categories were taken into account, some parameters were not statistically different (Figure 3, A and B superscripts; APPENDIX C). Considering plasma types for protocols comparisons, only eFFP showed higher maximum clot strength (MA, G, E) with cryo in relation to protamine precipitation ($P \leq 0.001$).

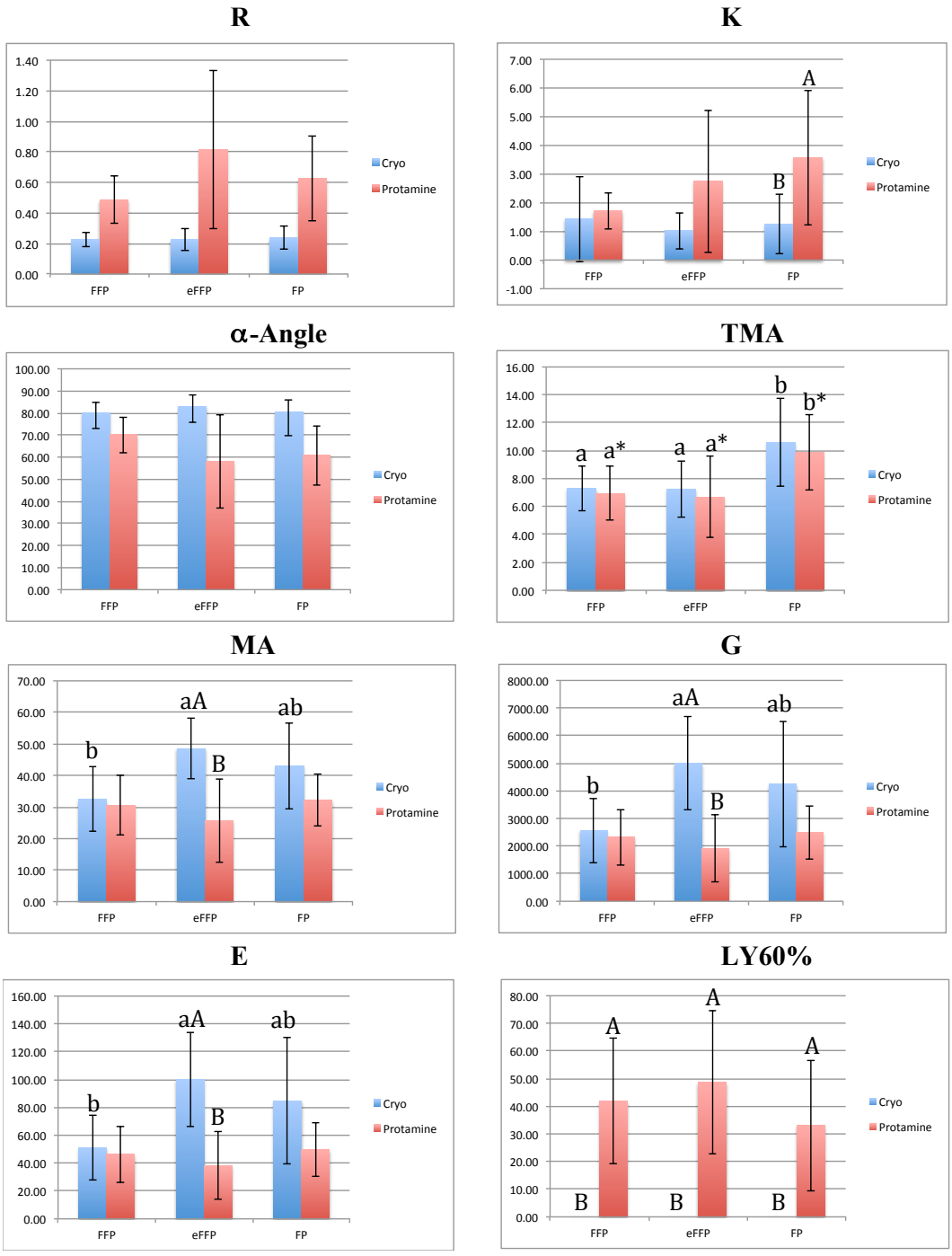

Figure 3 – Comparisons between rheological measurements performed with thromboelastography (TEG) of FG clots obtained from cryo- and protamine protocols considering the different categories of canine plasma

Results are presented as means (columns) and standard deviation (bars), calculated from all replicates in TEG. Differences were considered significant when *P* value was <0.05. Superscripts indicate statistical difference (A, B: between protocols within a specific plasma category; a, b: among plasma types within a protocol; a*, b*: FFP^{a*} *versus* FP^{b*}, *P*=0.0770; $eFFP^{a*}$ *versus* FP^{b*}, *P*=0.0509). **R** = reaction time, in minutes (first evidence of clot formation); **K** = kinetics, in minutes (time taken from beginning of clot detection to achieve a certain level of clot strength); **α-angle** = rate of clot formation, in degrees (speed at which fibrin build up and cross linking takes place); **TMA** = time to maximum

amplitude, in minutes; **MA** = maximum amplitude, in millimeters (maximum stiffness of the clot); **G** = shear modulus strength, in dynes/cm^2 (clot elasticity); **E** = extensibility, in dynes/second (clot elasticity constant); **LY60** = lysis at 60 minutes, in percentage (clot disintegration). ***FFP***: fresh frozen plasma; ***eFFP***: expired fresh frozen plasma; ***FP***: frozen plasma.

Within cryo protocol, the maximum amplitude and elasticity (MA, G, E) were greater in eFFP than in FFP samples (P=0.02, P=0.03, respectively). In clot strength parameters, FP did not differ significantly from any of the other plasma categories. Nevertheless, the time to achieve maximum strength (TMA) was longer with FP than with FFP and eFFP fibrin clots (P=0.026 and P=0.023, respectively). No other TEG variables showed statistical differences among plasma types in cryo-FG. Fibrinolysis (LY60) was not detected in any of cryo samples.

In the protamine protocol, differences on clot initiation and strength variables (R, K, MA, G, E) did not differ statistically among plasma types. Yet not significantly, FP seemed to take longer than FFP and eFFP to reach MA (TMA; P=0.077 and P=0.051, respectively). With protamine protocol, fibrinolysis (LY60) did not differ among plasma types.

Correlations of clot kinetics with clottable fibrinogen, measured by Clauss, were also accessed. In cryo, K was negatively correlated (r^2=-0.685; P=0.002), while MA and G were highly positively correlated with fibrinogen concentration (r^2=0.829 and r^2=0.836, respectively; P<0.001). With protamine, K and Clauss did not correlate (P=0.2), but MA and G also correlated positively with fibrinogen content (r^2=0.826, P=0.043; r^2=0.817, P=0.047, respectively). K values were negatively correlated with MA and G, only in cryo (P<0.01).

4.4.3 Scanning Electronic Microscopy Imaging of FG Clots

SEM images obtained with canine FG from eFFP (1:1 Ratio), through cryo and protamine precipitation, are depicted in figure 4. The overall appearance of protamine-FG was similar to that of cryo-FG. Both eFFP-derived cryo- and protamine-FG had a regular lattice-like arrangement of fibers and pores on scanning electron micrographs. However, some ultrastructural features differed between cryo-FG and protamine-FG. When comparing to its counterpart cryo, protamine-FG consisted of more discretely separated and slightly thinner fibers, arranged in a more randomly heterogeneous meshwork. The globular debris

that appeared more tightly attached within fibrin strands in both FGs, seemed to be more evident in cryo-FG. However, protamine-FG images showed an additional type of crumpled amorphous material, with a cluster pattern not seen in any of the cryo-FG. These clumps in the protamine clot did not seem to be adhered within the fibrin fibers as the more globular scattered kind that was present in both FGs.

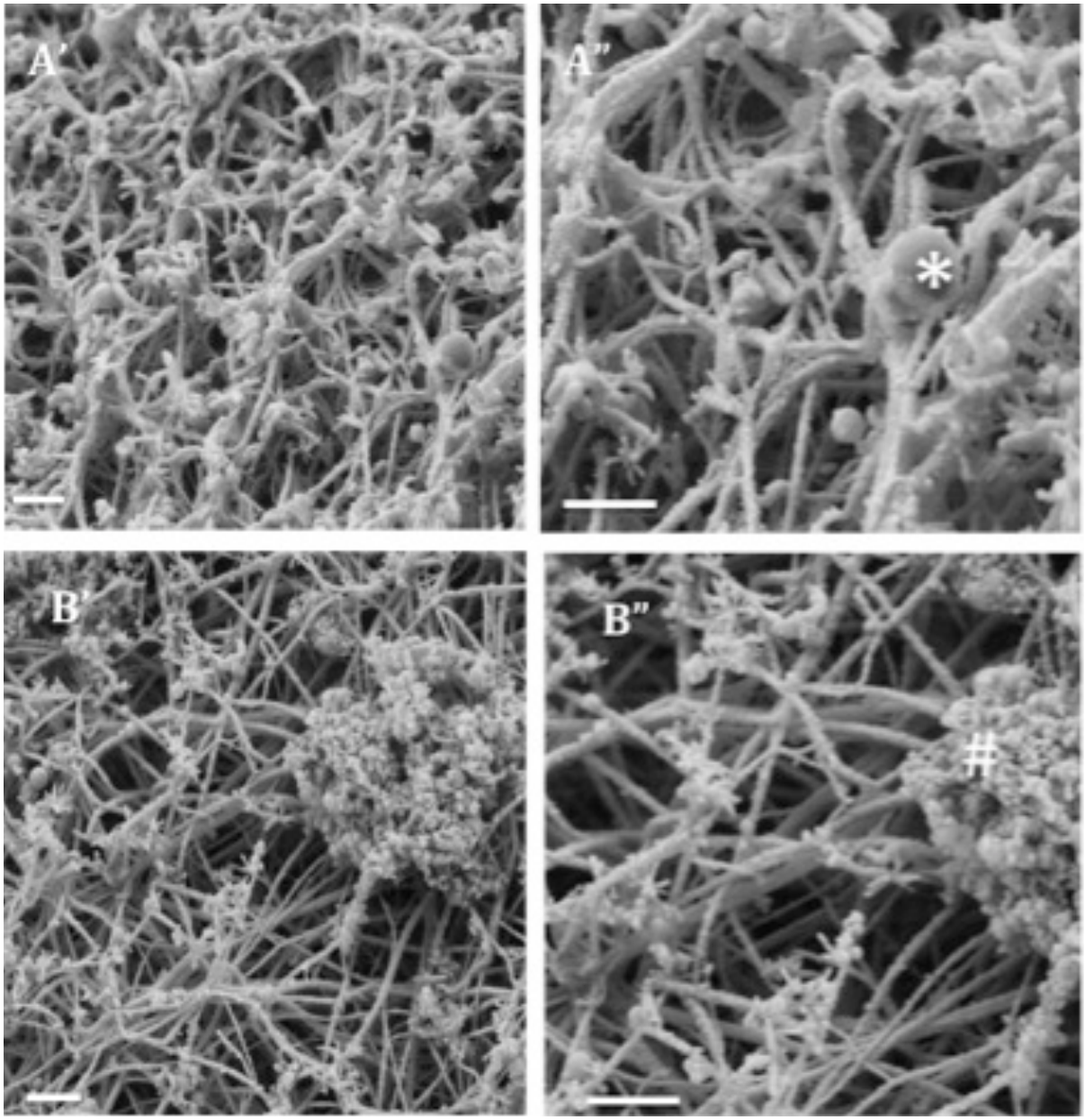

Figure 4 – Scanning electron micrographs of cryo-FG and protamine-FG clots obtained from canine expired fresh frozen plasma (eFFP).
A', **A"**: FG clot with fibrinogen obtained by cryoprecipitation of canine eFFP. **B'**, **B"**: FG clot with fibrinogen obtained by protamine precipitation of canine eFFP. White asterisks: plasmatic debris (*); remains from protamine protocol (#). Fibrinogen concentration in the precipitates used:

cryo=23.32mg/mL; protamine=11.92mg/mL. Magnification bar =1μm. Resolution of microscope images: 25nm (A', B') and 12.5nm (A", B").

With cryo- and protamine-FG derived from FFP, a different pattern of fibrin mesh density was seen from that of eFFP (Figure 4). The clots were dense and tightly packet. The debris was not so evident and differences between cryo and protamine could not be well appreciated.

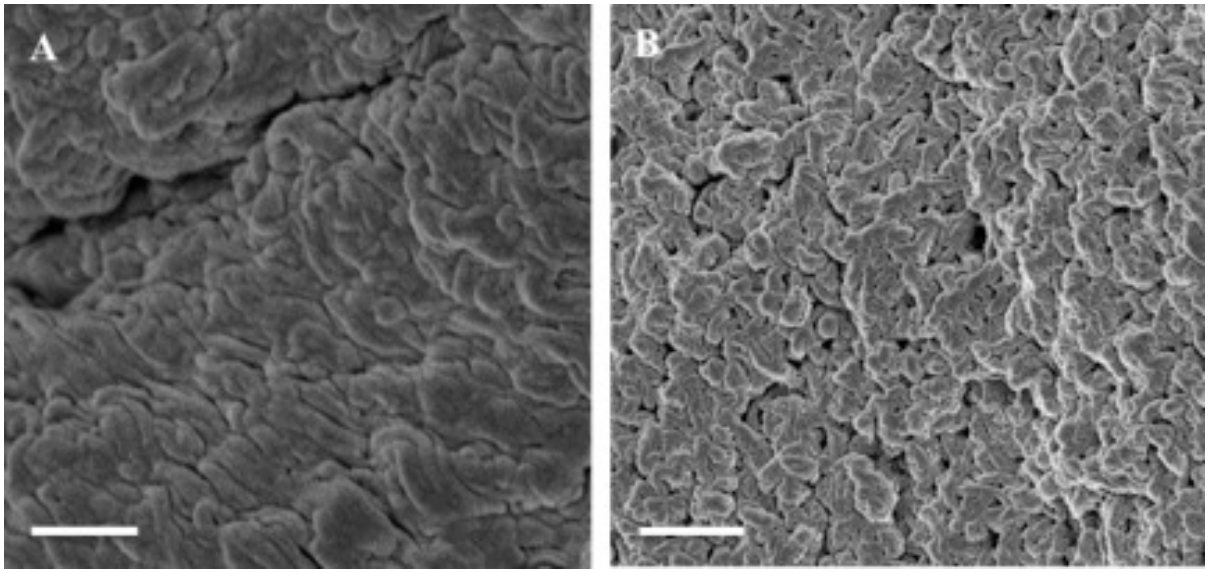

Figure 5 – Scanning electron micrographs of cryo-FG and protamine-FG clots obtained from canine fresh frozen plasma (FFP)
A: FG clot with fibrinogen obtained by cryoprecipitation of canine FFP. **B**: FG clot with fibrinogen obtained by protamine precipitation of canine FFP. The FFP-FGs from both cryo and protamine have large diameter fibers with small sized pores. Fibrinogen concentration in the precipitates used: cryo=13.09mg/mL; protamine=not detected. Magnification bar =1μm. Resolution of microscope images: 25nm.

When the same cryo-FG was coagulated in a three-fold increased fibrinogen to thrombin solution ratio, the density was the most prominent difference (Figure 6). The clot, with the higher fibrinogen content, presented thicker fibers, smaller pore sizes and a more heterogeneous mesh density pattern.

