- UNIVERSITA' DEGLI STUDI DI TORINO -

Department of Veterinary Science

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CYCLE XXX

TITLE OF THE STUDY PROJECT:

THROMBOASTOMETRIC ASSESSMENT OF HEMOSTASIS FOLLOWING HYDROXYETHYL STARCH (130/0.4) ADMINISTRATION AS A CONSTANT RATE INFUSION IN HYPOALBUMINEMIC DOGS.

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CONTENT INDEX

PART I: HEMOSTASIS

PART II: COLLOIDAL SOLUTION

PART III: RESEARCH PROJECTS

- **3.3 ADMINISTRATION OF HYDROXYETHYL STARCH (130/0.4) AS A CONSTANT RATE INFUSION IN HYPOALBUMINEIC DOGS: EVALUATION OF EFFECTS ON PLASMA COLLOID OSMOTIC PRESSURE.**
    - Abstract
    - Introduction
    - Material and Methods
    - Statistical analysis
    - Results
    - Discussion

ADDITIONAL RESEARCH PROJECT

PART I: HEMOSTASIS

1.0 INTRODUCTION

Hemostasis is a physiological protective mechanism, present in all vertebrates that occur during damage, rupture or alteration on the vascular wall. The responses of the coagulation system are coordinated with the formation of the platelet plug that initially occludes the vascular lesion. Anticoagulant mechanism ensure careful control of coagulation and, under normal condition, they prevail over the procoagulant fores. Disturbances of the natural balance between the procoagulant and anticoagulant systems may result in bleeding or thrombotic diseases.

In addition to a vascular rupture, infectious agents, acidosis, hypoxia, inflammation and hypotension could also cause endothelial damage [1]. Therefore, the triggering of coagulation process can be caused by soluble factors, by factors related to the vessel wall or by changes in blood flow, as described by Virchow.

Normal hemostasis occurs in 3 steps: **primary hemostasis**, **secondary hemostasis** and **fibrinolysis**.

During the primary hemostasis is it possible to identify 2 different phases: a vascular phase, where vasoconstriction occurs in order to favour the slowing of blood flow, and a platelets phase. During this phase platelet adhere to the damaged endothelium and undergo morphological shape changes that allow platelets to adhere to each other, thus forming a temporary platelet plug [2].

The platelet plug is stable only for a few hours if the secondary hemostasic forces do not solidify and reinforce the plug with a crosslinked fibrin meshwork. The last stage in coagulation occurs when plasminogen is activated to plasmin, which breaks down the fibrin and removes the clot once the integrity of the vessel has been restored. The mechanisms involved to maintain the normal blood flow and the integrity of the vascular system, in order to be efficient, must be rapidly activated and remain as confined as possible to the site of the injury, in order to avoid a widespread of the coagulation process [3]. This balance is achieved through a complex control system of specific inhibitors of both coagulation factors and those involved in fibrinolysis.

During physiological condition there is always a certain degree of activation of hemostatic system, because the absolute vascular integrity doesn't exist; in fact continuous micro traumas cause minimal endothelial lesion and therefore a small amount of fibrinogen is continually converted into fibrin activating the so-called "physiological hemostasis". During functional hemostatic balance, the fibrinolysis process continually removes the fibrin previously formed [3].

1.1 PRIMARY HEMOSTASIS

1.1.1 Vascular phase

The first event that occurs during hemostasis is a vascular contraction in correspondence of the endothelial injury. The vasoconstriction mechanisms are more efficient in vessels with a thick vascular

tunica and smooth muscle cells (medium tunica, but also occur at capillary level by contractile proteins present in the endothelial cell [2]. Vasoconstriction is due to several factors: direct response of muscle fibrocyte to the stretch caused by the trauma itself, neuro vegetative vessel motor reflex (nerve vasorum stimulation), local release of vasoconstrictive substances by the endothelial cells, such as endothelin, and finally from the platelets by the release of serotonin contained in the granules [2]. This mechanism would be inappropriate without the mechanisms of platelet adhesion, aggregation and activation, with the release of contenets from the granule and the exposure of the membrane procoagulant surface.

Therefore, the vascular phase allow to:

- reduce the blood flow through the damaged vessel, thus reducing bleeding
- platelet margination and subsequent activation (platelet phase)
- promotes local accumulation of activated coagulation factors as a response of subendothelial tissue exposure or following the release of tissue factor (TF) as a consequence of vascular injury.

1.1.2 Platelet phase

Platelets are small discoidal and anucleate cells that originate from the cytoplasma fragmentation of megakaryocytes produced by the bone marrow. Human platelets have a 5-7 day lifespan, and are approximately 2-5 μm in diameter, while canine platelets have an average lifespan of approximately 6 days; platelets are subsequently sequestrated by the hemocateretic organs (mainly from the spleen and the liver) where they are phagocytized by the cells of the mononuclear phagocyte system [2, 4]. Platelets synthesis is regulated by total platelets mass rather than the total platelet number and occurs predominantly in response to thrombopoietin, which is synthetized in bone marrow and smooth muscle cell. Elimination of thrombopoietin from the circulation occur via adsorption onto platelets, providing a negative feedback mechanism [5].

The platelet plasmatic membrane surface is characterized by a layer of polysaccharides, the glycocalyx, which includes receptors involved in the most important platelet function and mediate the mechanisms of platelet adhesion and aggregation. Moreover, platelet membrane has also a procoagulant activity due to the presence of the Platelet Factor 3 (PF3), which provide the surface for the interaction between the various coagulation factor and cellular elements when the coagulation is activated.

The ability of the platelet to respond to such a wide variety of stimuli, despite its lack a nucleus, is due, in part, to the abundance of substances contained in platelets [2]. Preformed sunstances exist in platelets within alpha and delta (dense) granules. The granules content is directly secreted during the platelet response, through a canalicular system, in correspondence of the endothelial injury in order to allows the subsequent platelets aggregation.) Alpha granules contain platelet derived growth factor, fibronectin, transforming growth factor β, platelet factor 4, fibrinogen, factor V and VIII, and adhesive proteins such as von Willebrand factor and the β-thrombomodulin, inhibitor of endothelial prostacyclin synthesis, released during the activation phase. Dense granule represents the site of adenyl

nucleotide deposition [adenosin diphosphate (ADP) and adenosin triphosphate (ATP)], as well as containing serotonin, histamine, catecholamines and calcium ions; dense granules and are less numerous the α granules [2].

Prevention of adherence to normal endothelial cells is facilitated by a combination of physical and biochemical factors. Physical factors include repulsive forces from electronegative charges on platelets and endothelial cells. Biochemical factor include synthesis of inhibitors of platelet activation such as nitric oxide (NO) and prostacyclin. Nitric oxide and prostacyclin are released constitutively from the endothelium and act locally to prevent platelet adhesion to normal endothelium [6].

1.1.3 Platelet adhesion

The circulating platelets, as a consequence of the reduction of blood flow secondary to the vasoconstriction, move from the centre to the periphery of the vessel and, therefore, can more easily adhere to the exposed structures following the vessel injury.

Among the different groups of membrane proteins, integrins play a very important role during the process of cell adhesion and, in particular, they appear to be the primary mediators of platelet adhesion to the extracellular matrix [7]. The integrins are formed by two subunits (α and β) linked together by non-covalent bonds; both subunits have an extracellular domain able to bind bivalent cations, in particular calcium ions. The high specificity of the integrin to bind with different ligands mainly depends on the extracellular portion of the α subunit [8].

More than seven β subunits have been identified, but only two, β_1 and β_3, are present on platelet surface. The subunit β_1 is associated with three different α subunits that allow binding with collagen ($\alpha_2\beta_1$), fibronectin ($\alpha_5\beta_1$) and laminin ($\alpha_6\beta_1$) [8]. The subunit β_3, expressed exclusively on megakaryocytes and platelets ($\alpha_{IIb}\beta_3$), has affinity for fibrinogen and von Willebrand factor (vWF), thus playing an important role during platelet adhesion and aggregation [7].

When an insult occurs to the vascular wall, the subendothelial matrix is exposed to the blood flow and platelet promptly adhere to it in order to limit bleeding and promote the tissue repair [6]. (Figure 1)

The subendothelial matrix presents different adhesive macromolecules such as collagen, vWF, laminin, fibronectin and thrombospondin, which are all ligands of different platelet receptors [6].

Initial platelet adhesion to exposed subendothelial connective tissue is primarly mediated by collagen and vWF. vWF contributes to thrombus formation in 2 ways: by mediation of adhesion of platelet to the extracellular matrix and each other, and by protecting factor VII from rapid clearance from plasma [9]. The vWF is an adhesive protein synthesized by megakaryocytes and endothelial cells [10]. It is stored in secretory vesicles called Weibel-Palade bodies in endothelial cells and in α granules in magakaryocytes and platelets [9]. Release of vWF can be stimulated by several substances including thrombin, fibrin, vasopressin, collagen, platelet-activating factor (PAF), ephinephrine and histamine. After release from Weibel-Palade bodies, vWF may enter the bloodstream or become bound to collagen in the subendothelium. There are 2 distinct receptors for vWF on platelets, the Integrin $\alpha_{IIb}\beta_3$ receptor (formerly called the GP IIb-IIIa complex) and the GPIbα in the GP Ib-IX-V complex [9].

(Figure 1). After endothelial damage, exposure of subendothelial collagen occurs and vWF rapidly binds to the exposed collagen through specific binding sites located at the level of the domain A1 and A3 [9]. After its immobilization has occurred, it is able to binds the platelets in the circulation between the binding between its A1 domain and GPIbα, which is the only receptor, exposed on the inactivated platelet surface that has a very high affinity for vWF [9]. (Figure 1).

Under normal condition there is no interaction between vWF and GPIbα because the binding site for GPIbα on the A1 domain of vWf is hidden.

Once platelets attach to the endothelium via vWF and collagen they undergo a conformational change with exposure of the Integrin $\alpha_{IIb}\beta_3$, which binds the plasma circulating vWF. Stimulation by endothelial collagen causes platelet to expose and assemble membrane glycoprotein, which can then bind fibrinogen and circulating vWF, thus favouring the aggregation mechanisms of platelets by the link between the vWF and the GPIbα receptor present on incoming platelets. In this case, their activation is not possible through the interaction between the GPVI receptor and the collagen, and therefore occurs through the activators released from the degranulation of the first layer of platelets [9].