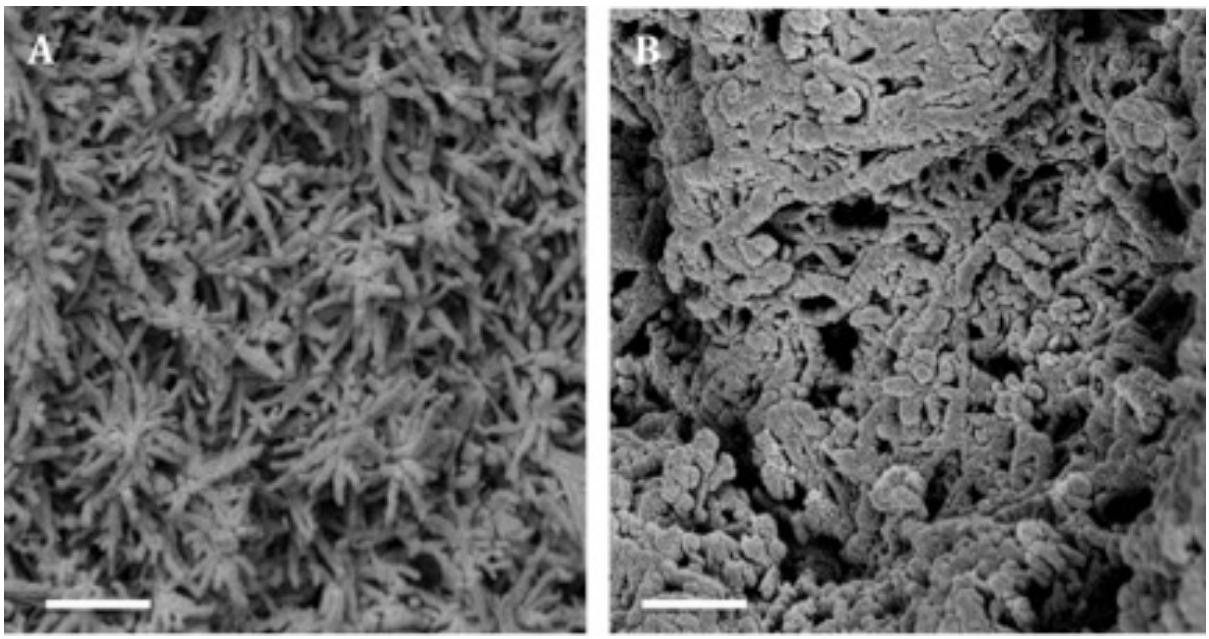

Figure 6 – Scanning electron microscopy of FG clot formed with reaction between cryo-precipitated fibrinogen and thrombin solutions in different volume ratios (1:1 *versus* 3:1).
The cryo-precipitated fibrinogen solution (eFFP) was from the same sample in both clots, but the proportion of volume was different to increase final fibrinogen concentration in the final mixture. **A**: volume 1:1 (11.12mg/mL). **B**: volume ration 3:1 (3x11.12mg/mL). The organization and packing of the fibers seemed to be different. Magnification bar =1μm. Resolution of microscope images: 25nm.

4.5 Discussion

4.5.1 Fibrinogen Concentration

Fibrinogen has been considered the most determinant component on efficacy of fibrin sealants in most surgical applications (DICKNEITE *et al.*, 2006; KAETSU; TAKANORI; SHINYA, 2000). Different types of fibrinogen precipitation methods have been reported to concentrate plasmatic fibrinogen aiming this purpose (AKSOY *et al.*, 2009; ALSTON *et al.*, 2007; SILVER; WANG; PINS, 1995a; DURHAM *et al.*, 1987).

In this study, the mean clottable protein content of protamine was lower than that of cryo, and only one sample reached results over 20mg/mL of fibrinogen measured by Clauss method (29.4mg/mL, FP). More fibrinogen should be present in the protamine precipitate according to the study that describes the protamine protocol for FG production (Alston *et al.*, 2007; p.190). Without mentioning the reasons, the cited authors used Clauss to measure the initial fibrinogen concentration in plasma but not in the final fibrinogen solution precipitated

with protamine. Instead, they used enzyme-linked immunosorbent assay (ELISA). ELISA would be able to determine the amount of fibrinogen precipitated, but not whether that fibrinogen was available or functional for clotting (MACKIE *et al.*, 2003).

Longer periods of plasma storage were associated with higher fibrinogen yield in a preliminary study with cryoprecipitation (Article 1, Chapter 3). Since such effect of storage time upon fibrinogen precipitation was not found in literature before, evaluation of the ability of each protocol to precipitate clottable fibrinogen from each kind of plasma available in blood banks was warranted. Veterinary blood banks, in general, follow the guidelines of the American Association of Blood Banks (AABB; ABRAMS-OGG; SCHNEIDER, 2010; GIBSON, 2007). According to AABB classification of plasma types, FFP is platelet-poor plasma that has been frozen within 8 hours of collection and stored up to a year at temperatures lower than -18^{o}C. FP is defined as a platelet-poor plasma that either stayed longer than 8 hours at room temperature before freezing or a FFP that has been stored for more than a year (BRETCHER, 2005). Since the plasma units obtained for the study could be distinguishable among all three situations, and literature supports more of a negative effect of room temperature storing than the freezing period upon fibrinogen concentrations, it seemed wise to study all three categories separately (MENG *et al.*, 2003; SIMON, 1988).

With cryoprecipitation, the higher fibrinogen yield was observed in older plasma units (eFFP and FP). The FFP plasma was drawn 4 months before the fibrinogen precipitation process, while eFFP and FP were collected much earlier, being 23±1- and 28-month old, respectively. Protamine-precipitated fibrinogen measurements by Clauss were, overall, inconsistent within and among plasma sources. The only category that had all samples measured was eFFP. This inconsistency hindered reliable comparison and conclusions about clottable fibrinogen obtained by protamine precipitation. The mechanism by which low-temperature storage positively affects fibrinogen precipitation and/or clottability is not known. Additional tests would be needed to elucidate whether these results are due to changes in fibrinogen molecular charge, size, or conformation, and influenced by pro-coagulant or inhibiting factors activity (SIMON, 1988; HALL; SLAYTER, 1958).

The high variation among FP bags fibrinogen yield supports both negative and positive influence of time prior to and after freezing. Since the time these bags stayed at room temperature during blood collection and separation is not known, the FP bags that showed lower fibrinogen concentration could indeed be the ones that stayed for much longer

after the 8-hour limit period. The detrimental effect of room temperature on coagulation factors is well reported (SMITH, 2010; SIMON, 1988; MILLER *et al.*, 1986; PALMER; GRALNICK, 1982). Although the depletion of labile coagulation factors, like V and VIII are not paramount for FG efficacy, minor activation of factor VII may consume fibrinogen and affect negatively final fibrinogen content for clotting in FG (MILLER *et al.*, 1986). The initial plasmatic fibrinogen concentrations did not differ among plasma categories. Also, no correlation was detected between the initial and final fibrinogen concentrations, although a tendency seemed to occur with the upmost and lowest values (data not shown). Further studies with canine plasma from same donor and day of collection could give some new insights about the influence of room and low-temperature storage on fibrinogen precipitation and clottability for FG usage.

Different reasons could have influenced the difficulty of Clauss assay with protamine protocol samples (BAILEY; KOENIGSHOF, 2014; GIBBS, 2006). Clauss detection of fibrinogen relies on polymerization of fibrin monomers after cleavage of fibrinogen molecules by thrombin. Normal fibrin polymerization happens in a half-staggered manner as the negative D-domains of other fibrin or fibrinogen molecules become attracted to the positive center of the just cleaved fibrin (LAURENS; KOOLWIJK; DE MAAT, 2006; MEDVED *et al.*, 1993; MEDVED *et al.*, 1990; SMITH, 1980). The way protamine precipitates fibrinogen is by binding to at least one of the two D-domains of the fibrinogen molecule (OKANO *et al.*, 1981). After protamine precipitates fibrinogen, it stays bonded to the D-domain of fibrinogen, at the periphery. Yet, it does not inhibit the action of thrombin in cleaving fibrinopeptides A and B at the central part of the molecule (RIEDEL *et al.*, 2011; MEDVED; LITVINOVICH; PRIVALOV, 1986; OKANO *et al.*, 1981). Since the central domain of fibrinogen becomes positively charged after cleavage (AINLE *et al.*, 2009; RYAN *et al.*, 1999), polymerization would still occur in the presence of protamine. However, whether the configuration among fibrin strands would be altered if protamine were still attached to fibrinogen's D-domain is not known.

According to Alston *et al.* (2007), the highest amount of fibrinogen precipitation was reached with a protamine concentration of 10mg/mL. Then, this quantity of protamine would be the amount needed to have at least one D-domain in each fibrinogen molecule connected to protamine (ALSTON *et al.*, 2007, OKANO *et al.*, 1981). This optimum protamine concentration for precipitation of fibrinogen was determined using human plasma, which had

about 3mg/mL of fibrinogen (ALSTON *et al.*, 2007). In this study, the fibrinogen concentration in canine plasma was rarely higher than 2mg/mL, containing at least 30% less fibrinogen than that used by Alston *et al.* (2007; p.190). If more sites became unavailable in the same fibrinogen molecule, this possible excess of relative protamine could have also worsened the impairment over fibrin polymerization. When Alston *et al.* (2007, p.190) evaluated the amount of protamine that remained in the precipitate, they found that only 0.32% of the protamine added to plasma stayed bounded to fibrinogen after centrifugation. However, the authors rinsed the clot three times with water before evaluation (ALSTON *et al.*, 2007). This step was not included in the protocol for precipitation of fibrinogen for use as FG (ALSTON *et al.*, 2008). Considering that Clauss results are dependent on quantity and clottability of fibrinogen, it is possible that persistent protamine binding to fibrinogen would be hindering polymerization in standard Clauss reading.

Furthermore, fibrinogen coagulability with protamine precipitates was only detected when extra calcium was added to Clauss assay. Additional calcium was used aiming neutralization of the citrate employed in the protamine protocol (THIJSSEN, 2011; QUICK and STEFANINI, 1948). In normal coagulation cascade, calcium is needed for generation of thrombin and for activation of factor XIII (SMITH, 2010; SMITH, 1980). For normal fibrinogen measurement by Clauss, calcium is not essential if activated thrombin is added (STANG; MITCHELL, 2013). Normal polymerization of fibrin monomers happens after fibrinopeptides release by thrombin, while cross-linking of factor XIII serves only to stabilize the polymerized network (MOSESSON *et al.* 1995). The dependence of extra calcium for fibrinogen detection in the protamine samples supports the impression that the detection of a clot by Clauss assay relied more on factor XIII cross-linking and less on normal fibrin polymerization in these samples. Moreover, while cryo samples were only once or twice diluted prior to Clauss assays, protamine samples had to be diluted up to four times. This could be associated with high fibrinogen content in these samples, but yet, not available for clotting due to a possible impairment by protamine. Indeed, Clauss technique was not able to properly determine the amount of fibrinogen precipitated with the protamine protocol used in this study.

The inability to perform all of Clauss readings with protamine samples limited statistical evaluation of cryo and protamine protocols with FFP and FP samples and among plasma types in protamine protocol. Moreover, the small number of samples read by Clauss

in some plasma categories may have diminished the power of statistical analysis. The reason for FFP and FP being the ones to have the less readings within protamine protocol is not known. Hindrances of the protamine protocol with different plasma types, including fresh plasma (data not shown) need further investigation.

A higher CV was observed with Clauss in this study than the normally reported. A reasonable degree of precision for normal plasma was considered to be with a CV of typically 3–7% by optical methods and 6–9% by mechanical (MACKIE *et al.*, 2003). However, the intra-assay variability in this study was not so different from reports using Clauss to measure concentrated fibrinogen samples, where concentration of 7.5mg/ml had a CV of 14.1% (OOSTING; HOFFMANN, 1997). With such variations, subtle differences among groups may appear less significant. These may reflect the still required standardizations to use this assay for high concentrated fibrinogen solutions as the ones intended for FG production.

4.5.2 Viscoelastic Properties of FG Clots

Clots formed with mixing of FG's components must achieve sufficient strength to resist shear and rupture stress during healing, which is achieved by a balance between stiffness and elasticity (CARLSON *et al.*, 2014; DICKNEITE *et al.*, 2003). The rheological properties of clots formed with cryo-FG and protamine-FG were measured mechanically by TEG and compared considering the different canine plasma sources mostly available in veterinary blood banks. The dynamics of clot formation was accessed through R, K, α-angle and TMA values, while clot strength (stiffness and elasticity) was analyzed through MA and G results. Fibrinolysis was observed with LY30 and LY60 measurements.

Similarly to the physiological events, the beginning of clot formation detected by TEG is first dependable on cleavage of fibrinogen by thrombin and subsequent assembly of fibrin strands, followed by factor XIII cross-linking (GENTRY; BURGESS; WOOD, 2008; NIELSEN; GURLEY; BURCH, 2004; MUSZBEK; YEE; HEVESSY, 1999). Although the amount of thrombin in cryo-FG was the same in the protamine-FG, the clot formed by protamine-FG took longer to be detected by TEG, seen with the increased R times. This could be reflecting the possible high interaction of protamine with the D-domains of fibrinogen, impeding the newly formed fibrin to prompt polymerize with other fibrin monomers and be cross-linked (ALSTON, 2006; OKANO *et al.*, 1981). The rate of clot

formation after polymerization had started, seen through K and α-angle values, was also slower in protamine-FG. This could be also due to the polymerization impairment by protamine binding to fibrinogen's D-domain as well to a possible diminishing of factor XIII activity (NIELSEN; GURLEY; BURCH, 2004). To perform cross-linking, factor XIII must be activated by thrombin and calcium before performing its own enzymatic function (SMITH, 2010). Calcium chelation by the additional citrate in the protamine protocol could have induced a calcium deficiency or misbalance, reducing proper factor XIII activation (THIJSSEN, 2011). Thus, factor XIII cross-linking activity, in the protamine protocol, could have been diminished by both lower accessibility of factor XIII's binding and substrate sites on already polymerized fibrin, due to protamine, and by a possible calcium insufficiency.

Overall, cryo-FG had significantly stronger clot than protamine-FG. Although, clot stiffness (MA) in FGs is mostly determined by fibrinogen concentrations, it is also influenced by factor XIII cross-linking (CARROL *et al.*, 2008; ROCK *et al.*, 2007). Since TEG variables are derived from an increase in viscoelastic resistance mediated by fibrin mesh formation, FXIII-mediated fibrin cross-linking would indeed affect K, α-angle, A, G and E (NIELSEN; GURLEY; BURCH, 2004). Although much more fibrinogen should have been precipitated with protamine protocol according to previous reports (ALSTON *et al.*, 2007), it could be that the probable hindrance of protamine binding upon fibrin polymerization and cross-linking, added to a possible diminished activation of factor XIII, as previously discussed, could have been synergistically decreasing clot strength.

Considering plasma types, differences on clot strength values between cryo-FG and protamine-FG could only be appreciated in eFFP. With the protamine protocol, not all the samples from FFP and FP types were able to generate fibrinogen measurements in Clauss. Although TEG was able to assayed them all, the possibility of having unidentified interferences, which could had also interfered with FFP and FP readings in Clauss, should not be discarded as potential influences in TEG values to a point of enabling statistical capability to detect differences. Another reason that can possibly explain the lack of significant difference of FFP and FP between cryo and protamine is that these plasma types had inferior performance in cryo; thus, a real difference would not exist in relation to their protamine counterparts. Such findings could motivate protamine precipitation in cases when only FFP and FP are available and equipment and time favor processing with protamine protocol.