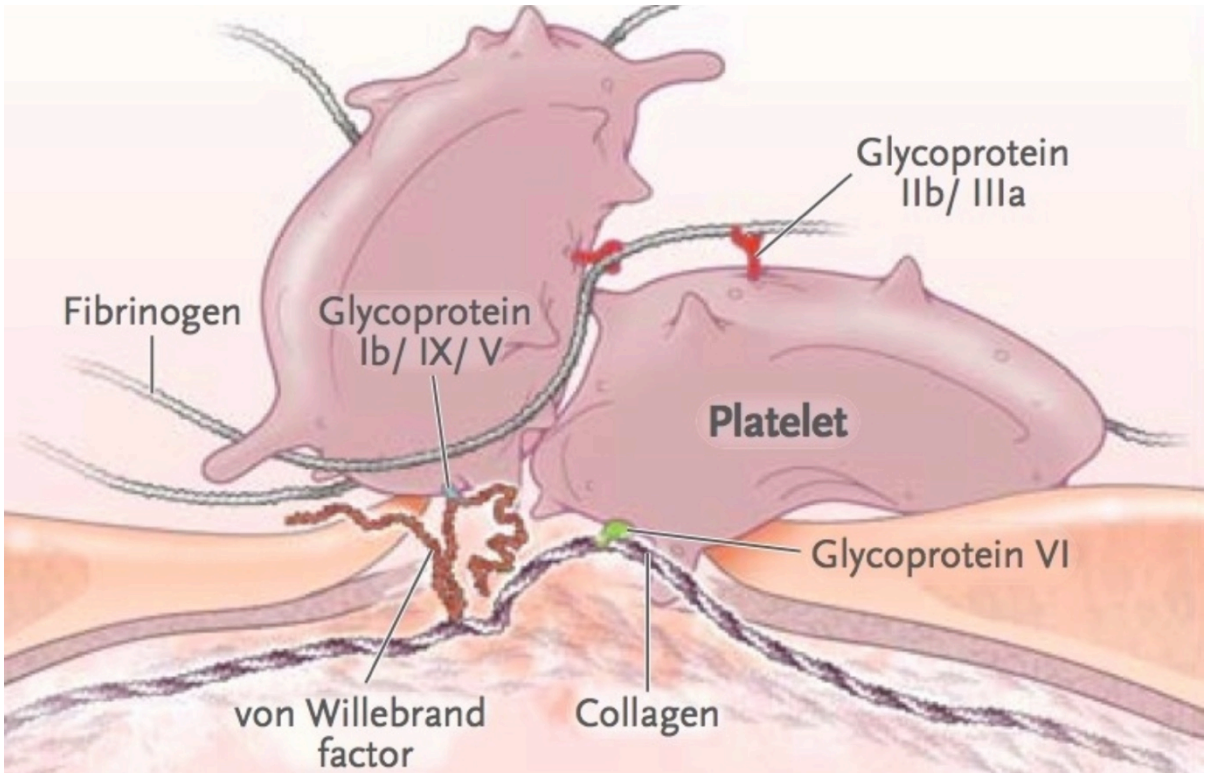

Figure 1: Representation of platelet adhesion to the sub endothelial collagen after a vascular insult has occurred. There are 2 distinct receptors for vWF on platelets, the Integrin $\alpha_{IIb}\beta_3$ receptor (formerly called the GP IIb-IIIa complex) and the GPIbα in the GP Ib-IX-V complex. After endothelial damage, exposure of subendothelial collagen occurs and vWF rapidly binds to the exposed collagen through specific binding sites. After its immobilization has occurred, it is able to binds the platelets in the circulation between the binding between its A1 domain and the complex GP Ib-IX-V. Once platelets attach to the endothelium via vWF and collagen they undergo a conformational change with exposure of the Integrin $\alpha_{IIb}\beta_3$, which binds the plasma circulating vWF and fibrinogen thus allowing the adhesion of further platelets.
From: usmle287 Platelet adhesion/aggregation/activation.

1.2 SECONDARY HEMOSTASIS

Secondary hemostasis is activated within 15-20 seconds if the vascular damage is extended, or within 1-2 minutes if the damage is less severe, due to the presence of activators deriving from vascular wall, platelets and plasma. This process leads to the formation of a fibrin network that stabilizes and strengthens the platelet clot. Anticoagulant mechanism ensures an accurate coagulation control and under normal circumstances prevails over the procoagulant forces thus allowing a normal blood flow. However, in case of vascular wall damage, the procoagulant agents become activate and predominate on the anticoagulant factors, thus allowing the blood clot formation. Defects in the normal balance between the two system can leads to hemorrhagic phenomena or results in thrombotic state [11].

In the 1960s, two independent and similar reports were published, introducing the **cascade model of blood coagulation** that defined a series of steps where enzymes and their cofactors cleaved subsequent proteins [12,13].

Most of the plasma proteins involved in the hemostatic process circulate as inactive proenzymes (zymogens) and are converted into their active form by other enzyme in a sequences of successive reaction referred as a “cascade reaction” which have as a final result the conversion, catalysed by thrombin, of fibrinogen into fibrin; the majority of these steps occur in the presence of calcium on the phospholipid surface of cell membranes [14]. Secondary hemostasis is subdivided in two pathway: the **intrinsic** and **extrinsic pathway** respectively; either pathway could activate factor X (FX) to FXa, which in turn (with its cofactor FVa) could activate prothrombin to thrombin, which then cleaved fibrinogen to form fibrin. This latter portion was referred as **common pathway.** (Figure 2)

The extrinsic system was localized outside (or extrinsic from) the vasculature lumen and consisted of tissue factor (TF) and FVIIa[1] [14]. The component of the intrinsic system are localized within the blood (or intrinsic to) and the main event for the initiation of blood coagulation is the activation of Hageman's FXII, the first cascade serine-protease zymogen that subsequently activates other components [14].

Originally, the intrinsic pathway, or the contact activation system, was considered the primary pathway to trigger thrombin generation following a trauma affecting the vascular endothelium surface, whereas the extrinsic pathway was instead considered a complementary pathway through which thrombin could be formed [12,13].

It later became evident that the extrinsic pathway plays the major role in *in vivo* blood clotting, bringing the physiological relevance of the contact pathway into question [15].

The intrinsic coagulation pathway is activated when blood contacts a strongly negatively charged surface exposed as a consequence of damage to endothelial cells [16]. It is composed of 2 zymogens, FXII and pre-kallikrein (PK), and one cofactor (high-molecular-weight-kininogen) (HMWK).

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1 FVII is the only coagulation factor present in the circulation in both active and inactive forms; it can be activated by FIXa, FXa, FXIIa, thrombin and plasmin or by the FVII activating protease. However the real mechanism of activation of FVII is still unknown and for this reason its autoactivation is thought to exist. Factor IX certainly plays an important role in the activation of FVII because patients affected byhaemophilia B have low levels of FVIIa

The initial triggering event leading to clot formation is represented by the interaction between the positively charged amino acid residues of FXII and the negatively charged endothelial surface; the HMWK behaves in the same manner, positioning itself near the FXII [16]. Both plasma PK and FXI are linked to the non-adhesive surface of the high-molecular-weight-kininogen (HMWK) and are thus presented to FXII. The *in vivo* activation mechanism of FXII is not fully understood: it is believed that when the zymogen FXII binds to the subendothelium its activation occurs, thus assuming its self-activation, resulting in the formation of FXIIa [16]. Regardless of the initial triggering event, during the subsequent steps, the zymogen FXII is activated by the callicrein formed from the PK, which it is in turn activated by FXIIa [16]. Once a threshold amount of FXIIa is formed, it activates FXI to FXIa. Factor XIa then activates FIX. Factor IXa, along with calcium, phospholipids, and FVIIIa, activate FX. Factor Xa, along with FVa, calcium and phospholipids activate prothrombin (FII) to thrombin (FIIa).

While this mechanism is useful to explain the *in vitro* coagulation triggering, it has been observed that congenital deficiency in FXII and FXI are not related to bleeding disorder, thus indicating how the intrinsic pathway is not the main mechanism of *in vivo* activation [11]. Only the deficiency of FIX or its cofactor FVIII is associated with hemorrhagic diathesis, haemophilia B and haemophilia A respectively.

Studies have therefore demonstrated that the interaction between the FVIIa and the TF, the event that determines the initiation of the extrinsic pathway, represent the effective initiator mechanism of *in vivo* coagulation while the intrinsic pathway is now considered an amplification and propagation pathway of hemostatic process [17].

Tissue factor, also called tissue thromboplastin or FIII, is a membrane protein abundantly express by fibroblast of the subendothelial matrix, and then exposed to the blood flow in when vascular wall injury occur, or by endothelial cells and macrophages, when stimulated by cytokines and endotoxins. It binds both zymogen and activated forms of FVII (which in itself would not have enzymatic activity) forming, in association with calcium ions, the TF-FVII-Ca^{2+} complex, an enzymatic complex with high affinity for FX of which catalyse the activation to FXa [11].

Furthermore the TF-FVII complex activates FIX, proving to be the fundamental mechanism with which the coagulation is activated and demonstrating how the two pathways do not operate independently of each other, but showing how the reactions of the extrinsic mechanism affect the intrinsic pathway. Indeed, a study conducted in 1982 reported that plasma deficient in either FVIII or FIX showed significantly less activation of FX by TF compared with normal plasma, suggesting some form of dependency between the 2 pathway [18].

The activation of FX determines the initiation of the common pathway of coagulation that is characterized by two events: thrombin and fibrin formation.

Factor Xa, interacting with FVa, platelet factor-3 (PF-3) and calcium ions, form a multimolecolar complex, the **prothrombinase complex**, which convert prothrombin (FII) into thrombin. Thrombin exerts its proteolytic action convertin fibrinogen into soluble fibrin. Maximum thrombin generation occurs after the formation of fibrin clot; this thrombin is important for additional fibrin generation as

well as for activation of FXIII and the thrombin-activable fibrinolysis inhibitor. Activated factor XIII, or fibrinoligase, is a transglutaminase that stabilises the clot by covalent cross-linking of fibrin [11].