Within cryo protocol, there were significant differences in clot strength among plasma types. Canine plasma that stayed frozen for longer periods (eFFP) had better clotting performance than fresher ones (FFP). Although FP showed numerically higher MA values than FFP, statistical differences were not verified. Just as seen with Clauss evaluations, FP-derived FG had no detectable differences from the other plasma types. Such TEG results could be a reflection of the same factors that influenced fibrinogen measurements with these samples. The higher fibrinogen yield of eFFP, and/or higher clottable fibrinogen, could also explain the higher clot strength (MA) with this plasma source. FP had a little bit more storage time then eFFP, but it had the detrimental effect of staying at room temperature for more than 8 hours during blood collection. This period could have increased the risk of factor VII-induced clotting and consequently lessened fibrinogen availability (MENG *et al.*, 2003; MILLER *et al.*, 1986). The supposedly beneficial and the undesirable characteristics of FP could have accounted for weaker detection of statistical differences. However, the negative effect of room temperature period may not have interfered in clot strength in FP to the same extent that shorter freezing storage time influenced FFP.

In the other hand, time to reach maximum clot strength (TMA) was longer for FP than for FFP and eFFP fibrin clots, more evidently in cryo samples. Among other factors that may diminish at room temperature storage, the labile factors V and VIII have been shown to be the most affected ones (WANG *et al.*, 2014; GROCHOWSKY *et al.*, 2014). These cofactors are important in the propagation phase of clot formation, increasing thrombin activity up to 300.000-fold via factor X (AINLE *et al.*, 2009; GENTRY; BURGESS; WOOD, 2008). Their possible diminished levels in FP could have accounted for longer time to achieve maximum clot strength in TEG assays of FP samples. Yet, the significance of this parameter in practical surgical applications of FG needs to be better established, since thrombin concentration can be easily tailored. Studies with bettered defined periods of processing and freezing storage and specific tests could help elucidate the effect of such processing characteristics on cryoprecipitation of fibrinogen aiming FG production.

Within the protamine protocol, differences of clot strength among plasma types could not be appreciated. Although there could have been detrimental effects of protamine itself on clot formation in all plasmas types, the presumably high fibrinogen yield in the protamine-precipitated solution (ALSTON *et al.*, 2007) may explain why the marked difference seen among plasma sources in cryo-FG was not so greatly appreciated in the protamine protocol.

Thus, the factors that favor cryoprecipitation and/or fibrinogen clotting in older plasma may not have much influence with protamine precipitation. However, TMA showed similar tendencies with protamine and cryo-FG with the same plasma type. Yet not significantly, FP seemed to take longer than FFP and eFFP to reach MA in protamine samples as well.

In order to better evaluate the clot among plasma categories and for longer periods, tissue plasminogen activator (t-PA) was not used to induce fibrinolysis in these TEG analyses. Thus, the assay features would be more related to the clinical application of FG, where, frequently, fibrinolysis is inhibited by addition of anti-fibrinolytic substances like aprotinin (GLIDDEN; MALASKA; HERRING, 2000). Different from cryo, protamine showed spontaneous induction of fibrinolysis. Such aspect of protamine-FG was not expected and the reason for the onset of fibrinolysis is not known. Poor incorporation of fibrinolysis inhibitors and plasminogen by a possibly reduced cross-linking activity of factor XIII has been considered (LORD, 2011; NIELSEN; GURLEY; BURCH, 2004; PHILLIPS; DICKNEITE; METZNERC, 2003; RYAN *et al.*, 1999a). Just as it happens with the cross-linking among fibrin molecules, when protamine is bonded to some D-domains of fibrin or fibrinogen fewer sites on these fibers would be available for factor XIII to incorporate fibrinolysis inhibitors, such as α2-PI, PAI-2, TAFI, into the fibrin network (SMITH, 2010; GENTRY; BURGESS; WOOD, 2008; MUSZBEK *et al.*, 2008). Also, in normal fibrin assembly, factor XIII mediates plasminogen entrapment to reduce its activation rate (WEISEL *et al.*, 1994). Thus, fibrinolysis could have been promoted and enhanced through the protamine binding effect, by diminishing cross-linking of fibrin-strands and incorporation of fibrinolysis inhibitors and plasminogen to fibrin (OLSON, 2015; STANDEVEN *et al.*, 2005; COLLET *et al.*, 2003; RYAN *et al.*, 1999b). Old literature (OLESEN, 1961 *apud* DEMPFLE; HEENE, 1987) has mentioned that protamine also precipitates plasminogen activator, which could explain the onset of fibrinolysis. No additional information about precipitation or direct effect of protamine on plasminogen was found. Alston *et al.* (2007) did not evaluate fibrinolysis directly. Yet, using a different system from that of TEG for evaluation of the “cure time” to reach maximum clot tensile strength, the clots lasted at least 60 minutes without showing significant changes in tensile strength values. In their study, there was no mention about clots being observed for longer periods or whether fibrinolysis was detected (ALSTON *et al.*, 2007). In the present study with TEG, fibrinolysis started earlier than 30 minutes after maximum strength was reached. Correlation of TEG

observations of FG fibrinolysis with *in vivo* FG application and behavior is difficult to make. TEG was developed to predict fibrinolysis with whole blood and in a different situation than that of FG. The mechanisms by which the forces are applied in TEG were intended to simulate the pressure existing during physiologic hemostasis after a natural clot has formed (WHITING; DINARDO, 2014). Moreover, TEG engine was developed to assay whole blood considering the effect of red blood cells and platelets on clot rheology. Also, cellular and enzymatic condition of the tissue, type of stress that is set upon the FG clot, and even the presence of the patient's own platelets and blood may influence fibrinolysis of FGs (OLSON, 2015; OVERBEY; JONES; ROBINSON, 2014; BEDUSCHI *et al.*, 1999). Thus, the addition of anti-fibrinolytic agents in FGs is commonly performed depending on the intended use, regardless the FG protocol (FATTAHI; MOHAN; CALDWELL, 2004; DICKNEITE *et al.*, 2003). Different FG protocols, including commercial FGs, would not have platelets and would contain different concentrations of adhesive proteins like fibronectin and von Willebrand factor (WOZNIAK, 2003; KJAERGARD *et al.*, 2000; OKADA *et al.*, 1985). The effect of adhesive proteins in FG and whether their possible lower concentration in the protamine protocol could have induced detachment of the clot from the cup and influenced fibrinolysis onset in TEG will need further elucidation. Nevertheless, these results show that, in comparison to cryo-FG, protamine protocol may generate a clot that is more susceptible to fibrinolysis.

Lower correlation between Clauss and TEG's clot strength variables in the protamine protocol increases the suspicion of protamine-induced impairment of proper clot formation and calcium misbalance (NAIR; SAHA; DHALL, 1986). In another hand, with cryo protocol, fibrinogen content highly correlated with clot strength in TEG. This correlation of fibrinogen and clot strength has also been observed and discussed earlier by other authors studying FG, whole blood, platelet rich plasma, and platelet poor plasma (WHITING; DINARDO, 2014; URBAN; COUTO; IAZBIK, 2013; HICKERSON; NUR; MEIDLER, 2011; ; CARROL *et al.*, 2008; NIELSEN; GURLEY; BURCH, 2004).

The coefficient of variation for MA between replicates in this study reached up to 31% in cryo samples and 42% in protamine. The standard intra-assay coefficient of variation for the TEG in literature has been reported as 7.9% to 39.9% (WHITING; DINARDO, 2014, UK NEQAS data). TEG assays for FG analyzes would benefit from better standardization to diminish coefficients of variance and increase statistical power of results.

TEG parameters of clot strength have been related to FG performance *in vivo* when applied for hemostatic purposes (HICKERSON; NUR; MEIDLER, 2011). For other applications, like sealing, adhesive or wound healing support, different methods have been developed and combined (LACAZE *et al.*, 2012; KULL *et al.*, 2009; PERRIN *et al.*, 2009; SIERRA; EBERHARDT; JACK, 2002; SILVER; WANG; PINS, 1995a). FGs' properties need to be accessed by one or more tests that replicate the forces and environment of a specific tissue and application goal (DICKNEITE *et al.* 2003). Nevertheless, TEG helps to elucidate clot formation and behavior in real time, which has not been available by any other assay so far (WHITING; DINARDO, 2014; HICKERSON; NUR; MEIDLER, 2011; GLIDDEN; MALASKA; HERRING, 2000). Moreover, it allows fast and simple evaluation of different protocols and variation of components on FG rheology (CARLSON *et al.*, 2014; CARROL *et al.*, 2008; ROCK *et al.*, 2007).

4.5.3 Ultrastructure of FG Clots

For some applications, FG structure may be particularly relevant and a clot arrangement may be more appropriate to a specific use than to another (HO *et al.*, 2006; COX; COLE; TAWIL, 2004). It can influence clot strength, cellular migration, and neovascularization, impacting the outcome of an intended FG usage (CECARELLI and PUTNAM, 2014; FERGUSON; NURNBERGER; REDL, 2010; HO *et al.*, 2006). SEM imaging can be used to evaluate and adjust components in FGs aiming the identification of most suitable mesh works (FERGUSON; NURNBERGER; REDL, 2010; WEISEL, 2005; RYAN *et al.*, 1999a). The present study intended to image clots obtained with fibrinogen concentrated solutions derived from cryo- or protamine-precipitation of canine plasma, and with FG formulations mostly used during *in vivo* applications. The plasma sources were fresh frozen plasma within a year of collection and fresh frozen plasma that elapsed a year of freezing storage, the later being usually considered a surplus material in veterinary blood banks (NICHOLS *et al.*, 2009).

Although the ultrastructure of cryo- and protamine-FG were somewhat alike, some aspects differed between their clots. It seemed that the protamine had slightly thinner and less packed fibers, besides the presence of more preeminent debris. The apparently lower density presented mainly by wider pores in the protamine-FG clot, could be related to factors

underlining the lower fibrinogen concentrations measured by Clauss and the decreased clot strength detected by TEG, when compared to its cryo-counterparts (WOLBERG, 2007, RYAN *et al.*, 1999a; RYAN *et al.*, 1999b; OKADA; BLOMBACK, 1983). Although Alston *et al.* (2007) reported precipitation of more than 90% of the plasmatic fibrinogen, perhaps, not all of this protein was capable of adequate polymerization. No rheological profiles or imaging of the resultant clot were performed with protamine samples in Alston's study, which hinder inferences and interpretations of the proposed protamine protocol (ALSTON *et al.*, 2007). Moreover, no imaging of canine fibrin clots derived from cryo- or protamine-FG has been reported. An electron microscopy study with protamine present in normal human plasma did not present suitable parameters for comparison (HORN; HAWIGER; COLLINS, 1969). The clotting of protamine FG, believed to be somewhat troublesome by TEG and Clauss evaluations, could be further appreciated with SEM imaging. The marked differences detected by Clauss and TEG between cryo and protamine protocol with eFFP did not seem so apparent in SEM imaging. In studies where clot strength analysis were associated with SEM images, the presence of factor XIII increased strength of the clot, but did not change the structure of the fibrin network (RYAN *et al.*, 1999a). As other authors have suggested, they attributed this observation to the fact that cross-linking of fibrin strands promoted by factor XIII happens only in already polymerized fibrin, whether normal or abnormally assembled (STANDEVEN *et al.*, 2005; RYAN *et al.*, 1999a; MOSESSON *et al.*, 1995). Although fibrin polymerization seemed to occur in the presence of protamine, when residual protamine is bonded to some D-domains of fibrin, there could be fewer sites on these fibrin strands available for factor XIII to perform cross-linking of fibrin network. This possibility could explain the significant difference seen in clot strength measured by TEG and not so much distinction observed in fibers arrangement of SEM images between cryo and protamine FGs.

The globular and smooth debris identified among fibrin fibers in both cryo and protamine-FG has been previously described as possible cellular fragments and plasmatic proteins (WEISEL, 2005). During the separation of blood cellular components, some platelets and leukocytes can remain in the plasma. With the fibrinogen precipitation process, along with the fibrinogen-associated factors, heavier proteins and cellular remnants may precipitate with fibrinogen (TOBIAS; JOHNSTON, 2011; FATTAHI; MOHAN; CALDWELL, 2004; SHAH; NAIR; DHALL, 1987). This type of residues looked more

pronounced in cryo-FG, where coarseness of fibrin strands surfaces seemed more evident. This fact could be associated with the longer centrifugation cycle and higher *g* force performed in cryoprecipitation. Considering the SEM evaluations performed with FG clots in other studies, most images were acquired with pure fibrinogen solution or commercial FG preparations. These images do not show the debris seen in the present study (NURNBERGER *et al.*, 2010; HO *et al.* 2006). When evaluating FG gel derived from equine plasma, Textor *et al.* (2014) also reported observation of cellular debris within the clots, but their SEM images could not be correlated. A different type of clustered residue was seen in protamine-FG. In the study Alston *et al.* (2007), it was reported that some of the protamine added to the human FFP was precipitated with the fibrinogen during the concentration process. The debris observed in the protamine-FG clot could indeed be residual protamine. Whether this possible protamine remains would be detrimental to *in vivo* applications is not clear.

Some authors consider changing the fibrinogen to thrombin ratio to increase fibrinogen final concentration of FGs and enhance its effectiveness on certain purposes (KAETSU; TAKANORI; SHINYA, 2000; DICKNEITE *et al.*, 2003; BRENNAN, 1991). A sample of increased proportion of fibrinogen to thrombin was imaged with SEM. Two clots from the same fibrinogen solution and of equal final thrombin concentration were prepared with different proportions of fibrinogen to thrombin solutions volume (1:1*versus* 3:1). The organization and packing of the fibers of these clots seemed different, with ticker fibers and smaller pore size being evident in the samples with increased ratio. Such higher density may or may not be desirable, depending on the objective with the usage of FG. Besides the increased risk of poor homogeneity, this approach may convey possible detrimental effects on tissue regeneration. In applications where migration of cells and vessels is required, dense clots may hinder the physiologic invasion of cells into the fibrin matrix (CECARELLI and PUTNAM, 2014; FERGUSON; NURNBERGER; REDL, 2010; HO *et al.* 2006). These aspects have been considered to influence the success or failure on detecting the beneficial effect of FG in some skin grafting or wound healing applications with high fibrinogen-content FG (HOLZAPFEL *et al.*, 2013; FERGUSON; NURNBERGER; REDL, 2010; RADOSEVICH; GOUBRAN; BURNOUF, 1997). For hemostasis, sealing effects and slow delivery of substances, however, this tightness among fibrin fibers may be desirable (OVERBEY; JONES; ROBINSON, 2014; NURNBERGER *et al.*, 2010; SPICER; MIKOS,

2010; MARONE *et al.*, 1999). Nevertheless, the benefit of clot structure and polymerization patterns would be better analyzed in association to elasticity and clot strength evaluations of FG, considering each specific application, type o tissue to be applied, and mode of forces to be resisted (LAURENS; KOOLWIJK; DE MAAT, 2006; FATTAHI; MOHAN; CALDWELL, 2004; DICKNEITE *et al.*, 2003). Still, higher fibrinogen to thrombin solution ratio, can be an approach for increasing fibrinogen concentration in the final FG clot when fibrinogen precipitation may yield fibrinogen contents considered suboptimum to a specific use.

Components concentrations and ratios that influence clot rate may alter fibrin clot structure, mostly branching and fiber diameter, and consequently clot stiffness and elasticity (LORD, 2011; STASIO *et al.*, 1998; COLLET *et al.*, 1996; WEISEL; NAGASWAMI, 1992). Among the ones reported are fibrinogen, factor XIII, calcium, and thrombin (WOLBERG, 2007; WEISEL, 2005; RYAN *et al.*, 1999a; OKADA; BLOMBACK, 1983). Some studies mention that release of fibrinopeptide A happens first and faster than the release of fibrinopeptide B. And that, the rate and amount of cleavage of each site can alter the structure of the clot, by the way half-staggered polymerization, lateralization and branching will happen (BLOMBACK; BARK, 2004; WEISEL; VEKLICH; GORKUN, 1993). In higher thrombin concentration, the cleavage of fibrinopeptides would happen faster, and more branching and less lateralization would occur (WOLBERG, 2007; BLOMBACK *et al.*, 1994; MOSESSON *et al.*, 1993). Thus, the pore size and fiber diameter would be smaller. Since cross-linking happens concomitantly, this type or configuration would be stabilized as such, and elasticity could be decreased. The overall clot strength in such situations will be more positively related to fibrinogen concentration (LORD, 2011; COLLET *et al.*, 1996; WEISEL; NAGASWAMI, 1992). In the present study, the thrombin concentration used would not be considered as high if compared to fibrinogen concentrations (NURNBERGER *et al.*, 2010). Thus, major changes in clot structure due to thrombin content were not expected. With the thrombin concentration used, clot formed within 10 to 15 seconds. The following ten minutes of resting prior to sample fixing was meant to allow stabilization of the clot and circumvent shrinkage and misleading image results.

Ultrastructure characterization of most appropriate proportions of clottable protein to other FG components aiming specific purposes is important considering the differences of tissue architecture and movement (FERGUSON; NURNBERGER; REDL, 2010; PARK *et*

al., 2002). For example, if FG is to be applied in the subcutaneous for adhesive support between muscle and skin, during reconstructive surgery, a tick and stiff clot with low elastic modulus may not be desirable, because it can hamper neovascularization, cause the adherence between tissues to fail with movement of muscles, increasing death space and accumulation of serum. In situations like these, porosity of the clot may be more desirable to allow cellular migration through the fibrin glue matrix, what is diminished in high fibrinogen clot content (DUARTE et al., 2012; WHEAT; WOLF, 2009). Yet, in situation, like orthopedic reconstructive surgery, for compounding small fragments together to fill a bone gap, some stiffness of the clot may be necessary to counteract the weight of the fragments themselves and stand friction from the surrounding soft tissues (KIM *et al*., 2014; YOU *et al.*, 2007).

Although the effect of clot structure on strength and fibrinolysis has been studied considering physiologic hemostasis (COLLET *et al.*, 2003; WEISEL *et al.*, 1994), not much has been published associating FG clot structure with surgical performance in more defined applications. Adequate clot arrangement studies could enhance appropriate and successful usage of FGs by helping direct formulations to specific uses.

In this study, SEM images of cryo-FG were not much different from those of protamine-FG. Yet, higher resolution images were difficult to be evaluated due to the gold coating. Cutting the clot could be a strategy to analyze better the inner structure of these samples. Objective comparisons by SEM analytical software are planned for better assessing the ultrastructure of FGs derived from cryo and protamine protocols.

4.5.4 Final Considerations

Complementarily multimodal tests are important to reach a comprehensive and reliable FG characterization. Knowledge of important FG features and of well-defined options for changing FG composition to reach appropriate formulation for distinctive usages would enhance FGs' effectiveness and extend its use to other therapeutic fields in veterinary medicine. The commercial sealants do not have much permutation of components and are expensive to support further studies on the different formulations for each proposed application. Although the blood plasma may have individual variations, with precise and controlled laboratory protocols, it could be possible to obtain a uniform fibrinogen solution to

be tailored into a custom-made product aimed at each different surgical or research setting.

While cryoprecipitate demanded less preparation of special solutions and appeared more consistent regarding homogeneity and capacity to increase final fibrinogen concentration, protamine protocol showed to be faster and simpler considering the equipment required (APPENDIX B).

Due to the lesser therapeutic need, fresh frozen plasma stored at veterinary blood banks, can become a surplus material of inconvenient storage even before expiration (considering a year from collection). Thus, its use for FG production implies an economical approach to avoid biological material wastage, increase income, and provide a valuable tool for a variety of surgical circumstances.

Additional studies are needed to improve protamine precipitation and better elucidate the effect of freezing storage time on precipitation and/or clottability of fibrinogen intended for canine FG production. FG production with fresh and small volumes of plasma is still to be improved to allow production of FG in emergency situations.

4.6 Conclusion

Cryoprecipitation produced higher clottable fibrinogen content with a more prompt polymerized and stable FG clot in comparison to protamine method. Both precipitation techniques produced FG clots with similar fibers arrangement. Older platelet poor plasma appeared to be a superior source of fibrinogen for canine FG production by cryoprecipitation.

Fibrinogen concentrated through both cryo- and protamine precipitation seams feasible for canine FG production. Veterinary hospitals, blood banks, and patients can benefit from usage of surplus plasma units for FG production aiming surgical and scientific applications.

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4.8 Acronyms and Abbreviations

FFP fresh frozen plasma
FG fibrin glue
FP frozen plasma
E elasticity constant (dynes/sec)
eFFP expired fresh frozen plasma
G shear elastic modulus strength (dynes/cm^2)
IU international units
K kinetics, time to achieve a certain clot firmness
LY60% clot lyses at 60 minutes after maximum strength in percentage
MA maximum amplitude, maximum clot strength
P significance level
R reaction time for clot formation
r^2 Pearson's product-moment correlation coefficient
SD standard deviation
SEM scanning electronic microscopy
TEG thromboelastography
TMA time to maximum amplitude

4.9 References

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5 ARTIGO 3 – CANINE FIBRIN GLUE PRODUCED WITH FIBRINOGEN CONCENTRATED BY CRYO- AND PROTAMINE PRECIPITATION: *IN VIVO* EVALUATION OF HEPATIC HEMOSTASIS AND INTESTINAL WALL ADHESIVENESS IN A RABBIT MODEL

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5.1 Abstract

Canine-derived fibrin glue (FG) can become a valuable material to be used by veterinary surgeons. FG's effect on hemostasis and sealing of suture lines, to block liquid or air leakages from hollow organs that are naturally under peristalsis or content distention, could benefit a variety of troublesome surgical situations. This study aimed on production of FG from canine plasma with two different fibrinogen precipitation protocols and evaluation its use as surgical hemostat and sealant on intestinal serosa. Cryo- and protamine precipitation were performed with canine fresh frozen plasma (FFP) from veterinary blood banks. Aliquots containing an average of 15±3mg/mL of fibrinogen, determined by Clauss method, were used, in a volume ratio of 1:1 of fibrinogen to thrombin solutions. Thrombin solutions were prepared to have 100UI/mL of thrombin with 40μmol/mL or 80μmol/mL of calcium chloride to be used with cryo- or protamine-FGs, respectively. When used, aprotinin was added to have a final FG concentration of 1.500UI/mL. Six rabbits were subjected to general anesthesia and treatments were randomized among hepatic and intestinal incised regions in a complete block design. In each animal, three hepatic lobe incisions were carried out to evaluate the hemostatic treatments: manual compression (control), cryo-FG, and protamine-FG. In the colon-rectal region, four full-thickness 1.5-cm incisions were carried-out and closed, receiving: sutures only, sutures and cryo-FG, sutures and protamine-FG, and sutures and protamine-aprotinine-FG. Hepatic hemostasis was significantly faster with FG

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treatments than with control. Time to polymerization in contact with organ, time to achieve hemostasis, feasibility and efficacy of FG application, volume of FG applied, and need for reapplications did not differ among FG treatments. At post-mortem evaluations performed after three or seven days post-operatively, macroscopic signs of hemorrhage were absent. Presence of unfavorable visceral adhesions and microscopic evaluation of detection of fibrin, inflammatory cells and tissue healing process did not differ among treatments in both liver and intestine trials. The use of aprotinin in the protamine clot did not impair its application and seamed beneficial in diminishing the intestinal adherences evaluation. The canine FG produced with cryo- and protamine-precipitation showed feasible processing and similar performance in the *in vivo* evaluations with the rabbit hepatic biopsy-size incisions and intestinal anastomosis models. Veterinary hospitals, blood banks, and patients can benefit from usage of surplus plasma units for FG production aiming many surgical and scientific needs.

Keywords: fibrin sealant, hemostat, platelet poor plasma, dog

5.2 Introduction

Fibrin glue (FG) has become a useful tool in many surgical specialties worldwide (SPOTNITZ, 2014). FGs' effectiveness in controlling hemorrhage and supporting healing process has been reported by various studies (ZUCCHELLI; SILVESTRI, 2004; REISS; OZ, 1996; FALSTROM et al, 1999). Since FG promotes local hemostasis independently of the coagulation factors profile of the patient, it can contribute to control bleeding not only in parenchymal organ surgery, but also in different surgical interventions when patients may have hepatic dysfunctions, inherited and acquired coagulopathies, or have received high volume of fluid therapy (OVERBEY; JONES; ROBINSON, 2014; KUNIO; SCHREIBER, 2013; DHILLON, 2011). If bleeding can be reduced with the use of FG during parenchymal organ resection or traumatic wound management, transfusion of blood bags and erythrocytes concentrates due to excessive trans or post-operatory hemorrhage can be prevented. Thus, the risk of adverse reaction with transfusion would be avoided providing more safety for the patient (ZUCCHELLI; SILVESTRI, 2004). Also, in periods of scarcity of blood donors, the erythrocyte-containing units can be directed to other patients in need. The use of FFP units for FG production increases its applicability and diminishes its wastage after a prolonged

period of storage in blood banks (GROCHOWSKY et al, 2014). Evading transfusion and diminishing hospitalization time may also entail a more affordable budget for the owner (MINTZ et al; 2001).

FGs have also been used for sealing of suture lines, blocking liquid or air leakages in different clinical and controlled studies (BORIN et al, 2008; LIPPERT et al, 2011; RICE; BLACKSTONE, 2010). Thus, patients that are under certain diseases processes, either generalized or localized to an organ, can benefit from the sealing property of FGs over the incised or damaged tissue (PORTILLA-DE BUEN et al, 2014; DUNN; GOA, 1999). Intestinal anastomosis is a commonly performed surgical procedure in dogs (WILLIAMS, 2012). Following this type of surgeries, the risk of peritonitis increases when deterioration of the tissue or patient condition may develop in the post-operative period, especially when proper diagnosis and or treatment were delayed (CORNELL, 2012). FG has been used for support of gastro-intestinal lesions and high-risk anastomosis, applied in mucosal, submucosal and serosa layers, providing a sealant effect during a concerned healing process (LIPPERT el al, 2011; RUTGEERTS et al, 1997; BRADY et al, 1993).

Due to the scarce literature on the production and use of FG in veterinary medicine, the employment of this sealant is still dependent on production protocols directed to the needs of the veterinary surgical setting and meticulous evaluation of its properties and biological characteristics (WHEATHON et al, 1994). From the different protocols for attaining plasmatic fibrinogen cited in literature, the most used is the cryoprecipitation method (SPOTNITZ 2014; REDOSEVICH et al, 1997; SPOTNITZ, 1987). Although it is considered more efficient and safer than other methods, biologically and chemically, it has some process-related hindrances, mostly related to often low fibrinogen content, volume of plasma required, and accessible equipment for its processing (SILVER; WANG; PINS, 1995b). Protamine precipitation method has been recently described as able of precipitating higher amounts of fibrinogen with a simpler and faster processing method (ALSTON et al, 2007; ALSTON et al, 2008).

Canine FG availability could benefit different areas of veterinary surgical therapy. However, the employment of this sealant is dependent on a production protocol directed to the needs of the veterinary surgical setting and meticulous evaluation of its biological properties. Aiming to evaluate the hemostatic property and tissue adhesiveness of canine FG

derived from cryo- and protamine-precipitated fibrinogen, hepatic wedge excisions and intestinal anastomosis were performed in a rabbit model.

5.3 Material e Methods

All animal handling and procedures were in accordance with the guidelines from the Brazilian[20] and U.S.[21] national research councils and approved by the Committee of Ethics on Animal Use and Research Commission of the University of Rio Grande do Sul (CEUA-UFRGS, registration number 24362).

5.3.1 Fibrin Glue Solutions

Canine FG consisted of a clottable protein solution contained plasmatic concentrated fibrinogen alone or with aprotinin ("fibrinogen solution") and a catalyzer solution containing thrombin and calcium chloride ("thrombin solution").

5.3.1.1 Fibrinogen Concentrate

The canine plasma obtained from a commercial veterinary blood bank[22] was processed through standardized techniques (ABRAMS-OGG; SCHNEIDER, 2010) for separation of the cellular components of whole blood. Within eight hours from collection, the platelet poor plasma units were transferred to 50mL-polypropylene centrifuge tubes into final volumes of 50mL (BRENNAN, 1991). All 50-mL aliquots were refrozen at -80°C for a minimum of 6 months preceding the fibrinogen precipitation processes (GONCALVES et al, 2005, chapter 3). From each bag, at least one 50mL-sample of fresh frozen plasma (FFP) was directed to the cryo protocol and another was used for the protamine precipitation process. The concentrated fibrinogen solutions obtained through cryo- and protamine-precipitation of canine (FFP) followed the methodologies previously described for human FG (SILVER; WANG; PINS, 1995a; ALSTON et al, 2007).

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[20] Brazilian Federal Law No 11.794, 8/OCT./2008

[21] Guide for the Care and Use of Laboratory Animals; Animal Welfare, 7 USC/7 SFR

[22] Blut's, Porto Alegre, RS, Brazil (Privately owned veterinary blood bank)

Briefly, cryoprecipitate was obtained by slowly thawing the plasma at 2°C for 11 to 13 hours. When almost no ice crystals could be seen among fibrinogen flakes, the plasma was centrifuged[23] at 4,000 *x g* for 10 minutes, at temperatures between 1°C and 3°C, to form a sediment at the bottom of the tube. Before decantation of the supernatant plasma, 1.6 mL of the solution was recovered for dilution of the cryo-precipitated sediment. The cryo aliquots were apportioned in 0.5mL aliquots and stored at -20°C.

For the protamine precipitation of fibrinogen, the 50-mL samples were thawed at 37°C for about 10 minutes, until all fibrinogen was solubilized, and were then left at room temperature of 22°C for another 10 minutes. The amount of 13.4 mL of a protamine[24] stock solution (40mg/mL) was added to the thawed plasma to reach a final concentration of 10mg/mL. After mixing the content by lightly rotating the tube five times up side down, the solution was centrifuged at 1,000 *x g* for five minutes to sediment the precipitate (20°C). The supernatant was discarded and the remaining sediment was redissolved in 1.6mL of 0.2M sodium citrate (pH 7.4), on a vortex. The protamine-precipitated fibrinogen solution was stored at -20°C until later use.

Prior to use or assaying, the cryo solutions were thawed at 37°C in water-bath for three minutes. Samples from cryo- and protamine- precipitates were submitted to fibrinogen measurement and standard microbial culture for assuring biological safety. Fibrinogen solutions used in the rabbit model had a concentration of 12 to 18mg of clottable protein (*mean*=15.14mg/mL, *SD*=3.1), determined by the Clauss method. When present, aprotinin[25] was added to have a final FG concentration of 1.500UI/mL.