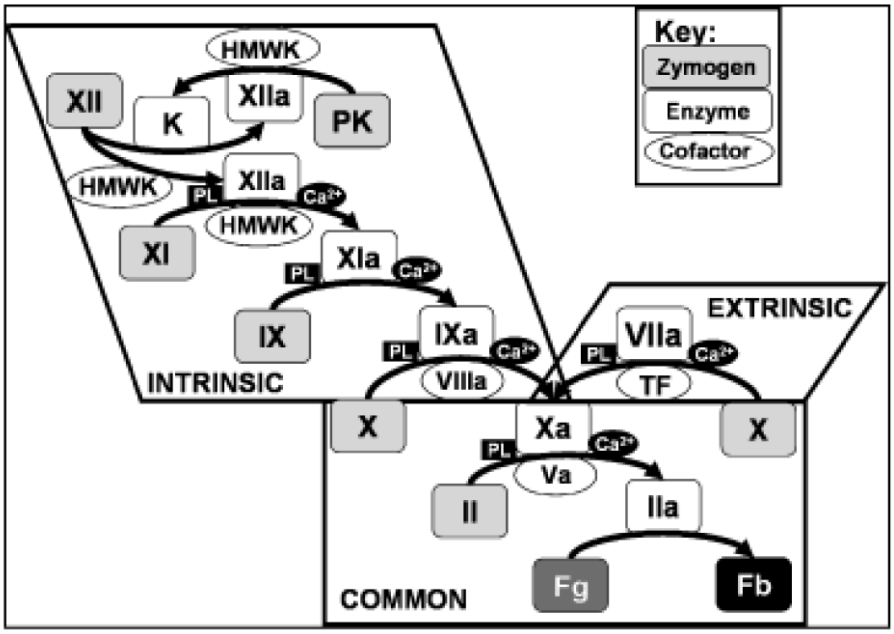

FIGURE 2: The cascade model of fibrin formation. This model divides the coagulation system into separate pathway (extrinsic and intrinsic) either of which results in generation of FXa. The common pathway results in generation of thrombin and subsequent cleavage of fibrinogen to fibrin.
From: Smith SA. The cell-based model of coagulation. J Vet Emrg Crit Care 2009:19(1);3-10

1.3 DEFICIENCIES IN THE CASCADE MODEL AND THE CELL-BASED MODEL OF COAGULATION

While separating the various enzymatic processes of coagulation into the cascade model was useful to understand how hemostatic process occur in plasma-based *in vitro* coagulation, this model does not adequately explain how the coagulation process occur *in vivo* [14, 19].

Clinical studies conducted in mice and humans have shown that deficiencies of FXII, HMWK or PK, the initial components of the intrinsic pathway, cause marked prolongation of the activated partial thromboplatin time (aPTT), but this deficiency is not associated with an *in vivo* increased bleeding tendency [20]. Furthermore , FXII is clearly not required for normal hemostasis because some mammalian species (such as whales and dolphins) do not have this protein. Deficiency of the next downstream enzyme FXI (haemophilia C) is associated with variable hemostatic deficits in humans, with some individuals experiencing bleeding. In contrast, deficiency in either of the next downstream components of the intrinsic pathway (FVIII and FIX) results in serious bleeding tendency, known as haemophilia A and B respectively, despite an intact extrinsic pathway. Similarly, deficiency of the primary enzyme of the extrinsic pathway (FVII) can be associated with severe bleeding, despite the presence of an intact intrinsic pathway [19].

The cascade model of coagulation presents a further limitation: it describe in detail how blood coagulation occurs in plasma, but does not take into account the interaction between plasma components and cellular elements who actively participate during the hemostatic process. These limitations led to a revised theory of coagulation, which includes the vital contribution of cellular elements to hemostasis. Coagulation complex formation requires a phospholipid membrane surface and the addition of calcium. *In vivo* , the procoagulant membrane is currently though to consist mainly of activated platelet. In fact, in addition to undergoing a shape change, activated platelets expose on their surface anionic phospholipids, mostly in the form of phosphatidylserine [19]. Other cells (monocytes, smooth muscle cells, endothelial cells) can also function as the procoagulant surface.

In 1992, Mann proposed what has become known as the “cell-based model” of coagulation [21]. (Figure 3)

Evaluation of this model suggests that coagulation actually occurs in vivo in distinct overlapping phases: initiation, amplification and propagation and requires the participation of 2 different cells type: a cell-bearing TF, and platelets [14].

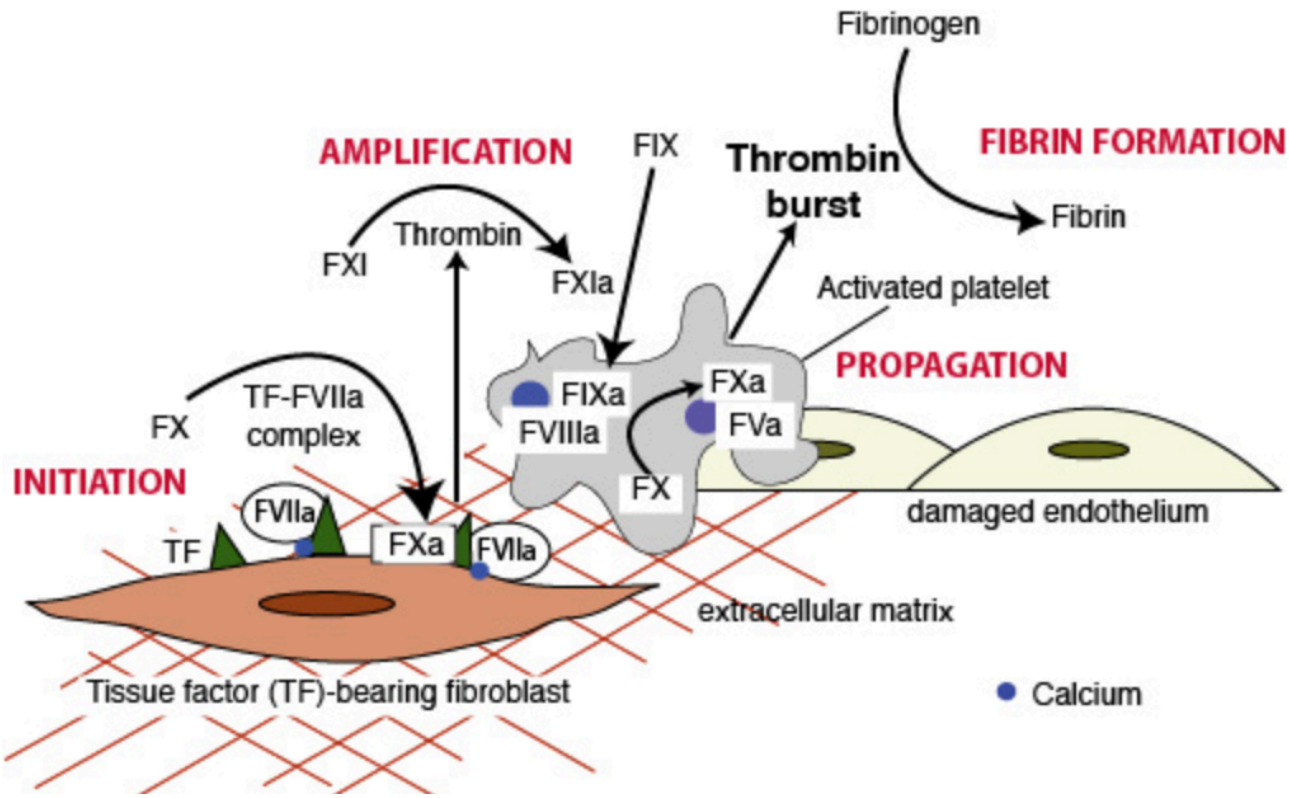

Figure 3: The cell-based model of fibrin formation. The cell-based model incorporates the contribution of various cell surfaces to fibrin formation. In this model thrombin generation occurs in overlapping phases.
From: http://www.eclinpath.com/hemostasis/physiology/secondary-hemostasis/secondary-haemostasis/

1.3.1 Initiation

All evidence to date indicates that the sole relevant initiator of *in vivo* coagulation is TF. Cell expressing TF are generally localized outside the vasculature, which prevents initiation of coagulation under normal flow circumstances with an intact endothelium. Some circulating cells (eg, monocytes or tumor cell) and microparticles may express TF on their membrane surface, but this TF under normal condition is thought to be inactive or encrypted.

Once an injury occurs and the flowing blood is exposed to a TF-bearing cell, FVIIa rapidly binds to the exposed TF. Factor VII, a vitamin K-dependent protein produced in the liver, is the only coagulation factor that circulates in both the active and inactive forms, with approximately 1% of total FVII circulating as FVIIa [22]. The TF-FVIIa complex then activates additional FVII to FVIIa, allowing for even more TF-FVIIa complex activity, which then activated small amounts of FIX and FX. Therefore, the TF-FVIIa complex appears to be the only physiological activator of coagulation *in vivo* [19]. Although it occurs slowly, FV can directly be activate by FXa. The FXa generated by TF-FVIIa binds to the few generated molecules of its cofactor FVa to form the prothrombinase complex, which subsequently cleaves prothrombin and generates a small amount of thrombin. This activity is tightly monitored by the tissue factor pathway inhibitor (TFPI) and antithrombin (AT) in order to prevent an abundance of thrombin generation for a false alarm. The TF-FVIIa complex is rapidly inhibited by the TFPI while the AT rapidly inactivates any FXa that dissociated from the membrane surface of the TF-bearing cell.

Procoagulant triggering only proceeds if TF is exposed at high enough levels to overcome inhibition by TFPI and AT. (Figure 4)

1.3.2 Amplification

Once a small amount of thrombin has been generated on the surface of a TF-bearing cell during the initiation phase, that thrombin diffuses and actives platelets that have leaked from the vasculature at the site of injury. Binding of thrombin to platelet surface receptors lead to a change in their shape, release of their granule content and the exposure of membrane phospholipids to create a procoagulant surface. In addition to activating platelets, the thrombin generated in the initiation phase cleaves FXI to FXIa and activates FV to FVa on the platelet surface. Thrombin also cleaves von Willebrand factor off FVIII (they circulate bound together), releasing it to mediate platelet adhesion and aggregation. The released FVIII is subsequently activated by thrombin to FVIIIa [23]. (Figure 4)

1.3.3 Propagation

Once a few platelets are activated in the amplification phase, the release of granule contents results in recruitment of additional platelets to the site of injury. The propagation phase occurs on the surface of these platelets. Factor IXa that was generated by TF-FVIIa during the initiation phase can bind to FVIIIa (generated in the amplification phase) on the platelet surface. Factor XIa, generated during amplification on the platelet surface, generates additional FIXa by cleaving FIX. In the presence of

Ca^{2+} the intrinsic tenase complex (FIXa-FVIIIa) forms on the activated platelet surface and it rapidly begins to generate FXa. Factor Xa was also generated during the initiation phase on the TF-bearing cell surface. As this FXa is rapidly inhibited if it moves away from the TF-bearing cell surface, it cannot easily reach the platelet surface. Therefore the majority of FXa must be generated directly on the platelet surface through cleavage by the intrinsic tenase complex. The FXa generated on platelets then rapidly binds to FVa (generated by thrombin in the amplification phase) and cleaves prothrombin ti thrombin. This prothrombinase activity results in a burst of thrombin generation leading to cleavage of fibrinopeptide A from fibrinogen. When enough thrombin is generated with enough speed to result in a critical mass of fibrin, these soluble fibrin molecules will spontaneously polymerize into fibrin strands, resulting in an insoluble fibrin matrix [23].