5.3.1.2 Catalyzer solution

Thrombin[26] solutions were prepared to have a final concentration of 50UI/mL of thrombin with 20µmol/mL of calcium chloride in the cryo-derived FGs and with 40µmol/mL of calcium chloride in the protamine- and protamine-aprotinin-derived FGs. All preparations

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[23] Thermo® ALC-PK 121R refrigerated centrifuge, AM-10 rotor

[24] Sigma Chemical Co., St. Luis, MO; Protamine sulfate from salmon, grade II – P4380

[25] Merck Millipore, Darmstadt, Germany; Aprotinin, bovine lung – 616399

[26] Merck Millipore, Darmstadt, Germany; Thrombin, bovine plasma – 112374

were strictly performed under aseptic conditions.

5.3.1.3 Delivery System

Before each FG application, an operating-room assistant thawed the aliquots containing the fibrinogen and thrombin solutions, for 3 minutes in water-bath. The surgeon pulled the content from each microtube into sterile syringes. One-piece device held the two syringes linking their plungers for simultaneous discharge through a custom-made dual-chamber single-channel tip system (Figure 7).

For all groups tested, the ratio used was 1:1 of thrombin to fibrinogen solutions.

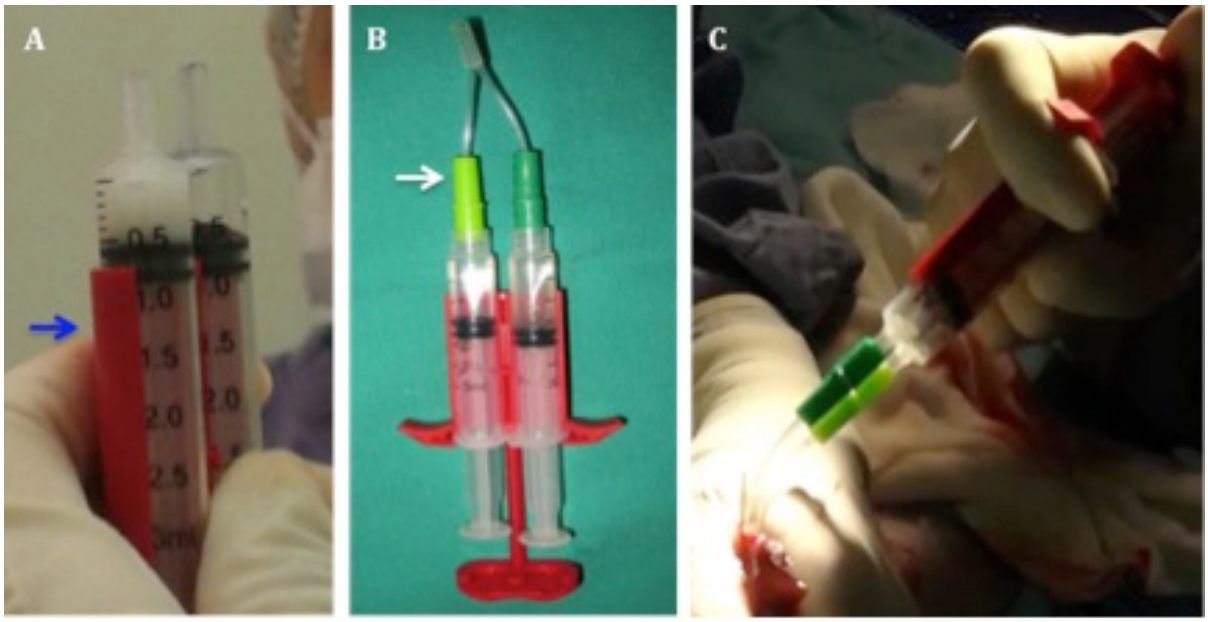

Figure 7 – Customized delivery system used for application of canine FG.
A: The system kept the fibrinogen and the activated thrombin in separate chambers. The holding device (blue arrow) was obtained from a human commercial FG (Tisseel®) and was autoclaved before each use. **B**: Customized delivery tip (white arrow): built with two 4-gauge urethral catheters and a small piece of a drip set line. Inner diameter x length of delivery channels: individual tip = 1mm x 2cm, common-end= 3mm x 2mm. **C**: As the surgeon depressed the linked syringe plungers, the protein solutions were mixed in a common channel and ejected from the syringe tip into the wound.

5.3.2 Experimental Design

To determine and compare hemostatic efficacy between canine cryo-FG and protamine-FG, a liver excisional biopsy model was established. The methods applied for hepatic hemostasis were: FG with cryo-precipitated fibrinogen (cryo-FG), FG with protamine-precipitated fibrinogen (protamine-FG), and digital compression (control).

To assess the behavior of cryo-FG and protamine-FG with or without aprotinin, considering adherence to intestinal serosa, colorectal incision and anastomosis were performed. Four treatments were executed for intestinal wall closure: suture with FG with cryo-precipitated fibrinogen (cryo-FG), FG with protamine-precipitated fibrinogen (protamine-FG), protamine-precipitated fibrinogen with addition of aprotinin (protamine-aprotinin-FG), and suture only (control).

In both liver and intestine, the order of the treatments was defined by causality design.

5.3.3 Animals

Six adult (female) New Zealand rabbits, weighing 3.5±0.5kg, purchased from a registered breeder, were subjected to only one-time surgery. In order to minimize variables and reduce the number of animals, all the treatments were tested in all rabbits. All rabbits underwent about a 1.5-hour (*mean*=88.1minutes, *SD*=13.3) surgical procedure in which all treatments were performed. The animals were fasted for 12h, to diminish intestinal gas content, and restricted to water access six hours before surgery (MENEZES, 2012; ALVES; LOPES; SASASAKI, 2011).

5.3.4 Surgical Procedure

5.3.4.1 Anesthesia

Each animal received ketamine (10mg/Kg), midazolam (0.5mg/kg), and meperidine (5mg/kg) in a single intramuscular injection as pre-anesthetic medication. An intravenous access was placed in an auricular vein for isotonic fluid and medication administration. After mask induction, oral endotracheal intubation was accomplished and anesthesia was maintained with isoflurane vaporized in 100% oxygen using an open system, universal

vaporizer (BALBINOTTO et al, 2010). Arterial pressure was recorded in two animals for reference. Ventilation was monitored to be around 6 to 8 breaths/min to assure an end-tidal pCO_2 of 30 to 35 mmHg.

5.3.4.2 Application of FGs in the Liver

After aseptic preparation and ventral midline incision, the liver lobes were exteriorized. The treatments were randomly assigned among the left medial, quadrate, and right medial liver lobes. After excision of a 1cm x 0.3cm x 0.3cm fragment at the caudal border, one of the following procedures was immediately employed: cryo-FG, protamine-FG, or manual compression. The sealant being tested was applied immediately into the lesion until bleeding stopped and a 1 to 2-mm fibrin cap formed on and around the cut surface. In order to avoid excessive arterial bleeding, the lobe was digitally compressed before FG application in a progressive manner until bleeding stopped and was immediately released as the FG was applied and a clot was observed. Only one FG application per lesion was anticipated. Fibrin glue that had adhered to surgical gloves or gauze was separated using gentle blunt dissection. The hemostasis in the control group was achieved with bilateral compression around the incised edge for a minimum of 10 minutes, without release. When bleeding had not completely stopped by that time, digital compression was continued with partial releases every 2-3 minutes. The biopsy site was observed for a minimum of 10 minutes after hemostasis had been achieved in all groups.

During surgery, the feasibility and efficacy of FG application, time to achieve hemostasis, volume of FG applied, and need for reapplications were recorded. Three observers inferred visual hemostasis scores and graded the quality of FG coagulation.

5.3.4.3 Application of FGs on Intestinal Serosa

For evaluation of FG's adhesiveness on intestinal serosa, four longitudinal, full-thickness, 1.5-cm incisions were performed in the anti-mesenteric border of the colorectal region, being 3cm apart from each other. The intestinal lumen was cleaned from fecal content through the first and most caudally incision. The treatments were randomly assigned among the four incisions prior to each trial. To keep uniformity and avoid excessive movement of

the intestinal loops after FG application, all incisions were created at first and in moments of ceased peristaltic contraction. Half way between each incision, a 4-0 polyamide (monofilament nylon) suture was placed on the serosa layer as a marker for post-mortem localization of each treatment. The control incision was closed with three simple interrupted sutures (4-0 polydioxanone). The FG treatments were closed with two simples interrupted sutures (4-0 polydioxanone) followed by 0.4ml of cryo-FG, protamine-FG, or protamine-aprotinin-FG. The concentration of thrombin and fibrinogen solutions and the dual-chamber single-tip system used for FG delivery were the same as those in the hemostasis trial.

Subjective scores on feasibility of application and adherence of FGs to the intestinal serosa were recorded during surgery.

5.3.4.4 Abdominal Cavity Closure and Post-operative Care

The abdominal wall was closed through standard 3-layer technique with non-absorbable suture (3-0 polyamide) in a continuous pattern. Following recovery from anesthesia, the animals received anti-inflammatory medication (meloxicam, 0.1mg/kg, IM, single dose), antibiotics (enrofloxacin, 2.5mg/kg, PO, *bid*, for 3 days) and tramadol (2mg/kg, PO, *tid*, for 3 days) as post-operative therapy (CALASANS-MAIA, 2009).

5.3.5 Macroscopic and Histological Evaluations

For macroscopic examination of the abdominal cavity and histopathology of hepatic and intestinal treatments sites, post-mortem assessment was carried out on either the 3rd or the 7th day after surgery. Three animals were included in each day. After sedation (12mg/kg of ketamine, 0.5mg/kg of midazolam), analgesia (6mg/kg of meperidine), and deep anesthetic induction (50mg/kg of thiopental, IV), cardiorespiratory arrest was achieve with intravenous injection of potassium chloride 10% (to effect).

After gross evaluation of the liver, intestine and surrounding tissues, specimens were collected, processed, and embedded in paraffin within 24h. Four-millimeter thick sections were stained with haematoxylin and eosin and viewed under a microscope.

The abdominal cavity was macroscopically examined for signs of hemorrhage, adhesions, peritonitis, and abscesses. The hepatic and intestinal biopsy sites were

histologically evaluated for signs of inflammation and tissue healing, presence of FG, and infection.

5.3.6 Statistical Analysis

Quantitative variables analyzed were time to polymerization in contact with organ, time to achieve hemostasis, volume of FG used, need for reapplication of FG, frequency of detrimental adherences. The data was evaluated for normality of distribution through graphical methods and submitted to subsequent statistical analyses by ANOVA considering a randomized complete block design (rabbit). Tukey test was used to identify differing groups.

Qualitative variables included quality of clot, signs of post-operative hemorrhage, detection of fibrin, presence of inflammatory cells, degree of tissue reparation or regeneration process, and overall easiness of application. Differences among the treatments used, regarding categorical (absence or presence – postoperatively: hemorrhage and adherences) and ordinal (nonexistent, low, moderate, prominent – trans-operatively: easiness of FG application and quality of clot; postoperatively: detection of fibrin, presence of inflammatory cells, and degree of tissue reparation or regeneration process) variables, were assessed by analysis of homogeneity of proportions through Fisher's exact test.

SAS® statistical program was used for data analyses. A P-value <0.05 was considered significant.

5.4 Results

5.4.1 Evaluation of Hepatic Hemostasis

Trans-operatory and post-mortem evaluations results with hepatic hemostasis are summarized in table 6. The average time for clotting with cryo-FG and protamine-FG was 2.7 seconds, after the mixed solution was released from the delivery system tip. The time for hemostasis in the control group varied from 10 to 14 minutes, which was significantly longer than that with cryo-FG or protamine-FG ($P<0.0001$). The total volume of FG used to stop bleeding did not differ between cryo-FG and protamine-FG groups, being around of 0.47mL and 0.48mL per bleeding area ($0.3cm^2$), respectively. In only one lobe, there was need for

extra volume due to misdirection during application. In the subjective evaluation of three observers, one being protocol-aware and the other two protocol-blind, the ability of the FGs to clot and adhere to tissue were considered as good in most of cases (66.6%, 4/6) for both groups. One cryo-FG was considered to have excellent quality.

Table 6 – Trans-operatory and post-mortem findings with hemostasis treatments applied on liver incisions of rabbits

	Control (compression)		Cryo-FG		Protamine-FG	
	Mean	***SD***	***Mean***	***SD***	***Mean***	***SD***
Time for hemostasis	12.3 min [b]	1.86	2.7 sec [a]	0.63	2.8 sec [a]	0.62
Volume applied per incision	-	-	0.47 mL	0.18	0.48 mL	0.15
	Frequency	***Observation***	***Frequency***	***Observation***	***Frequency***	***Observation***
Quality of clot/adherence to tissue	-	-	1/6, 4/6, 1/6	poor, good, excelent	2/6, 4/6	poor, good
Adherence of lobes	2/6	presence	2/6	presence	3/6	presence
Adherence of omentun	2/6	presence	2/6	presence	2/6	presence
Fibrin without cells	0/6	nonexistent	6/6	moderate	6/6	low to prominent
Fibrin with heterophils	6/6 [b]	low to moderate	3/6 [a]	low	2/6 [ab]	moderate
Giant cells in fibrin	0/6	nonexistent	4/6	low to moderate	3/6	low
Hemorrhage	3/6	moderate to prominent	4/6	low to moderate	3/6	low to moderate
Necrosis in margin	4/6	moderate to prominent	2/6	low to moderate	1/6	low
Macrophages	0/6	nonexistent	2/6	moderate	1/6	moderate
Lymphocytes	4/6	low to moderate	3/6	moderate to prominent	3/6	low to prominent
Heterophils	6/6	low to moderate	4/6	moderate	5/6	moderate
Connective tissue	6/6	low to moderate	6/6	low to moderate	6/6	low to moderate
Capsular integrity	4/6	low to moderate	4/6	low to prominent	4/6	low to moderate

Frequency is presented as number of observations per total of animals studied. *SD*: standard deviation. Superscripts indicate statistical difference (a, b: between treatments). Differences were considered significant when *P* value was <0.05.

At post-mortem evaluation, no signs of post-operative hemorrhage were detected in the abdominal cavity. Macroscopically, the scores of the adhesions between lobes and between lobes and omentum did not differ statistically among treatments. When considering the day of evaluation, adherences between lobes were more often seen on day 3 (85.7% of all inter-lobes adherences; P=0.024), while omentum adhesions were observed more on day 7 (83.3% of all omentum adherences; P=0.038).

Histological findings of hemostasis on liver resections with each technique are illustrated in figures 8 to 10. In the microscopic analyses, signs of surgical hemorrhage were more accentuated in the control group, but no significant difference was detected in comparison to the FG groups (Figure 8B and Figure 9B). Hemorrhage on day 3 was

significantly more pronounced than on day 7 in all groups (*P*=0.047). Presence of fibrin associated with heterophils was seen in all groups on days 3 and 7 after surgery. Although in the control group this reaction was more evident, only cryo-FG differed statistically from the control treatment (*P*=0.03). Overall, the presence of heterophils and lymphocytes was more evident on day 7 in all groups, without significant differences among treatments. Giant cells invasion into the fibrin layer and few macrophages was observed only in the FGs groups, remarkably on day 7 evaluations. Connective tissue was present over the lesion in the liver lobe margins of all treatments and significantly more pronounced on day 7 samples (*P*=0.004). The capsular integrity was not significantly different between groups in either day, although higher scores were observed on day 7 (*P*=0.001).

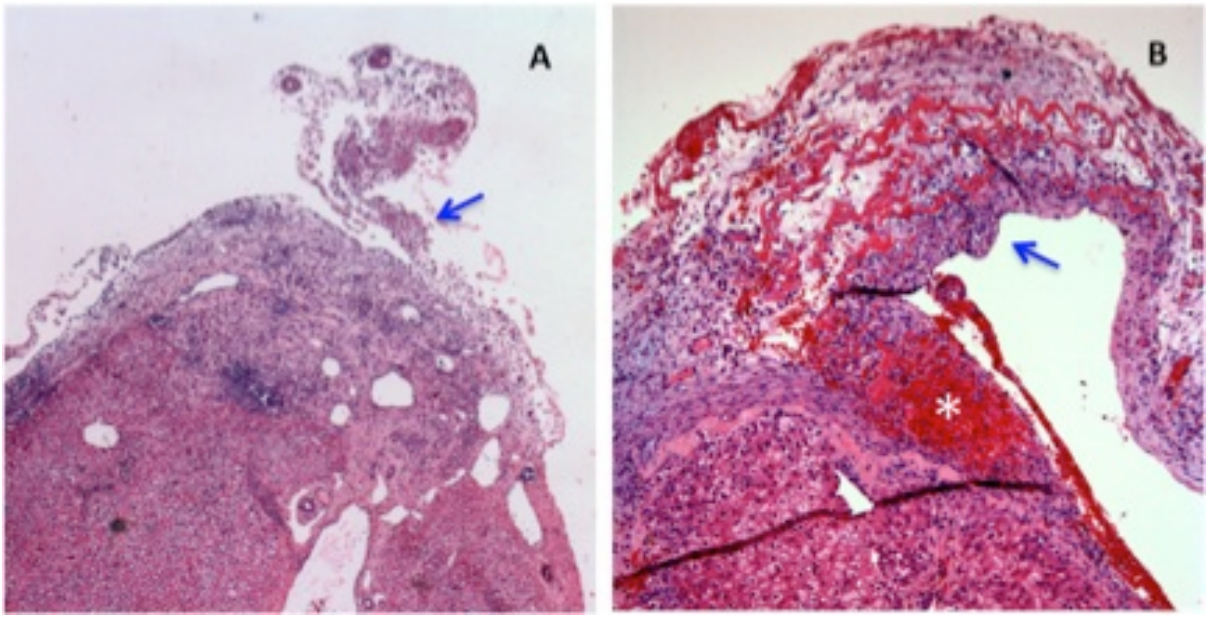

Figure 8 – Histological findings on slit edge of rabbit liver lobes from the control group, 7 days post-operatively.

Specimens from hepatic incisions without FG. Hematoxilin-Eosin (H&E) staining. **A**: Signs of adherence (blue arrow) and connective tissue are present. Magnification: 4x. **B**: Signs of hemorrhage (white asterisk) and omental adherence (blue arrow) are still pronounced. Magnification: 10x.

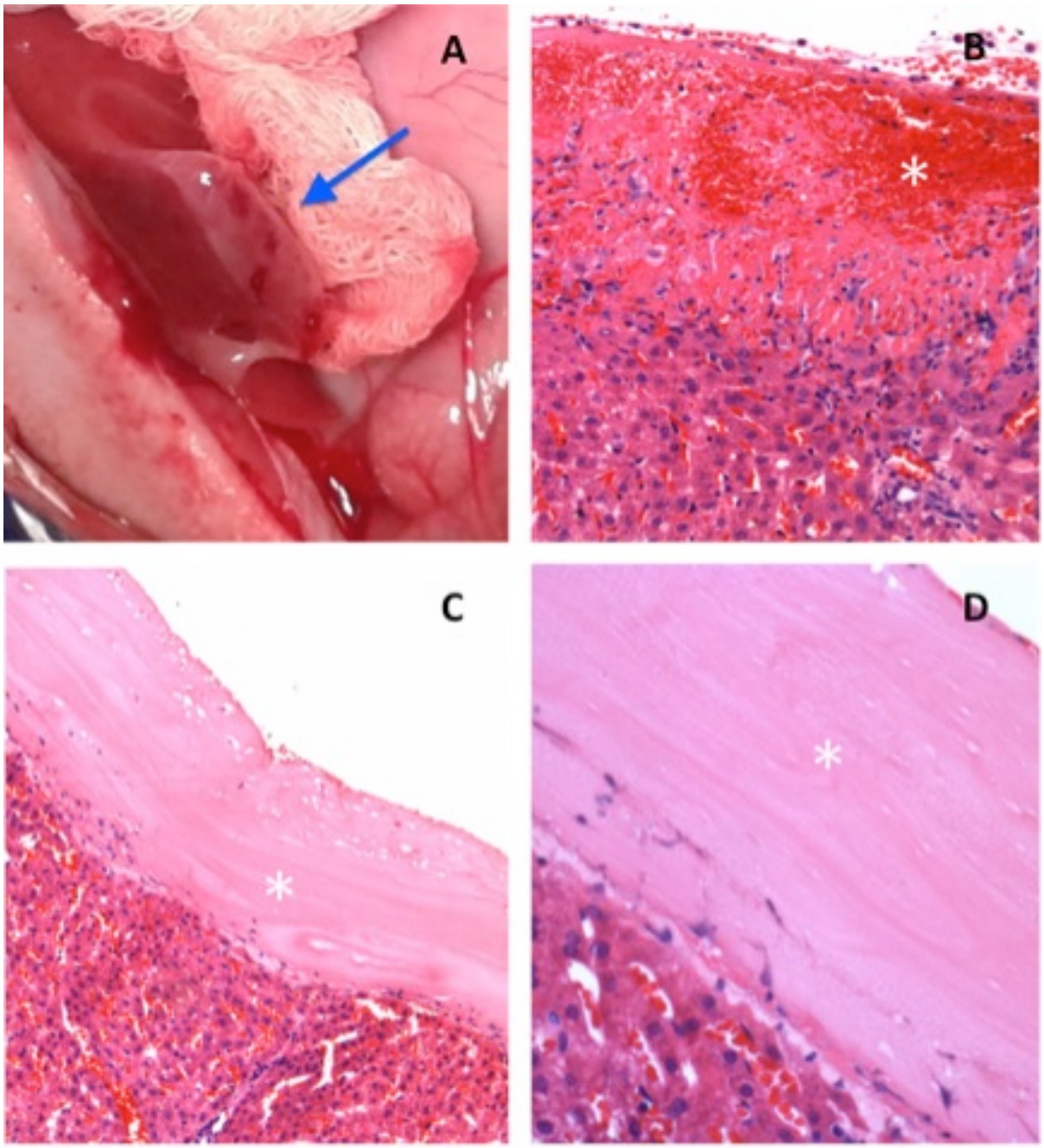

Figure 9 – Application and microscopic evaluation of rabbit incised hepatic edges treated with canine cryo-FG.

A: Cryo-FG applied trans-operatively. Blood was arrested underneath the FG cap (blue arrow). **B**: Histological signs of blood entrapped (white asterisk) within cryo-FG. Day 7, post-operatively; H &E staining; magnification of 20x. **C, D**: Noticeable layer of cryo-FG (white asterisk) at 3 days post-operatively. Magnification: 10x (C), 40x (D).

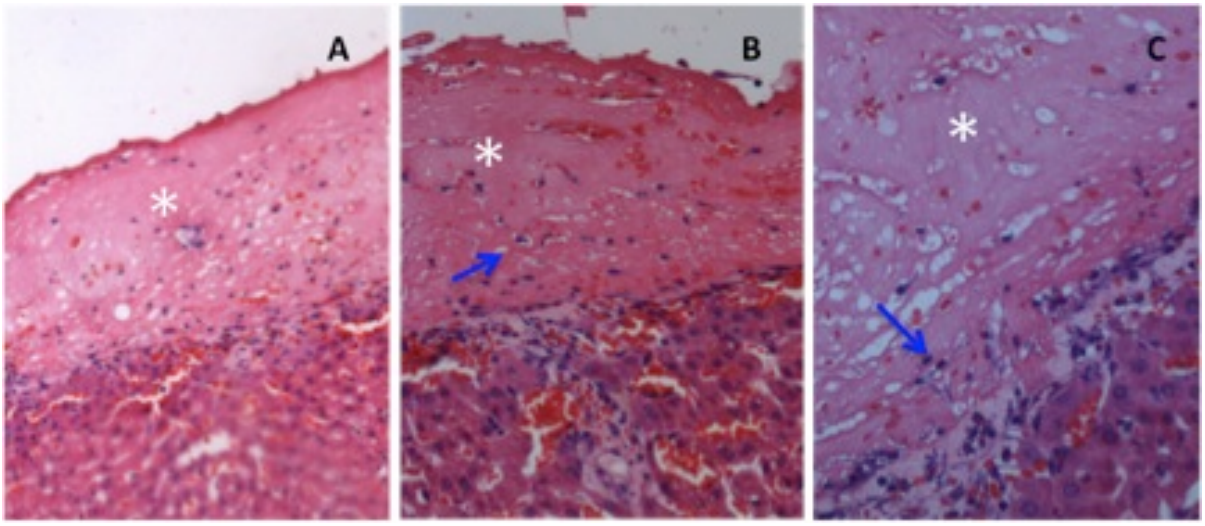

Figure 10 – Histological features of rabbit liver specimens that received protamine-FG for hemostasis.
Protamine–FG (white asterisks) with heterophils invasion (blue arrows) evidenced at day 3 post-operatively. H&E stain. Magnification: 10x (A), 20x (B), 40x (C).

5.4.2 Evaluation of Adhesiveness to Intestinal Serosa

Trans-operatory and post-mortem findings in the intestinal anastomosis trials are presented in table 7. The quality of coagulation and adhesion of cryo- and protamine-FGs to intestinal serosa was similar and graded as good by most of the evaluators. Only once, there was the need of reapplication of cryo-FG, with half of the volume (0.2ml), due to poor coagulation of a cryo sample. Abdominal adherences were observed in half of the control and cryo-FG samplings, and in only one third of the protamine-FG group. The protamine-aprotinin-FG group did not show any adverse adhesions, differing significantly from the control and cryo-FG (P<0.05). The control group was the only one to have omental attachments. The undesirable adhesions tended to appear more at day 7 (75%).

Table 7 – Trans-operatory and post-mortem findings with treatments used for intestinal wall closure in rabbits

	Control (compression)		Cryo-FG		Protamine-FG		Protamine+ aprotinin-FG	
	Frequency	*Observation*	*Frequency*	*Observation*	*Frequency*	*Observation*	*Frequency*	*Observation*
Quality/adherence to tissue	-	-	1/6, 4/6, 1/6	poor, good, excelent	2/6, 4/6	poor, good	1/6, 5/6	poor, good
Adherences	3/6 [b]	low to moderate	3/6 [b]	low to prominent	2/6 [ab]	low to moderate	0/6 [a]	nonexistent
Fibrin without cells	-	-	3/6	low to moderate	3/6	low to moderate	5/6	moderate
Fibrin with heterophils	6/6	moderate to prominent	4/6	low to prominent	4/6	low to prominent	4/6	moderate
Giant cells	1/6	low	1/6	low	1/6	low	2/6	low
Macrophages	0/6	nonexistent	1/6	low	0/6	nonexistent	1/6	low
Lymphocytes	2/6	low	2/6	low to prominent	2/6	low	2/6	low to moderate
Heterophils	6/6	low to prominent	6/6	moderate	5/6	low to prominent	5/6	low to prominent
Connective tissue	3/6	low to moderate	3/6	moderate to prominent	3/6	low to moderate	3/6	low to prominent

Frequency is presented as number of observations per total of animals studied. Superscripts indicate statistical difference between treatments (a, b). Differences were considered significant when P<0.05.

In conventional histological analyses, all samples showed heterophils invasion into the mucosa, submucosa and serosa layers. This granulomatous reaction was accompanied by fibrin deposition and no significant difference was observed among treatments. The control group had also high grades of fibrin buildup. Although the protamine-aprotinin group was the treatment with more often distinguishable enduring FG in the intestine, statistical difference among treatments was not detected (Figure 11). Lymphocytes, macrophages, and giant cells were present in all groups, especially surrounding fecal material debris. There was no bacterial proliferation in fibrin clots. Cocci, when present, were only observed around vegetal remnants. Presence of fibroblasts, connective tissue, and mucosa integrity scores did not differ among groups, but all treatments showed improved healing by day 7 of post-operative period (P=0.029).

The post-operative period was uneventful and none of the animals showed signs of discomfort or decreased appetite. The increased fasting time allowed adequate exposure and handling of liver and intestine loops and did not show detrimental effects in the trans or post-operative period. No bacterial organisms were identified in the cultured aliquots or at any of the histological sections.

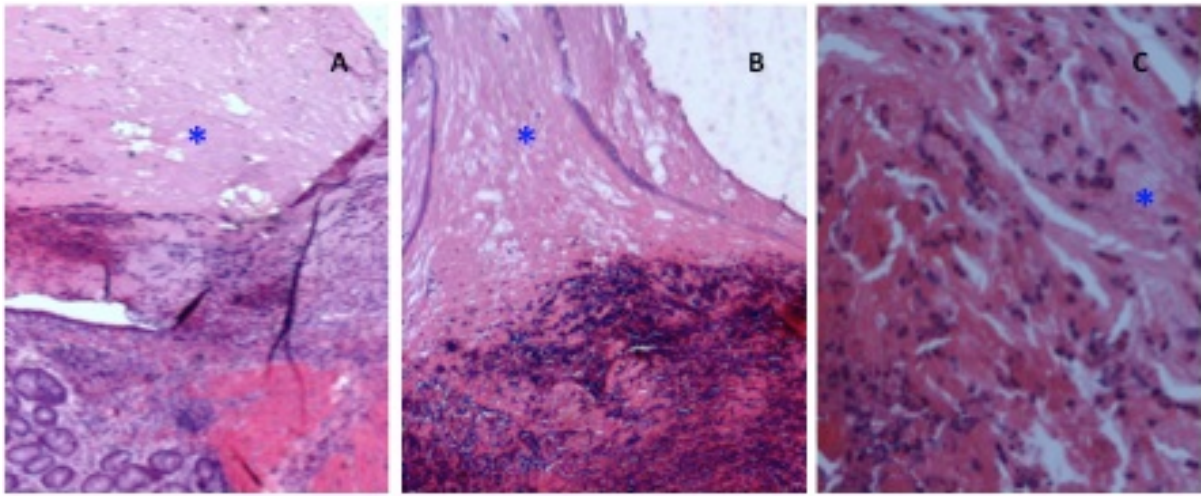

Figure 11 – Histopathology of intestinal region that received canine FG over the incision.
H&E staining of intestinal portions treated with FG produced by protamine-precipitated fibrinogen. Blue asterisks indicate presence of fibrin. **A**: Protamine-FG at day 7 post-operatively. Magnification: 4x. **B**: Protamine-aprotinin-FG, day 3 post-operatively. Magnification: 4x. **C**: Protamine-aprotinin-FG group, day 7 post-operatively. Heterophils invasion is noticeable. Magnification: 40x.