In conclusion it can be assumed that the cell-based model of coagulation answers to several important questions left unresolved by the waterfall/cascade models. Question such as why patients with haemophilia bleed if there are 2 indipendent pathways to clot formation, why deficiencies in FXII or PK do not cause coagulation abnormalities if the contact pathway is essential for hemostasis. Moreover this model suggest that the intrinsic and the extrinsic pathway are connected each other and therefore should not considered as 2 independent pathway. (Figure 4)

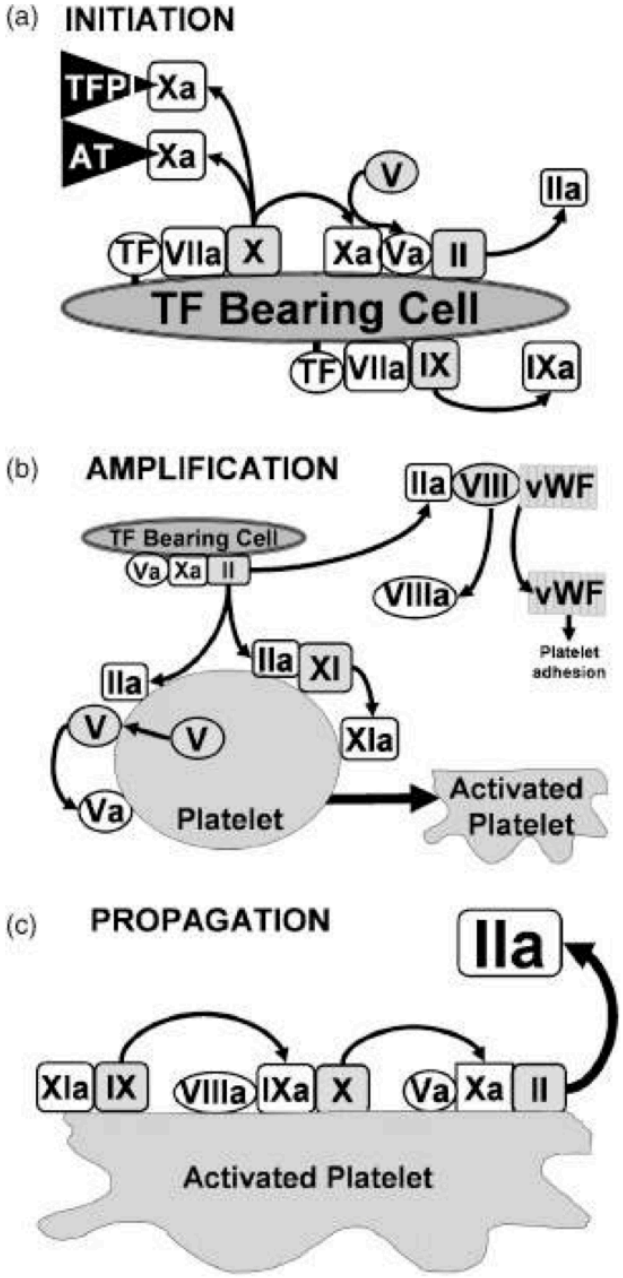

Figure 4: The cell-based model of fibrin formation.
(a) Initiation phase: this phase occurs on the TF-bearing cell. It is initiated when injury exposes the TF-bearing cell to the flowing blood. It results in the generation of small amount of FIXa and thrombin that diffuse away from the surface of the TF-bearing cell to the platelet.
(b) Amplification phase: in the second phase, the small amount of thrombin generated on the TF-bearing cell activates platelets, releases vWF and leads to generationof activated forms of FV, FVIII, and FXI.
(**c) Propagation phase:** in the third phase the various enzyme generated in the earlier phase assemble on the procoagulant membrane surface of the activated platelet to form intrinsic tenase, resulting in FXa generation on the platelet surface.
Smith SA. The cell-based model of coagulation. J Vet Emrg Crit Care 2009:19(1);3-10

1.4 FIBRINOLYSIS AND ANTICOAGULANT FACTORS

Simultaneously to the activation of coagulation, the mechanisms that govern the fibrinolysis process are also activated and through these the enzymatic degradation of the fibrin clot and the tissue repair occur. The fibrinolysis is therefore the main effector of clot dissolution and its action is coordinated by the interaction between activators, zymogens, enzymes and inhibitors, which provide for its local activation and regulation at the site where the fibrin is present. Fibrinolysis disorders may result in its reduced activation and therefore hesitate in thrombotic complication, or in its excessive activation and therefore an increased risk of bleeding [24].

This system includes the plasminogen, an inactive proenzyme synthesized in the liver, which is converted into its active form, plasmin, by the tissue plasminogen activator (TPA) and urokinase plasminogen activator (UPA)[2] (Figure 5). Plasminogen activation is enhanced by fibrin. Plasmin then degrades polymerized fibrin to form fragments reffered as fibrinogen degradation products (FDPs) [17].

Fibrinolysis inhibitors may acts on two different sites: by inactivating the plasminogen activators through the Plasminogen Activator Inhibitor-1 (PAI-1), or by inactivating plasmin through the α2-antiplasmin [24].

The main factors that prevent the activation of coagulation in the normal vascular system are represented by either some intrinsic characteristic of the endothelium and by circulating anticoagulants agent. During normal blood flow condition the endothelium glycocalix, a protein layer on the endothelium luminal surface, prevents the activation of the intrinsic pathway and rejects platelets and coagulation factors. Natural coagulation inhibitors are: the tissue factor pathway inhibitor, the components of antithrombin III (AT-III)- heparin pathway and the components of thrombomodulin (TM) – Prtotein C (PC) – S protein (PS) complex [24]. The TFPI inhibits the reaction involving tissue factor and factor VIIa [25]. The lack of TFPI may not be compatible with life, since no deficiency states have been described in human beings [11]. This idea is supported by the lethal phenotype found in TFPI knock out mice; in fact it has been reported that this animals showed uncontrolled activation of coagulation with subsequently consumption of coagulation factors [11].

Most of the enzymes generated during activation of coagulation are inhibited by AT-III, which preferentially inhibits free enzymes, whereas enzymes that are part of the tenase or prothrombinase complexes are less accessible for inhibition. The physiological role of AT is to limit the coagulation process to sites of vascular injury and to protect the circulation from liberated enzyme. Antithrombin is, in itself, an inefficient serine-protease inhibitor, but heparin and the heparin-like molecules that are

[2] TPA and UPA are named after the source from where they were originally isolated; TPA is the major plasminogen activator in the vasculature and UPA is the major activator in the extravasculature tissue. TPA is secreted by endothelial cells and its enzymatic activity is very weak in the absence of fibrin. UPA is synthesized by fibroblast-like cells, epithelial cell, monocytes and endothelial cells and, unlike TPA, can activate plasminogen in the absence of fibrin.

present on the endothelial cell surface stimulate its activity [11]. This mechanism is the molecular basis for the use of heparin as a therapeutic anticoagulant [11].

The protein C anticoagulant system regulates coagulation by modulation of the activity of two cofactors, FVIIIa and FVa; it is activated on the surface of intact endothelial cell by thrombin that has bound to the membrane protein thrombomodulin [26]. Thus, thrombin has the capacity to express both procoagulant and anticoagulant function depending on the context under which is generated. At site of vascular disruption, the procoagulant effects of thrombin are fully expressed. In contrast, in an intact system, thrombin has anticoagulant function since it binds to thrombomodulin and activates protein C. Activated protein C can cleave the phospholipid-membrane bound cofactor FVa and FVIIIa, which results in inhibition of the coagulation system. A vitamin-K dependent cofactor protein, protein S, supports the anticoagulant activity of PC [11,26].

Alterations of the balance between the procoagulant and anticoagulant mechanism that could occur as a consequence of reduction in the activity of coagulation inhibitors may results in hemostatic disorders known as thrombophilia.

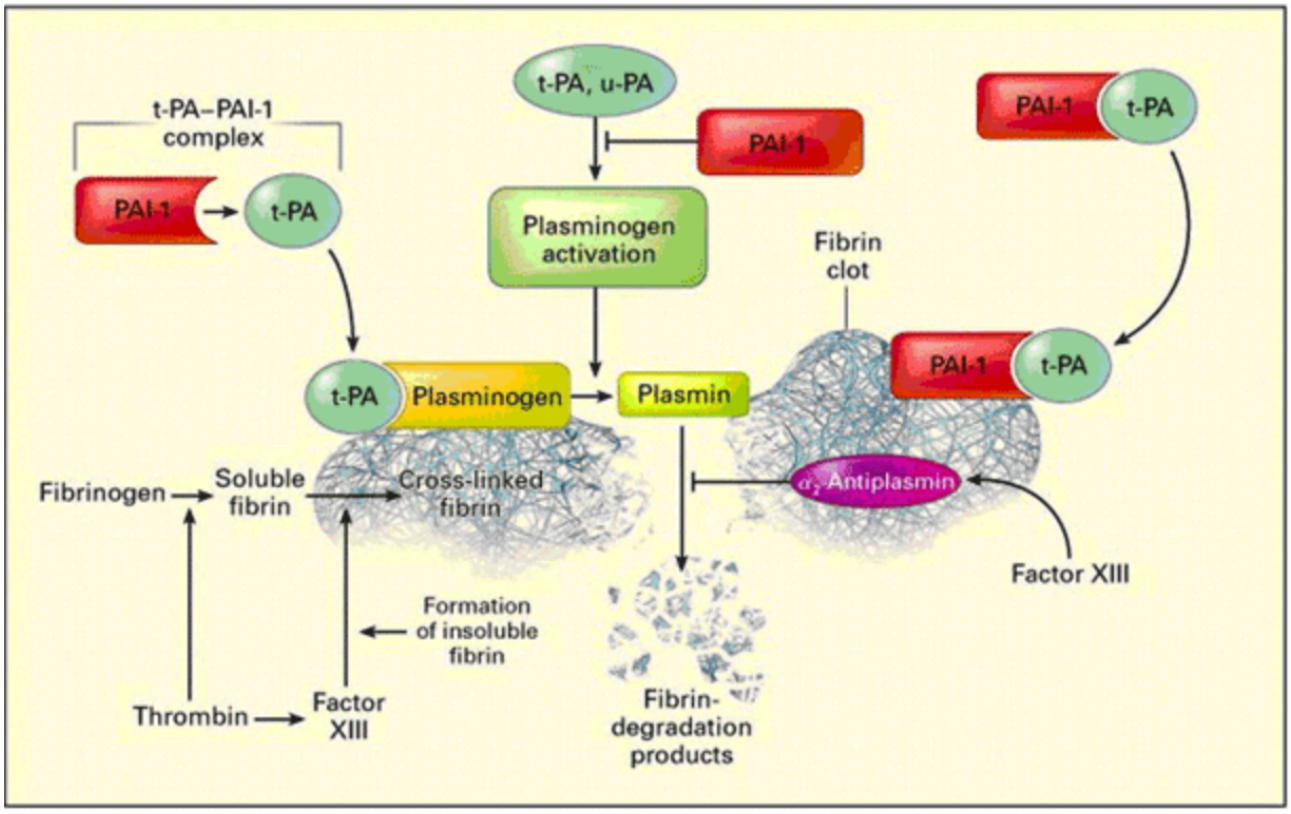

Figure 5: Schematic representation of the fibrinolysis process.