5.5 Discussion

5.5.1 Assessment of Hepatic Hemostasis

Hemostasis in parenchymal organs may be troublesome in many conditions related to systemic situation of patient, liver function, or specific surgical approaches, including organ region and equipment available (OVERBEY; JONES; ROBINSON, 2014; KUNIO; SCHREIBER, 2013). FGs have been reported to be effective as solely hemostat for simple biopsies in patients with coagulopathies by both open and minimally invasive techniques, and adjunctively in hepatobiliary procedures where standard techniques are not sufficient to reduce a high risk of bleeding or bile-leakage (KLINGLER et al, 2006; FALSTROM et al, 1999; RADOSEVICH; GOUBRAN; BURNOUF, 1997). Although canine cryo-FG has been used with success in experimental canine liver incision model (WHEATON et al, 1994), no evaluation of canine protamine-FG has been reported until now. The hepatic lobe incision model used in this study served not only to evaluate and compare the hemostatic properties of canine cryo and protamine-derived FG, but also to access the feasibility of their assembly and application in a sterile surgical setting.

The time needed for FG clot formation during hemostasis is important and is mostly determined by thrombin, being usually less than 5 seconds with final thrombin concentration above 50UI/mL (ALBALA; LAWSON, 2006). The hemostatic effectiveness of FGs, however, relies on clot adhesiveness to tissue and resistance to sheer forces. These properties are mostly dependable on fibrinogen content and are supported by factor XIII, calcium and fibronectin. These components need uniform mixing for proper polymerization and cross-linking of fibrin with concomitant contact to tissue during application (DICKNEITE et al, 2003; PHILLIPS et al, 2003; KAETSU; TAKANORI; SHINYA, 2002; KJAERGARD et al, 2000). In general, presence of blood is not a problem for FG clotting and adherence to tissue surfaces. However, under increased pressure or frank hemorrhage, the fibrin clot can be washed away before polymerization and strengthening of the clot is sufficiently established to resist blood flow. Thus, the efficacy of FG may change depending on the arterial pressure, size of vessels and regional blood pressure. For this reason, FG is not considered as solely hemostatic tool when ligation of bigger vessels is indispensable or a tourniquet needs to be applied momentarily (OVERBEY; JONES; ROBINSON, 2014; SPOTNITZ; BURKS, 2012;

WHEAT; WOLF, 2009; KLINDER et al, 2006; JACKSON, 2001; RADOSEVICH; GOUBRAN; BURNOUF, 1997). In a preliminary study, no compression of vessels or liver lobes was performed during biopsy incisions and application of FG in liver and kidney (GONCALVES, 2015, APPENDIX D). Although bleeding was twice of that observed in the present study, and higher volumes and reapplication of FG were required, hemostasis was successfully achieved with incisions deepened as far as 3cm into the hepatic parenchyma and amid arterial blood pressures of 70 to 90mmHg. The digital tourniquet used in the presented model, for all treatments, allowed less bleeding into abdominal cavity and, thus, prevention of excessive post-operative adherences. Also, more uniformity was achieved during application of fibrin glue in more difficultly accessed lobes in the rabbit. There are other forms of hemostasis available in human and veterinary surgical arsenal. However, they are not always able to fulfill the hemostasis depending on the localization, width, and configuration of the bleeding surface (SPOTNITZ; BURKS, 2012; KINDLER et al, 2006). Fibrin glue is moldable and temporary occlusion of big vessels is a procedure that is used in different circumstances for hemostasis or avoidance of massive bleeding during some surgical procedures (KITCHENS; LAWSON, 2013; JACKSON, 2001). Moreover, FG does not impede use of other hemostatic devices and may be an advantageous technique in dogs with severe liver disease, when liver is friable and/or coagulation factors may be deficient, increasing the risk of persistent bleeding (HACKNER; WHITE, 2012; WHEAT; WOLF, 2009; FALSTROM et al, 1999). The formation of FG clot does not depend on endogenous coagulation elements because all components needed for the conversion of fibrinogen to insoluble fibrin are present in the fibrinogen concentrate and thrombin solution mixture (WOZNIAK, 2003). Indeed, its adjunctive use in human surgery has been associated with less trans and post-operative bleeding and need for blood transfusion (MINTZ et al, 2001). This rabbit model served to appraise the ability of cryo- and protamine-canine FG, containing considerably low fibrinogen levels (12-18mg/mL), to stop bleeding from vessels of comparable diameter to those of liver biopsies of canine patients.

Although the volume used to stop bleeding (0.5mL/0.3cm^2) was more than twofold of that mentioned by literature to have 1-mm layer of fibrin clot (0.1mL/cm^2; SPOTNITZ, 2014; DUNN; GOA, 1999), the extra volume covered the edges around of the hepatic incision further, providing a capsule-anchoring surface and allowing later histological evaluation of the FG deposit. In less concentrated fibrinogen solutions, the total volume needed for the 1-

mm coverage may be higher due to loss of liquid after the clottable protein is polymerized (DICKNEITE et al, 2003). During FG application, more volume was prepared anticipating skidding of solutions due to limitations of the delivery device used.

There were no signs of postoperative hemorrhage in the abdominal cavity or secondary bleeding caused by the displacement of the fibrin clot or premature fibrinolysis. The histologic finding of bleeding at the incision sites in FG groups was associated with the entrapment of blood cells during the FG application, which is consistent with it being more evident on the third post-operative day.

Adherences were observed in some lobes from all treatment groups (control=2, cryo-FG=2, protamine-FG=3). While FG has been reported to diminish adhesions, it has also been advocated as an agent to be used for pleurodhesis (SPOTNITZ, 2014; FERGUSON; NURNBERGER; REDL, 2010; ALBALA; LAWSON, 2006). This controversy, is most likely due to the fact that strong primary adherences would happen during the process of polymerization and cross-linking of fibrin, which can take up to an hour to be completed. Hence, some authors suggest that, when adherences to be avoided, a minimum of 10 to 20 minutes should be wait before the site that received FG may be released and come in contact to adjacent body structures (SIERRA; EBERHARDT; JACK, 2002; RADOSEVICH; GOUBRAN; BURNOUF, 1997; LINDENBERG; LAURITSEN, 1984). In the present experimental model, the wait of more than ten minutes per incision site was not feasible, which could explain some of the adhesions observed between liver lobes. The adherence between lobes can be related also to the spillage of FG, as well to the hemorrhage that occurred from the control treatment. This is supported by the observation that some of the bonds between lobes that received FG were far away from the slit edge, while the control lobes had more incision-site related adherences. Also, the gross appearance of adhesions in the control lobes were consistent with accentuated hemorrhage observed in the microscopy in the same animals. Adherences between lobes without signs of hemorrhage were easily separated; possibly explaining reduced observation of this type of attachments on day 7.

Heterophils appearance in all histologic samples, surrounded by fibrin, were indicative that some inflammation occurred in all groups regardless of application of heterologous FG. The giant cells, however, appeared only on FG groups and mostly in the later stage of healing, most likely to clean the applied FG, as a foreign body reaction. Moreover, the inflammation did not extend into the hepatic parenchyma and adjacent

hepatocytes were preserved without signs of degeneration. Some necrosis was observed in spots where digital compression was applied, especially in the control group. Superficial sinusoidal spaces and central veins were intact.

Although the assessment of FG fibrinolysis and the effect of canine-FG on healing in the rabbit model is of limited value due to possible interspecies antigenic responses, neither delay in healing nor detrimental reactions was detected in the sites where FG was applied in comparison to control treatment. No signs of infection were seen, denoting the microbiologic safety of the product, as well supported in other studies (BUTCHA; SEMPLE, 2005).

5.5.2 Assessment of Adhesiveness to Intestinal Serosa

Intestinal anastomosis is a commonly performed surgical procedure in dogs. Following this type of surgery, the risk of peritonitis increases when deterioration of the tissue or of the patient condition is present in the trans or postoperative period. This is not uncommon, especially with delay on proper diagnosis or treatment (PORTILLA-DE BUEN et al, 2014; CORNELL, 2012). FG has been used to treat gastro-intestinal ulcers and to support intestinal anastomosis in humans (VAKALOPOULOS et al, 2013; LIPPERT et al, 2011; MOCCIARO et al, 2011; TRUONG et al, 2004; RUTGERERTS et al, 1997). FG produced from canine plasma was assessed for intestinal serosa adhesiveness in a rabbit model, aiming future applications on intestinal anastomosis and natural orifice trans-luminal endoscopic surgery (*NOTES*). Cryo- and protamine-FG's clotting, adhesion properties, and adverse reactions were evaluated.

Cryo and protamine-FG were able to attach easily to the intestinal serosa. The quality of clot formation and adhesion to intestinal serosa was similar among all FG groups. The FG batches used for liver hemostasis evaluation of a given treatment were the same used for the intestine-sealing model. Thus, similarities between clotting and adhesion were observed as subjective scores of intestinal application were in accordance to those given for the liver employment.

When present, adverse adherences of the descending colon to other organs were easily released. No adherences were seen in the protamine-aprotinin-FG. The main difference expected for the protamine-aprotinin group was a delay of fibrinolysis onset. Aprotinin can delay clot lysis up to nine days (DICKNEITE et al, 2003; FATTAHI; MOHAN;

CALDWELL, 2004). A slower fibrinolysis in the protamine-aprotinin-FG could have diminished the inflammatory stimulus from interspecies fibrin breaking down, thus possibly explaining the absence of adherences in the protamine-aprotinin-FG regions in neither day of evaluation (JENNEWEIN et al, 2011). Longer postoperative period evaluation would have elucidated better these findings. Also, the protamine-aprotinin-FG was the only one to have fibrin detected on day 7 in all samples. Degradation of FG would be desirable at the time of the proliferation phase of wound healing (CECCARELLI; PUTMAN, 2014). The time of this phase, however, can change considerably depending on the type and condition of the injured tissue. Indeed, different tissues have enzymes and cells that may degrade fibrin more easily or in faster rate (KITCHENS; LAWSON, 2013; MEIJEH; PANNEKOEK, 1995). Although for hemostasis, long lasting FG may not be necessary, a longer sealing effect of FG over an inflamed or unhealthy tissue may be beneficial (WHEAT; WOLF, 2009; COLLET et al, 2003; KHEIRABADI et al, 2002; BEDUSCHI et al, 1999). Nevertheless, fibrin fragments are thought to help with mechanisms of tissue reparation and regeneration processes (LAURENS; KOOLWIJK; DE MAAT, 2006). The effect of the protamine itself on FG fibrinolysis has not been studied *in vivo*. TEG detection of fibrinolysis in protamine-FG was significant. Yet, it would be difficult to correlate TEG's results with the *in vivo* findings due to differences on fibrinolysis mechanisms in which both situations were presented and assessed.

Although regeneration and reparation cells proliferation became more evident by day 7, no difference could be seen on tissue healing among treatments. It could have been due to the experimental model and small number of animals, which hindered adequate assessments and comparisons of qualitative variables. Although this model did not allow for complete assessment of the FG effect on intestinal healing and FG degradation time, it permitted evaluation and comparison of the feasibility of the two fibrinogen precipitation protocols and observation of on-site behavior of protamine-aprotinin-FG.

5.5.3 Final Considerations

Cryo-FG and protamine-FG application did not obstruct physiologic wound healing in the hepatic or intestinal tissue of rabbits and degradation of fibrin and invasion of cells into the clot was observed. Whether the components of canine cryo- and protamine-FG may

increase inflammation due to foreign body reaction, induced by specie-specific or allogeneic material, as well as their effects on tissue healing and time of degradation needs further investigations.

This study indicate that cryo-FG and protamine-FG exhibited hemostatic properties and adhesiveness to intestinal serosa in the rabbit model developed to provide surgical conditions of hepatic biopsy and intestinal anastomosis incision for trocar-access in NOTES. Likewise, canine FG is a promising tool to be used for parenchymal organ hemostasis and for suture line sealing in troublesome anastomosis of hollow organs that are naturally under peristalsis or content distention.

Determination of the plasmatic components precipitated through cryo- and protamine-protocols, regarding their concentrations' and ratios' influence on FG performance in specific surgical usages, will enable a more directed selection of this valuable tool by veterinary surgeons.

5.6 Conclusions

FG produced from canine FFP with cryo- and protamine-precipitation methods supported hemostasis of hepatic biopsy like-excisions and showed adhesiveness to intestinal serosa in an *in vivo* evaluation using a rabbit model. Production of canine FG with surplus plasma units from veterinary blood banks is feasible and economical for enabling surgical and scientific applications of this versatile biomaterial.

5.7 Acknowledgements

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The authors declare responsibility upon the content and no conflicts of interest. These data was presented as part of a PhD thesis in Veterinary Science and examined by an academic faculty commission.

5.8 Acronyms and Abbreviations

Bid *bis in die*, twice a day
FFP fresh frozen plasma
FG fibrin glue
H&E hematoxylin and eosin stain
IM intramuscularly
IU international units
IV intravenously
P significance level
PO *per os,* orally
SD standard deviation
Tid *ter in die,* three times a day

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6 FINAL CONSIDERATIONS

The feasibility of a fibrinogen precipitation protocol is appraised regarding its capacity to be performed with small volumes of blood, yield a high and clottable fibrinogen content, be easy and fast to be accomplished, stand free of infectious agents or nocuous substances, and have effectiveness *in vivo* considering the intended use. Depending on the usage purpose however, tensile strength, elasticity, porosity, tissue adhesiveness, and long-term stability may be of variable importance. Cryoprecipitation and protamine precipitation of fibrinogen possess these characteristics in different levels. The employment of the protamine protocol for canine plasma seems to need further adjustments to perform the expected superiority over other fibrinogen precipitation methods. Yet, due to some impairments of cryo processing, especially regarding time and volume required for an adequate final fibrinogen content, protamine precipitation is still a promising technique to make effective FG with small volumes of blood and in a very short period of time. Moreover, protamine protocol makes the production of FG feasible in some hospital settings with simpler equipment and from cryo-supernatant.

In an efficiency standpoint, considering the concept of *economical* as the best use of the available resources, the usage of FG in certain surgical settings favors the efficient usage of blood banks material. If FG is available during parenchymal organ resection or traumatic wound management, the risk of excessive bleeding can be reduced. By preventing trans and post-operatory hemorrhage, whole blood bags and erythrocytes concentrates can be spared. Besides of providing more safety for the patient, as the chance for adverse reaction would be diminished, this could be strategic for a blood bank when shortage of blood donors is a concern. This material could be directed to other patients in need while the usage of leftover frozen plasma units would be freeing storage space and avoid its wastage. Moreover, these measures may entail a more affordable budget to the owner.

For improvement of efficacy of fibrinogen precipitation methods and FG protocols, taking into account its contents and intended use, evaluations correlating intrinsic clot characteristics with *in vivo* performance need to be standardized and carried out considering clinical and research FG applications.

7 CONCLUSÕES

A precipitação de fibrinogênio por frio ou protamina é factível de ser executada em centros hospitalares e de pesquisa a partir de fontes congeladas de plasma pobre em plaquetas canino provenientes de bancos de sangue.

Plasma canino fresco demonstra precipitação de fibrinogênio coagulável inferior quando comparado a plasmas congelados para fins de produção de cola de fibrina.

O coágulo de cola de fibrina constituída com fibrinogênio precipitado por frio possui dinâmica de desenvolvimento, propriedades visco-elásticas e estabilidade *in vitro* superior daquele obtido com precipitação por protamina.