Plasminogen, an inactive proenzyme is converted into its active form, plasmin, by the tissue plasminogen activator (TPA) and urokinase plasminogen activator (UPA)

Plasmin then degrades polymerized fibrin to form fragments reffered as fibrinogen degradation products (FDPs).

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1.5 LABORATORY TEST FOR COAGULATION ASSESSMENT:

Currently available laboratory tests allow to investigate the different components of hemostasis; it is important to differentiate and correctly identify if the hemostatic alteration responsible for the clinical signs depend on primary, secondary hemostasis or fibrinolysis.

Laboratory tests currently available for the coagulation assessment will be illustrated in this section.

1.5.1 Test of primary hemostasis

Tests used for the evaluation of primary hemostasis are focused to identifying the presence of possible numerical (thrombocytopenia/thrombocytosis) and functional (thrombocytopathy) platelets abnormalities in addition to the evaluation of Von Willerbran factor.

Numerical evaluation of platelet is performed by automatized and manual platelet count and estimation, whereas the assessment of platelet function is performed by buccal mucosa bleeding time (BMBT) and by means of specific tools such as Platelet Function Analyzer (PFA) and aggregometer.

Specific test are used for quantitative and qualitative assessment of von Willerbrand factor.

1.5.1.1 Estimation and platelet count:

Evaluation of platelet count is a necessary step in diagnosing disorder of primary hemostasis. Thrombocytopenia may be due to defects in platelet production, diminished platelet survival, loss of platelet from the systematic coagulation or drugs- induced etiologies [27]. Automated platelet counts can be determined through a commercial laboratory analyser. However, errors deriving from in vitro aggregation, poor quality sampling, presence of giant cells, red blood cell or leukocyte fragments, or even EDTA-induced agglutination, are common and can overestimate or underestimate the automated platelet count. Therefore, a manual platelet counts with a microscopic reading of the blood smear is always recommended. Each smear is evaluated microscopically on 100X high power; each platelet observed is equivalent to 15 x 10^3/µL platelets. Therefore the platelet average in 5 fields can be multiplied by 15 x 10^3/µL to obtain platelet estimation [27].

In dogs normal platelet count is 200-500 x10^3/µL; patients with platelet count below 20 x 10^3/µL are at high risk of spontaneous bleeding, while trauma-induced bleeding or surgery may occur with platelet counts below 50 x 10^3/µL. By using the smear, platelet clumping, morphology and number can be recorded to verify the accuracy of machine counts.

1.5.1.2 Buccal Mucosal Bleeding Time (BMBT)

The buccal mucosal bleeding time (BMBT) is a test easy to perform and does not require expensive equipment and specialized staff, but is inaccurate and difficult to interpret. It should only be performed on a patient with a normal platelet count and coagulation panel. Therefore is no longer routinely recommended. It has been used as an in vivo screening test of platelet function, abnormalities in vascular integrity and von Willebrand disease (vWD) and it does not become prolonged with disorders

of secondary hemostasis [28]. There are multiple limitations with the BMBT test, including its operator dependency, poor reproducibility, and lack of sensitivity to detect mild bleeding disorder. In addition the BMBT test is not always able to correctly predict the extent of surgical haemorrhage.
This test is performed using a commercial available blade; a standardized length and depth incision is made on the oral mucosa of the upper lip with patients restrained in sternal or lateral recumbency. After the incision the blood is gently buffered with a gauze and time between the mucosal incision and cessation of bleeding is calculated.
Normal BMBT range from 1.7 minutes to 4.2 min in dogs, and from 1 min to 2.4 minutes in cats, but variability has been found [29,30].
The BMBT is prolonged in case of hereditary primary hemostasis disorder, thrombocytopenia, severe azotemia and in those patients on aspirin or other nonsteroidal anti-inflammatory drugs.

1.5.1.3 Platelet function testing

The diagnosis of thrombocytopathia can be aided by specific platelet function testing including the impendance whole-blood platelet aggregometry (WBA), plasma based light transmission aggregometry, and a commercial available platelet function analyser (PFA-100).
The evaluation of platelet aggregation represents the gold standard for the diagnosis of primary hemostatic defects and it is able to detect different aspect of platelet function through the use of specific agonist (ADP, alpha or gamma thrombin or collagen). It can be measured by means of specific tool, the aggregometry, using two different methods: the turbidimetric methods and the impendace aggregometry.
The turbidimetric method was first described by Born in 1962 and should be performed on platelet-rich plasma. This test measures the variation of light transmission during platelet aggregation in response to an agonist. However the preparation of this substrate is a laborious procedure that requires specialized personnel and high volumes of blood [31].
Impedance aggregometry measures changes in the electrical resistance of the whole blood during platelet aggregation in response to an agonist. This technique uses samples of whole blood that do not require any special preparation and better reflects *in vivo* platelet function [32].
The PFA-100 is an *in vitro* test that measure time to cessation of whole blood flow (closing time) through a central aperture (150 µm diameter) of a nitrocellulose membrane under high shear stress condition. The membrane is coated with platelet agonist such as collagen and either epinephrine or ADP. High shear stress rates with standard flow condition results in platelet attachment, activation and aggregation forming a stable platelet plug at the aperture [33]. Closure time (Ct; sec) is time needed to plug to occlude the central membrane aperture and stop the blood flow. Advantages of the PFA-100 include its simplicity, accuracy, rapid result interpretation and reproducibility. Platelet aggregation, that is responsible for the closing time, is dependent on platelet number, function and functional von Willebrand factor [33].

It is used as a screening test to detect abnormalities in primary hemostasis, but is not specific for any one particular disease. Therefore a normal Ct can allow to exclude a severe disorder of platelet function or severe vWD but would not be able to completely exclude milder forms of these disease [27].

1.5.1.4 Von Willebrand factor

Deficiencies in von Willebrand Factor (vWF) result in defective platelet adhesion and clinical signs of bleeding. Three type of vWD have been identified in dogs: types I and III representing deficiencies in level of circulating vWF while type III reflecting an abnormality in the factor's structure [34].

The vWF concentration and functionality should be assessed to differentiate between these variants. The vWF antigen (vWF:Ag) test is a quantitative enzyme-linked immunosorbent assay (ELISA) that measure the concentration of vWF in the sample [34]. The ELISA is a rapid, sensitive and reproducible test that uses anti- vWF antibodies to quantify vWF antigen [34]. Patients affected by vWD type II have a qualitative defects of vWF and thus cannot be identified by this test.

Determination of vWF function is the next step in the appropriate diagnosis of vWD. This test is performed by evaluating the total amount of vWF able to bound to the subendothelial collagen by means of the collagen-binding (vWF-CBA) assay [34]. This test, in association with the vWF:Ag test help to distinguish between type I and type II vWD.

Causes of Primary Hemostatic Disorder

Thrombocytopenia

Decreased Production

- Immune-mediated megakaryocyte aplasia
- Drug-induced
  - Estrogen
  - Antibiotics
    - Chloramphenicol
    - Trimethoprim-sulfonamide
  - Cytotoxic drugs
    - Cyclophosphamide
    - Doxorubicin
    - Azathioprine
    - Chlorambucil
    - Cytosine arabinoside
    - Methotrexate
    - Decarbazine
  - Methimazole
  - Thiazide diuretics
  - Griseofulvin (especially in FIV-positive cats)
  - Albendazolo
- Infection
  - Chronic rickettsial disease
  - Cyclic thrombocytopenia
    - *(Erlichia platys)*
  - Systemic mycosis
  - Canine parvovirus
  - Canine distemper virus
  - FeLV
  - FIV
  - FIP
  - Cytauxzoonosis

Increased Destruction/Consumption

- Immune-mediated
  - Primary/autoimmune
  - Secondary
    - Systemic lupus erythematosus
    - Drug- induced
    - Infection
      - Rickettsial
      - Fungal
      - Bacterial
      - Viral (Felv-FIV)
      - Protozoal
      - Dirofilariasis
      - Babesia canis
      - Neoplasia
- Nonimmune-mediated
  - Drug induced
  - Ehrlichiosis
  - Rocky mountain spotted fever
  - Dirofilariasis
  - DIC
  - Microangiopathies
  - Vasculitis
  - Hepatic disease
  - Heparin-induced
  - Profound acute hemorrage
  - Hemolytic uremic syndrome
  - Anticoagulant rodenticide
- Sequestration

Thrombocytopathia

- Inherited
  - Von Willebrand disease
  - Canine thrombopathi (basset hound)
  - Spitz dog thrombopathia
  - Storage pool deficienc (American cocker)
- Acquired
  - Drug-induced NSAID
  - DIC (due to FDPs)
  - Uremia
  - Hepatic disease
  - Pancreatitis
  - Myeloproliferative disorde
  - Myeloma
  - Immune-mediated thrombocytopenia

○ Sepsi • Neoplasia ○ Myeloproliferative disease ○ Lymphoproliferative disease ○ Metastatic disease ○ Estrogen-secreting tumor • Other causes ○ Myelofibrosis ○ Idiopatic bone marrow aplasia ○ Radiation theraphy	○ Rickettsial ○ Fungal ○ Systemic lupus erythematosus ○ Splenitis ○ Hypothermia ○ Sepsis ○ Splenic torsion	

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1.5.2 Test of secondary hemostasis

1.5.2.1 Prothrombin Time (PT)

The prothrombin time (PT) is the test used for the evaluation of the extrinsic and common coagulation pathway, specifically for factor VII, X, V prothrombin (II) and fibrinogen [35]. It is unaffected by platelets or intrinsic factor defects. This is an *in vitro* test performed on citrate plasma by means of automated analyser where coagulation is activated following the addition of an exogenous mixture of thromboplastin, phospholipids and calcium. The PT is the time from the addition of calcium and thromboplastin to the appearance of the first fibrin strands in the sample. The PT is useful as a screening test but carries a low sensitivity to detect individual factor abnormalities.

The PT may be prolonged in patients affected by factor deficiency or dysfunction, liver disease, disseminated intravascular coagulation (DIC), circulating anticoagulant such as heparin, vitamin K deficiency or anticoagulant rodenticide intoxication. The vitamin K-dependent coagulation factors (II, VII, IX and X) have variable half-lives (41, 6.2, 13.9 and 16.5 hours respectively). With anticoagulant rodenticide ingestion or vitamin K deficiency, factor VII depletion, because of its short half-life, results in prolongation of the PT [35].

1.5.2.2. Activated Partial Thromboplastin Time (aPTT)

The activated partial thromboplastin time (aPTT) is a screening assays that evaluates the intrinsic and common coagulation pathway, and it is useful to detect deficiencies or inhibition of all factors excluding FVII and FXIII. Similarly to the PT, the test is performed using plasma citrate, and coagulation is activated following the addition of mixture of phospholipids, Ca^{2+} and a contact phase activator such as kaolin. The time, in seconds, required for the appearance of fibrin strands in the sample, following its recalcification, represent the aPTT [27].

A normal platelet count and and PT level with an abnormal aPTT could indicate a dysfunction and/or deficiency of FVIII (haemophilia A), FIX (haemophilia B), FXII, FXI, FV, FX, heparin theraphy, liver disease or DIC.

Contact factor deficiencies (FXII, prekallikrein, and HMWK) are often benign and, although they result in an increase in aPTT, are not associated with clinical manifestations of bleeding [27].

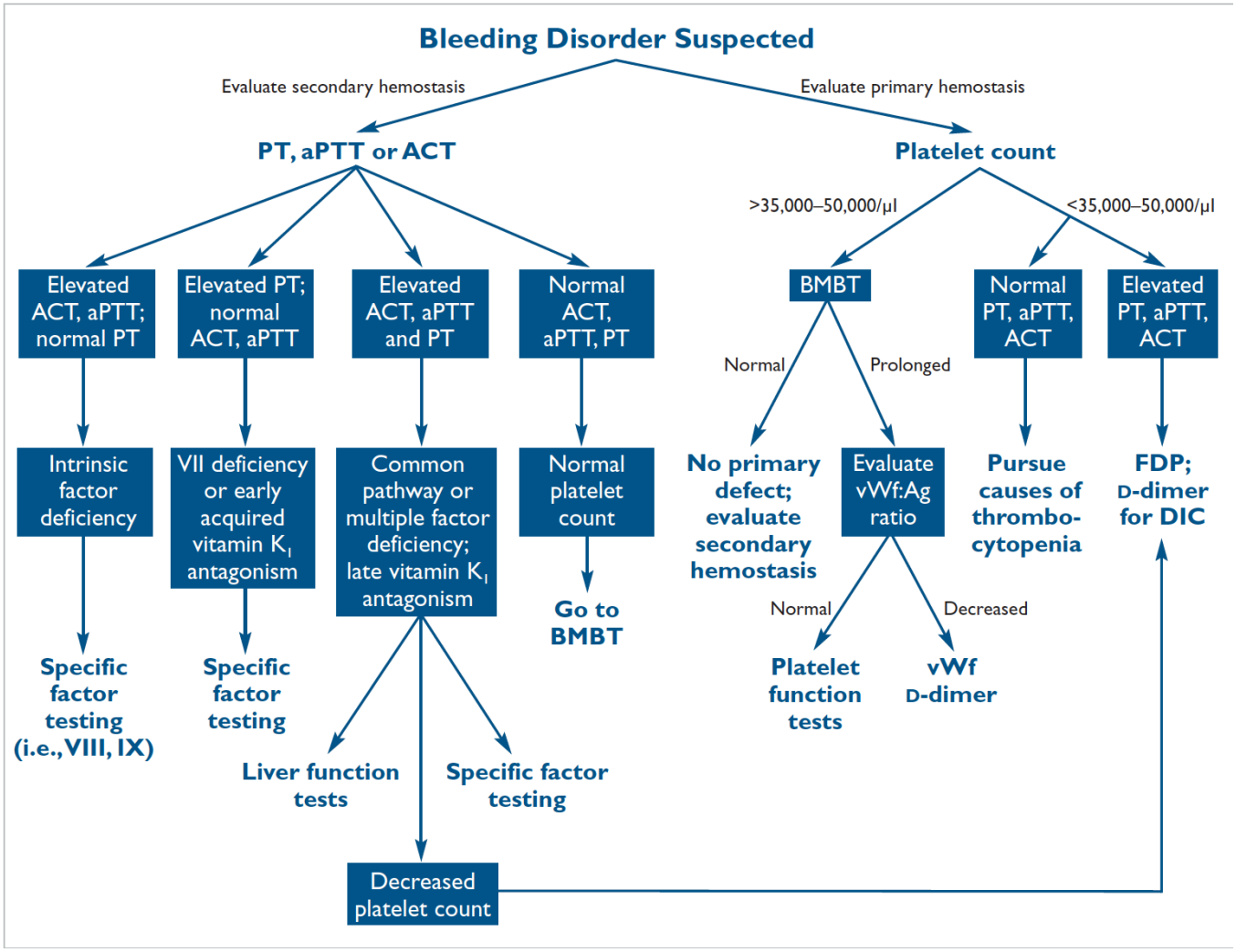
1.5.2.3 Activated Clotting Time (ACT)

The Activated Clotting Time (ACT) is a simple, rapid and inexpensive test performed on fresh whole blood, which provide information similar to aPTT, evaluating the intrinsic and common pathways. Unlike the aPTT, the ACT may be mildly to moderately prolonged in patients with severe thrombocytopenia and some thrombopathies [27]. The ACT is the time to detection of a fibrin clot in a whole blood sample after contact activation occurs [27].

Hereditary factors deficiency. (Brooks, 2010)

FACTOR DEFICIENCY	ABNORMAL SCREENING TEST RESULTS	BREEDS
Factor II (prothrombin)	aPTT, PT	Boxer, Otter hound, Cocker Spaniel
Factor VII	PT	Alaskan Klee Kai, Beagle, Deerhound, Alaskan Malamute, Schnauzer; domestic short haired cat
Factor VIII (haemophilia A)	aPTT	Any breed (German Shepherd [primarly], German shorthaired pointer, Labrador and Golden Retriver) and mixed breed dogs; domestic short-haired cats
Factor IX (haemophilia B)	aPTT	Any breed, mixed breed dogs, any breed and domestic short-haired cats
Factor X	aPTT, PT	Cocker Spaniel, Jack Russell Terrier; domestic short-haired cats
Factor XI	aPTT	English Springer Spaniel, Kerry Blue Terrier, Weimaraner and Great Pyrenees; domestic short-haired cats
Factor XII	aPTT	Miniature Poodle, Standard Poodle, German shorthaired pointer, Shar Pei; Domestic short-haired cats and domestic long-haired cats, Siamese and Himalayan.

Flow chart summarizing the use of screening tests of primary and secondary hemostasis in the investigation of a bleeding diathesis. (Ag= antigen)



From: **Compendium of Diagnosis of Bleeding Disorder**

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1.5.3 Test of Fibrinolysis

1.5.3.1 Fibrinogen

Fibrinogen is an acute phase soluble plasma glycoprotein synthesized in the liver and is a marker of the common pathway. Its biosynthesis increases with inflammation, stress, or infection due to the acute phase response. It is increased during malignancy, pregnancy, and in humans being, increasing age and female sex [36, 37].

Fibrinogen defects may be qualitative (dysfibrinogemia) or quantitative (hypofibrinogemia or hyperfibrinogemia), congenital or acquired such as in the case of hemodilution, blood losssor consumption, DIC or sepsi [36,37].

The fibrinogen value is obtained with the Clauss method by measuring the time taken by fibrinogen to convert into fibrin following the addition of a standard thrombin concentration to plasma; the time obtained is then converted into a concentration value through the use of appropriate tables [36].

1.5.3.2.Thrombin time (TT)

Thrombin Time (TT) is a surrogate test for fibrinogen because provide information about the reactivity of fibrinogen to exogenous thrombin. In this test thrombin cleaves fibrinogen into its fibrinopeptide A and B resulting in fibrin monomers that polymerize into the fibrin clot [27].

The TT is quick and easy to perform; the manual method is performed using 0.2 ml of platelet-poor plasma, warming to of 37 ° C in which 0.2 ml of human or bovine thrombin are added. The time needed to clot formation represents the TT and if prolonged, a congenital or acquired quantitative (fibrinogen definciency) or qualitative (fibrinogen dysfunction) defects should be considered [27].

1.5.3.3 Fibrin Degradation Products (FDP)

Fibrin degradation products (FDP) are generated by the action of plasmin on fibrinogen, soluble monomers of fibrin and insoluble crosslinked fibrin. They indicate plasmin activation but are not specific for crosslinked fibrin degradation. High levels of FDPs have been associated most commonly to DIC, especially when accompanied by increases in PT, aPTT, decreased in platelet count, AT activity and fibrinogen concentration. However increased in FDPs concentration have also been observed in canine patients affected by cerebral haemorrhage, warfarin toxicity, liver disease (due to a decrease in hepatic clereance) and in most disease associate with a prothrombotic state [38].

1.5.3.4 D-dimers

D-dimers represents the cross-linked degradation products that are produced by the action of plasmin on insoluble crosslinked fibrin. They have a half-life of approximately 5 hours and reflect recent or ongoing fibrinolysis. D-dimers can be increased in any disease that results in the generation and

degradation of fibrin and are most commonly associated with DIC and thromboembolic disease states but are not specific for these disorders [39].

1.6 VISCOELASTING COAGULATION TESTING: TECHNOLOGY, APPLICATIONS AND LIMITATION

Our current understanding of *in vivo* coagulation highlights the limitations of the standard coagulation tests, such as PT and aPTT. In particular these tests do not incorporate cellular elements and membrane surface, which are actively involved during clot formation, and only provide information on isolated components of the coagulation cascade. Although allows to evaluate the specific component of the enzymatic cascade these tests overlook such factors essential for the evaluation of global coagulation as rate of clot formation, the clot strength and the degree of clot dissolution [40].