A solução concentrada de fibrinogênio obtida de plasma canino por crioprecipitação ou com protamina gera coágulos com arranjo estrutural semelhantes e de capacidades hemostática e adesiva similares.

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APÊNDICE A – Aproveitamento de fibrinogênio plasmático por crioprecipitação

Volume inicial *(volume final)**	Tipo de plasma	*Intervalo*	*Média mg/mL*	Quantiade de aliquotas com 10mg/mL por bolsa de sangue**	Poder de precipitação	Poder de concentração
Crioprecipitado						
300 (*até 20mL*)						
	eFFP	*10-40mg/mL*	*18.732*	10 a 20 mL/bolsa	-	-
120 *(até 16mL*)						
	FFP	*5-11mg/mL*	*7.109*	-	22 a 37%	2.25 a 5.09x
	eFFP	*7-14 mg/mL*	*10.134*	12-30mL/bolsa	27 a 44(89)%	3.25 a 7.14x
	FP	*10-13mg/mL*	*10.270*	12-30mL/bolsa	23 a 31%	4.98 a 6.26x
50 (*2mL*) (BRA)						
	FR	*4-6mg/mL*	*4.675*	-	9 a 16%	2.4 a 4.0x
	FFP	*11-22mg/mL*	*15.142*	10-20mL/bolsa	23 a 53%	5.8 a 13.2x
40 (*1mL*)						
	FFP	*10-20mg/mL*	*15.538*	6-12mL/bolsa	16 a 36%	6.38 a 14.47x
	eFFP	*23-28mg/mL*	*25.500*	12-16mL/bolsa	25 a 54%	8.84a a 21.78x
	FP	*17-32mg/mL*	*25.200*	9-18mL/bolsa	21 a 25%	8.55 a 9.87x
40 (0.5mL)						
	FFP	*17-25mg/mL*	*20.496*	3-6mL/bolsa	16 a 20%	9.3 a 12.2x
35 (*0.8mL*)						
	FFP	*4.7-10.3mg/mL*	*7.248*	-	10 a 12%	4.4-4.6x
25 *(0.6mL)*						
	FFP	*<4mg/mL*	-	-	-	**<2x**
15 *(0.6mL)*						
	FFP	*<4mg/mL*	-	-	-	**<2x**
Crioprecipiação de sobrenadante de bolsas de 120mL e tubos de 50mL						
50mL (1mL)	FFP, eFFP	6.03-12.9mg/mL	*8.97*	4-10mL/bolsa	7%-23%	3.7 a 7.4x
Crioprecipitado de crioprecipitado de bolsas de 120mL						
10-25mL *(1mL)*	FFP, eFFP	4.5-31.3mg/mL	*15.229*	1mL/bolsa	5 a 18%	1.2-4.6x
Segunda centrifugação de crioprecipitado de bolsas de 120mL						
8-12mL *(1mL)*	FFP, eFFP	*2.68-12.13mg/mL*	7.65	1mL/bolsa	-	**<2x**

* volume final: até 15% volume inicial.

** bolsa de 450mL de sangue = 240mL de plasma pobre em plaquetas estimado: 6-15mL por cada bolsa de 120mL: ; 5 tubos de 50mL por bolsa, 6 tubos de 40ml por bolsa; 7 tubos de 35ml por bolsa).

eFFP: *expired fresh frozen plasma*, plasma fresco congelado vencido (com mais de um ano da colheita); **FFP**: *fresh frozen plasma*, plasma fresco congelado; **FP**: frozen plasma, plasma congelado; FR: *fresh plasma*, plasma fresco. **BRA**: processamento realizado nas dependencias do Laboratório de Tecnologia de Sêmen e Proteínas na Reprodução Animal – Faculdade de Veterinária – UFRGS, Porto Alegre, RS, Brasil; restante das amostras foi processado no Animal Health Diagnostic Center (AHDC), College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.

APÊNDICE B – Material utilizado para produção de cola de fibrina canina

MATERIAIS PARA PREPARO DE COLA DE FIBRINA	SOLUÇÃO DE FIBRINOGÊNIO			SOLUÇÃO DE TROMBINA	
	Crioprecipitação		*Precip. Protamina*	Trombina	Cloreto de Cálcio
	bolsas	*tubos*			
EQUIPAMENTOS					
Centrífuga refrigerada para bolsas de sangue	x				
Macrocentrífuga refrigerada para tubos		x			
Macrocentrífuga para tubos			x		
Extrator para bolsa de sangue	x				
Capela laminar de fluxo		x	x	x	x
Homogeneizador vortex			x		
Balança precisão			x	x	x
Freezer -20oC		x	x		
Freezer -80ºC (<30oC)	x				
Micropipetas (100uL, 1000uL)				x	x
Vidraria			x	x	x
Bailarinas magnéticas			x	x	x
Espátulas de inox			x	x	x
Phmetro			x		
Banho-maria	x	x	x	x	x
MATERIAL DE CONSUMO					
Tubo de ensaio Falcon de 50mL		x	x	x	x
Equipos	x				
Adaptadores para bolsa		x	x		
Ponteiras				x	x
Seringas (1, 3, 5, 10ml)	x	x	x	x	x
Tubos de 1,5ml		x	x	x	x
REAGENTS					
Citrato de Sódio (C6H7O7Na)			x		
Cloreto de Cálcio (CaCl2)					x
Tronbina bovina				x	
Sulfato de Protamina			x		
água pra injeção / milliq			x	x	x
TEMPO					
Congelamento*	24h a -80oC	18h a -80oC	-		
Armazenamento mínimo a temperaturas < -20oC**	6 meses (?)	4 meses (?)	(?)		
Descongelamento	16-28h	9-13h	15-20min		
Preparo	10-20min	10-20min	1.3h	10-20min	10-20min

* tempo de congelamento varia de acordo com a temperatura e quantidade do plasma; estudos divergem quanto ao tempo mínimo necessário.

** tempo de armazenamento estudado onde se conseguiu concentração mínima de 10mg/mL de fibrinogênio diluindo o precipitado em 2% do volume inicial. Nenhuma amostra de plasma fresco (<7 dias de armazenamento) diluido em 2% do volume inicial gerou concentração mínima de 10mg/mL.

APÊNDICE C – Resultados de tromboelastografia (TEG) de cola de fibrina canina

Parâmetros reológicos de tromboelastografia com géis de cola de fibrina obtida por crioprecipitação e precipitação por protamina a partir de diferentes categorias de plasma pobre em plaquetas canino.

Rheological measurements of fibrin glue clots obtained from cryo- and protamine protocols with different categories of canine platelet poor plasma performed by thromboelastography (TEG)

	R (min)		**K** (min)		**a-angle** (degree)		**TMA** (min)		**MA** (mm)		**G** (d/cm^2)		**E** (d/sc)		**LY60** (%)	
Samples	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Cryo																
FFP	0.23	0.05	1.43	1.49	80.04	4.54	7.29 ^{a}	1.59	32.50 ^{b}	10.2	2561.06 ^{b}	1164.49	51.23 ^{b}	23.28	0.00 ^{A}	0.00
eFFP	0.23	0.07	1.03	0.64	83.04	5.35	7.24 ^{a}	2.00	48.59 ^{a,A}	9.61	4994.94 ^{a,A}	1684.65	99.90 ^{a,A}	33.72	0.00 ^{A}	0.00
FP	0.24	0.07	1.26 ^{A}	1.04	80.34	5.83	10.60 ^{b}	3.14	43.00 ^{ab}	13.67	4241.53 ^{ab}	2266.99	84.84 ^{ab}	45.32	0.00 ^{A}	0.00
All plasma	**0.23** ^{x}	**0.06**	**1.23** ^{x}	**1.05**	**81.14** ^{x}	**5.22**	**8.38** ^{x}	**2.75**	**41.36** ^{x}	12.77	**3932.51** ^{x}	**1980.05**	**78.65** ^{x}	**39.60**	**0.00** ^{x}	**0.00**
Protamine																
FFP	0.49	0.16	1.71	0.63	70.13	8.15	6.95 ^{a*}	1.91	30.58	9.44	2322.13	1011.76	46.44	20.23	41.94 ^{B}	22.69
eFFP	0.81	0.52	2.75	2.48	58.09	21.28	6.69 ^{a*}	2.89	25.69 ^{B}	13.21	1915.83 ^{B}	1215.37	38.33 ^{B}	24.32	48.65 ^{B}	25.92
FP	0.63	0.28	3.56 ^{B}	2.34	60.79	13.44	9.89 ^{b*}	2.73	32.31	8.3	2489.78	960.80	49.79	19.24	33.09 ^{B}	23.68
All plasma	**0.64** ^{y}	**0.36**	**2.71** ^{y}	**2.05**	**63.00** ^{y}	**15.52**	**7.84** ^{x}	**2.85**	**29.53** ^{y}	**10.46**	**2242.58** ^{y}	**1050.07**	**44.85** ^{y}	**21.01**	**41.23** ^{y}	**23.96**

Mean: média considerando todas as duplicatas; ***SD****: standard deviation*, desvio padrão considerando todas as duplicatas. Diferenças com valor de P <0.05 foram consideradas significantes (ANOVA, Tukey); **x, y**: diferença significativa entre protocolos sem distinção do tipo de plasma; **A, B**: diferença significativa entre protocolos em considerando um tipo de plasma específico; **a, b**: diferença significativa entre tipos de plasma dentro de um protocolo de precipitação de fibrinogênio.

Results are presented considering all replicates in TEG. Differences were considered significant when P value was <0.05, except when. Superscripts indicate statistical difference (**x, y**: between protocols without plasma source distinction; **A, B**: between protocols within a specific plasma category; **a, b**: among plasma types within a protocol). Each plasma type had four samples with counterparts in both cryo and protamine processing from same plasma unit.*

a*, b*: FFP^{a*} *versus* FP^{b*}, P=0.0770; eFFP^{a*} *versus* FP^{b*}, P=0.0509.

FFP: plasma fresco congelado, *fresh frozen plasma*;

eFFP: plasma fresco congelado vencido, *expired fresh frozen plasma*;

FP: plasma congelado, *frozen plasma*;

R = tempo para início da formação do coágulo, *reaction time, in minutes (first evidence of clot formation);*

K = cinética durante a formação do coágulo, *kinetics in minutes (time taken from beginning of clot detection to achieve a certain level of clot strength)*;

α-angle = ângulo referente à velocidade da formação do coágulo, *rate of clot formation, in degrees (speed at which fibrin build up and cross linking takes place)*;

TMA = tempo para alcançar amplitude maxima, *time to maximum amplitude, in minutes;*

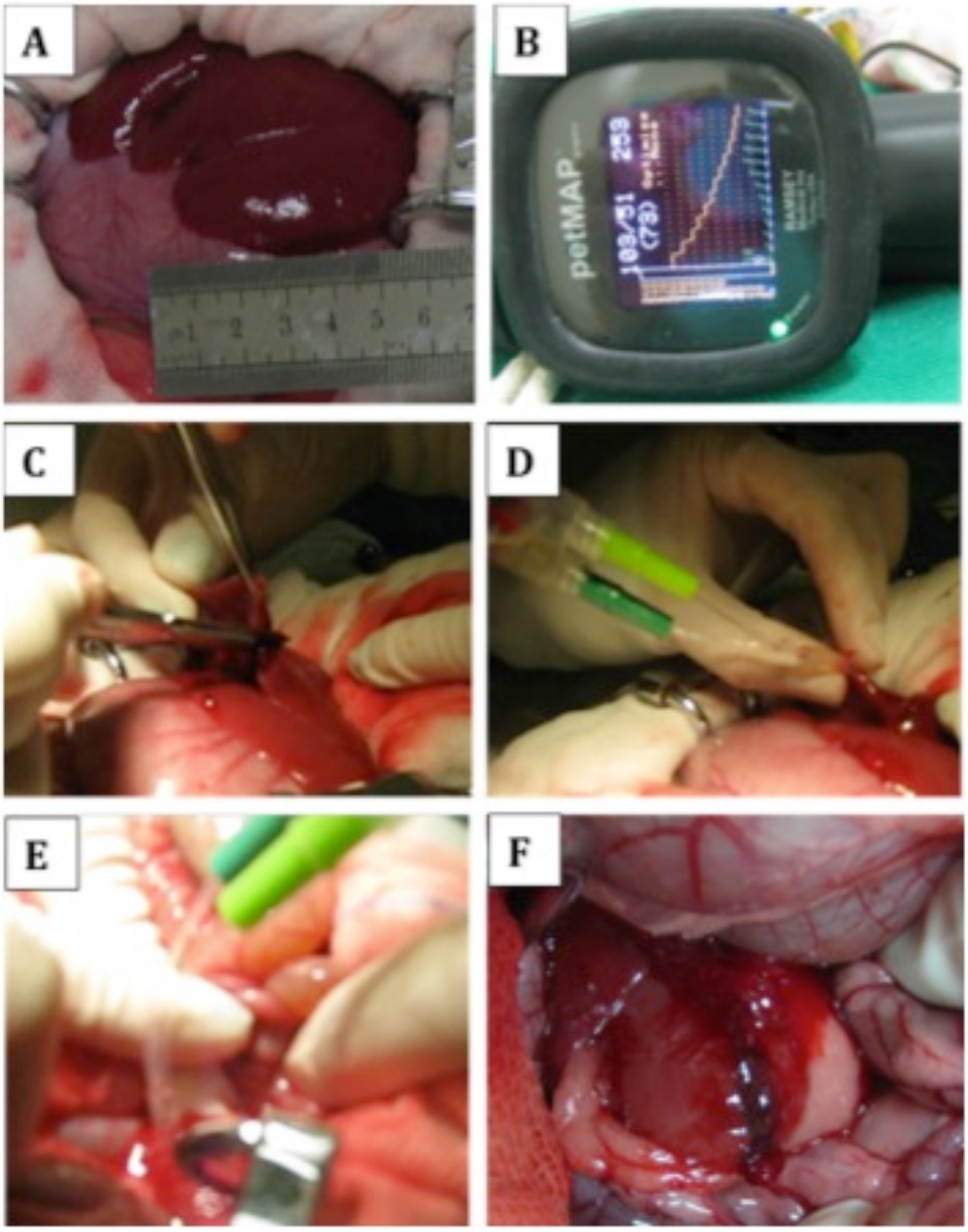
MA = máxima amplitude, *maximum amplitude, in millimeters (maximum stiffness of the clot)*;

G = resistência elástica, *shear modulus strength, in dynes/cm^2 (clot elasticity)*;

E = constante de elasticidade, *extensibility, in dynes/second (clot elasticity constant)*;

LY60 = lise do coágulo aos 60 minutos em %, *lysis at 60 minutes, in percentage (clot disintegration).*

APENDICE D – Hemostasia renal e hepática com cola de fibrina canina



Aplicação de cola de fibrina produzida por crioprecipitação de plasma fresco congelado canino em biópsia incisional hepática e renal sem garroteamento em coelho. **A**: Mensuração para incisão de 3cm no parênquima hepático. **B**: Pressão arterial média objetivada durante os procedimentos de hemostasia com cola de fibrina (FG). **C**: Manobra de retirada de fragmento hepático. **D**: Aplicação de FG na superfície hepática em sangramento. **E**: Aplicação de cola de fibrina em biópsia incisional de 1cm de profundidade no parênquima renal. **F**: Aspecto macroscópico do rim com sangue contido após aplicação da FG.