In 1889 Hayem suggested that quantification of changes that occur in blood viscosity during clot formation could be used as the basis for a test that investigate coagulation function. Blood clot has both elastic and viscous properties and technological methods have been developed to assess these properties [41]. The first coaguloviscometer, a tool designed to detect viscous changes in blood during clot formation, was introduced by Koffman in 1910. Since then, significant improvements have been occurred in the evolution of this technology.

Viscoelastic point-of-care (POC) devices provide *in vitro* assessment of global coagulation, from beginning of clot formation to fibrinolysis. Standard coagulation, such as PT and aPTT end when the first fibrin strands are developing in the sample, whereas viscoelasting coagulation test begin at this point and continue through clot development, retraction and lysis [40, 42]. Moreover this technology provides information about the kinetics of clot formation (the time needed fot clot to form), the mechanical properties of the clot (tenile strength), and the time required for its dissolution (fibrinolysis). The kinetics of clot determine the adequacy of the amount of factor available for clot formation while the tensile strength provides information about the ability of the clot to achieve hemostasis [40, 42].

The possibility of using whole blood sample in viscoelasting testing is an additional advantage for the evaluation of coagulation system; in fact whole blood contains cells with a phospholipid surface required for enzymatic reaction. It also provides platelets that further actively participate during coagulation by releasing granule contents and providing a surface for amplification and propagation of clotting process [14].

Viscoelastic technology was originally designed as a POC monitoring method using native whole blood run within minutes of collection. Since in the laboratory setting this approach is not practical citrated sample are frequently used.

Clinically relevant benefits from the use of viscoelastic tests have been reported in both human and veterinary medicine; improvements have been observed in the management of hemostasis during

surgery, decrease in the use of blood products, more accurate anticoagulation management and a rapid screening test for hypercoagulability [43, 44, 45]

Three instruments are currently used: Sonoclot, the thromboelastography (TEG) and thromboelastometry (ROTEM). The Sonoclot and ROTEM measure changes in impendance to movement of a vibrating probe immersed in a blood sample, whereas TEG utilizes and oscillating cup with a fixed probe or piston. As the clot start to form, fibrin strands develops between the pin and the wall cup. This torsion is converted into an electrical signal and graphically represented [40]. All 3 instruments measure the rate of fibrin formation, clot strength and clot lysis.

Thromboelastography and ROTEM are the most used instruments in veterinary medicine [40, 42].

1.6.1 Thromboelastography

Thromboelastography is an *in vitro* diagnostic technique initially introduced in Germany in 1948 by Hartet [46]. The technique is based on continuous detection and recording of changes in the viscoelastic properties of whole blood while it clots. Thromboelastographic tracing may better reflect the cell based model of coagulation and thus better predict the kinetics of coagulation compared with routine plasma based assays [42].

Thromboelastography consists of a pin held by a torsion wire and immersed in a cup heated to 37 °C, containing an aliquot of 360 µl sample of citrated blood, that oscillates for 10 seconds a cycle [40, 42]. As the clots starts to form, fibrin strands develop between the pin and the inner wall of the cup. This results in a rotation of the immersed pin that is transmitted to the torsion wire and converted, by means of a transducer, into an electrical signal that is displayed as a TEG tracing (Figure 6). As the clot lysis occurs the bonds between the pin and the clot are broken resulting in increased movement of the pin [40].

Thromboelastographic analysis is performed using samples of citrated whole blood with the addition of specific reagent.

A graphical tracing of viscoelastic changes displays initial fibrin formation (reaction time, *R*), kinetics of fibrin formation and development of the clot (*K* and *α* angle), and maximal strength of the fibrin clot (maximum amplitude [MA]). Two additional measurements representing fibrinolysis or clot lysis at 30 and 60 minutes (CL 30, CL60) indicate clot stability.

Reaction time (*R)* is the time in minutes from clot initiation until the first fibrin polymers are produced and the amplitude reaches 2 mm (that correspond to the point at which standard plasma-based clotting assays end). R is influenced by plasma concentration of coagulation factors and therefore a prolongation in R is correlated to deficiencies in coagulation factor, whese a shortening R could indicate a hypercoagulable condition.

K is the time in second fom the end of *R* until an amplitude of 20 mm is reached and represent the speed of clot formation. Alpha (*α*) represents the angle, expressed in degree, tangent to the curves as *K* is reached and represent the acceleration/kinetics of fibrin formation and crosslinking. K and *α* are

influenced by the activity of FII, FVII, platelet count and function, thrombin, fibrinogen concentration and HCT [40, 42].

The maximum amplitude, expressed in millimeters, is the maximum amplitude reached by clot and reflects maximal clot strength; is affected by fibrinogen, platelet count and function, thrombin, FXIII and HCT [40, 42, 47].

Clot lysis, represented by CL 30 and CL 60, indicates the percentage of lysis that has occurred at 30 and 60 minutes, respectively, after MA has been reached and provides information about clot stability. High CL percentage represents a rapid fibrinolysis.

Thromboelastography consist in 2 channels for cups that can simultaneously run.

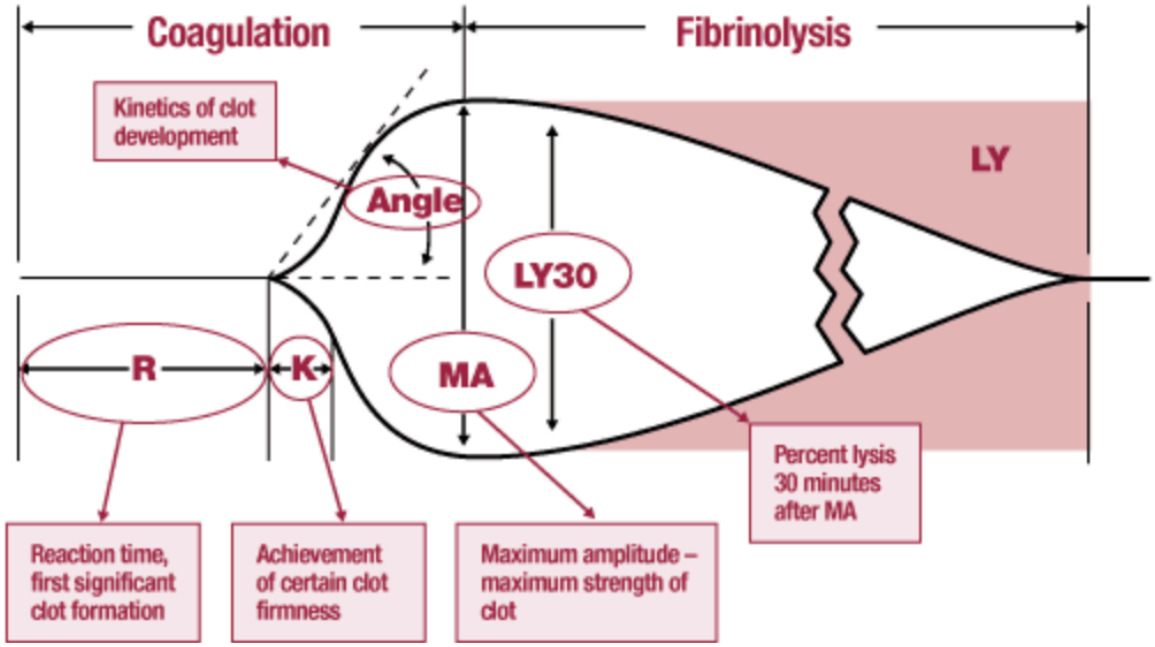


Figure 6: Example of thromboelastografic tracing:
Parameters of the thromboelastografic tracing are indicated in the upper part:
R: reaction Time; **K** Kinetics time; **MA**: Maximum Amplitude; **a angle**.

1.6.2 Thromboelastometry

Rotational thromboelastrography, termed thromboelastometry (ROTEM), also assess the viscoelastic properties of whole blood under low shear condition. The ROTEM represent the modification of a classic thromboelastography. Like the TEG, ROTEM provide information about the global hemostatic function from the beginning of clot formation through clot retraction and fibrinolysis.

The technical aspects of the ROTEM are slightly different from those of the TEG. There are 4 cylindrical cups and an optical detector system that detects the signal suspended in the blood sample cup (Figure 7-8). Differently from TEG, in ROTEM the cup is stationary and the pin oscillates. As a fibrin form between the cup and the pin, the impendence of the rotation of the pin is detected. As the blood clots the extent of the pin's oscillation is reduced; the movement of the pin is evaluated by an

optical detection system and the data obtained are processed and analysed by a computer through dedicated software (Figure 7). A ROTEM graphical tracing is recorded (Figure 10a,b). The 4 channels can be used simultaneously allowing multiple specimens to be sampled or the execution of several tests on the same samples [40, 42]. In addition, the ROTEM is equipped with an electronic pipette that permits consistency of dispensing the samples.

Figure 7: Thromboelastometry

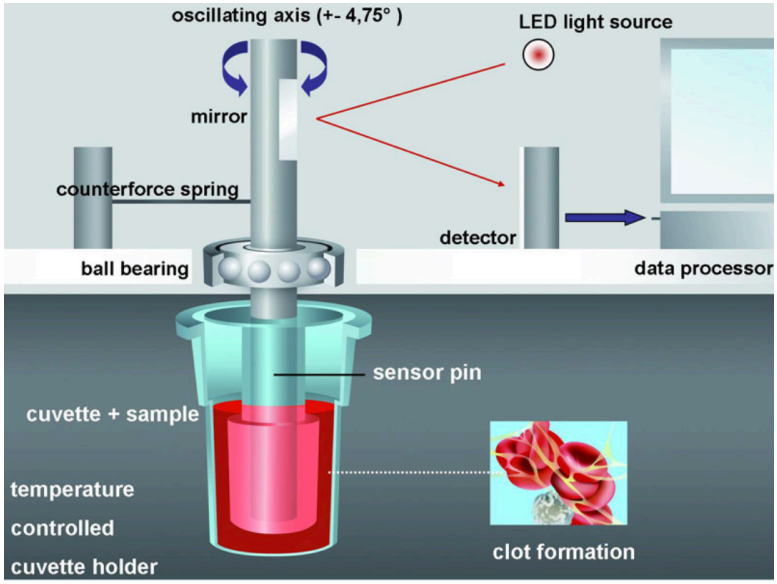


Figure 8: A whole blood sample is placed into a cuvette and a cylindrical pin is immersed. The pin is rotated by a spring to the right and left. As long as the blood is liquid, the movement is unrestricted. When blood start to clotting, the clot increasingly restricts the rotation of the pin with rising clot firmeness. This kinetics is detected by an optical detection system and the data obtained are processed and analysed by a computer through dedicated software and transform into to the typical curves (ROTEM tracing) and numerical parameters.
From: https://www.rotem.de/en/methodology/thromboelastometry

ROTEG and ROTEM evaluate the same variables, but using different terminology.

Graphical displays of viscoelastic changes indicate initial fibrin formation (clotting time, CT), the kinetics of fibrin formation and clot development (clot formation time [CFT]; α angle), the maximum strength of the fibrin clot (maximum clot firmness [MCF]), and fibrinolysis at 30 and 60 minutes (clot lysis. LY30; LY60) [40, 42].

The clotting time (CT) describe the time in seconds from clot initiation until the fibrin polymers are produced and the amplitude reaches 2 mm and is an indicator of plasma coagulation factor activity. This parameters is mainly influenced by plasma concentration of coagulation factors (intrinsic or extrinsic pathway depending on the activator used for test), and by coagulation inhibitors, such as AT, or drugs that prevent the thrombin formation or directly inhibit coagulation factor [48]. Clotting formation time, measured in seconds, is the time from initiation of clotting (2 mm) until an amplitude of 20 mm is reached and correspond to initial activation of platelets and fibrinogen; CFT and α angle provide information about the kinetics of clot formation and are mainly influenced by platelet count and activity and fibrinogen concentration [48].

Alpha angle is defined as a tangent towards the coagulation curve through the 2 mm and describe the kinetics of clot formation. This parameter is expressed in degree and represents the angle between the inclination of the tangent and the baseline [48]. Maximum clot firmness (MCF), expressed in millimeters, represents the maximum amplitude reached by the clot, reflects maximal clot strength and stability, and is affected by fibrinogen, platelet count and function, thrombin, FXIII, and hematocrit (Hct) (82) Maximum clot firmeness is the most important parameter of thromboelastometry because provide information about the quality and stability of the clot and, therefore, a reduced MCF indicates a decreased of clot dtability with consequent risk of bleeding [48].

Other values that can be calculated using ROTEM measurement include maximum clot elasticity (MCE). The MCE is a calculated value derived from MCF: MCE= (MCF * 100) / (100-MCF) [40]. This parameter describes more accurately the mechanical properties of the clot; above a certain level, small increases in MCE indicate over-proportional clot resistance, which may be associated with an increased of thromboembolic risk [48].

Additional calculated parameters include the platelets contribution to maximum clot elasticity ($MCE_{platelets}$), which evaluate the contribution of platelet component to clot strength and are calculated using the following formula: $MCE_{platelets}= MCE_{extem}-MCE_{fibtem.}$ [49].

The fibrinolysis is evaluated through the parameters LI30, LI45 and LI60 which evaluate the percentage of lysis of the clot respectively at 30, 45 and 60 minutes after the CT [40].

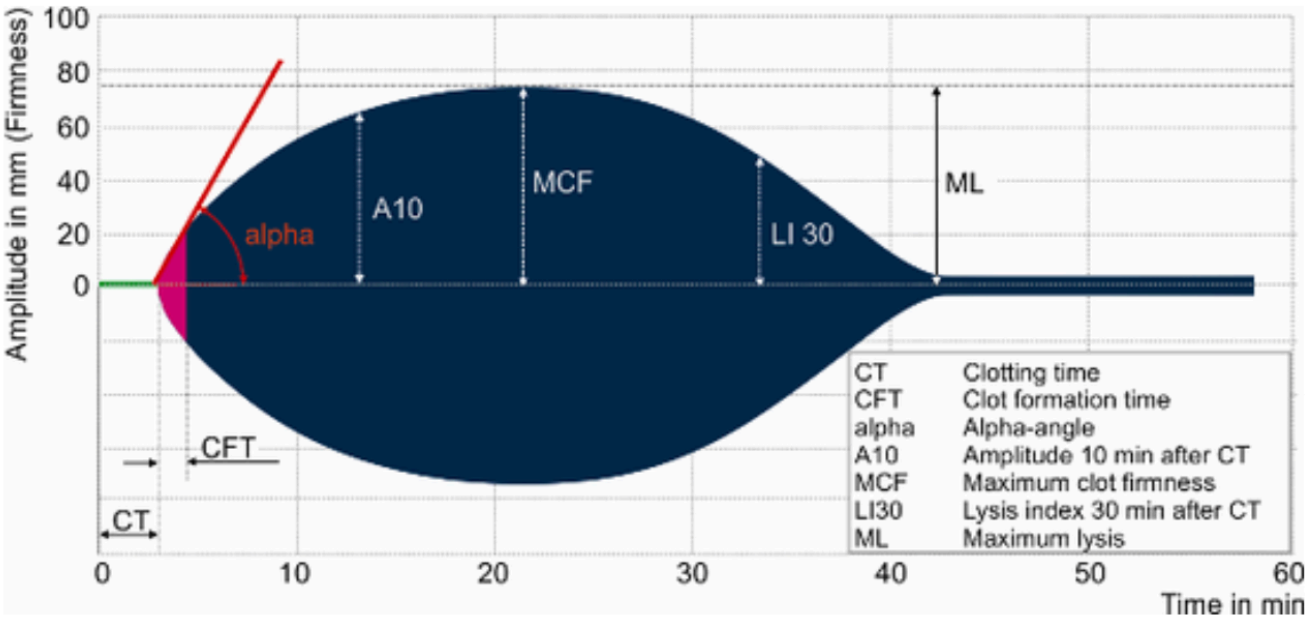


Figure 10a: Example of ROTEM tracing and ROTEM parameters.

CT: Clotting Time. Represent the plasmatic phase of coagulation and it's influenced by plasma concentration of coagulation factor of the intrinsic or extrinsic pathway depending on the profile that is being performed. The CT of the in-TEM profile it's influenced by plasmatic factors of the intrinsic pathway, whereas the CT of ex-TEM profile it's influenced by the plasmatic factor of the extrinsic pathway.

CFT:Clotting formation time, is the time needed for the clot to reach an amplitude of 20 mm and correspond to initial activation of platelets and fibrinogen; CFT and α angle provide information about the kinetics of clot formation and are mainly influenced by platelet count, activity and fibrinogen concentration.

MCF: Maximum clot firmness expressed in millimeters, represents the maximum amplitude reached by the clot, reflects maximal clot strength and stability, and is affected by fibrinogen, platelet count and function, thrombin, FXIII, and hematocrit

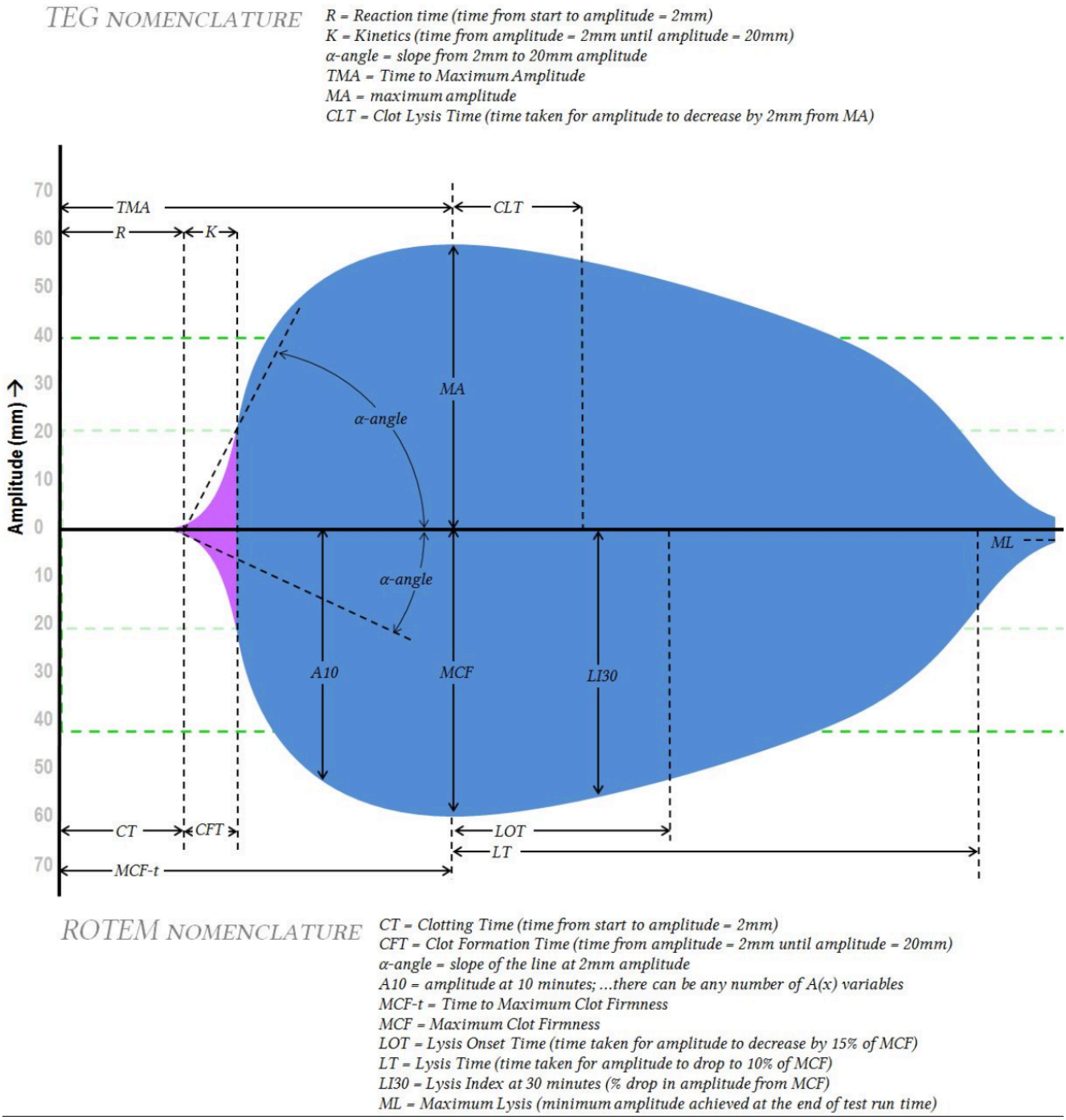


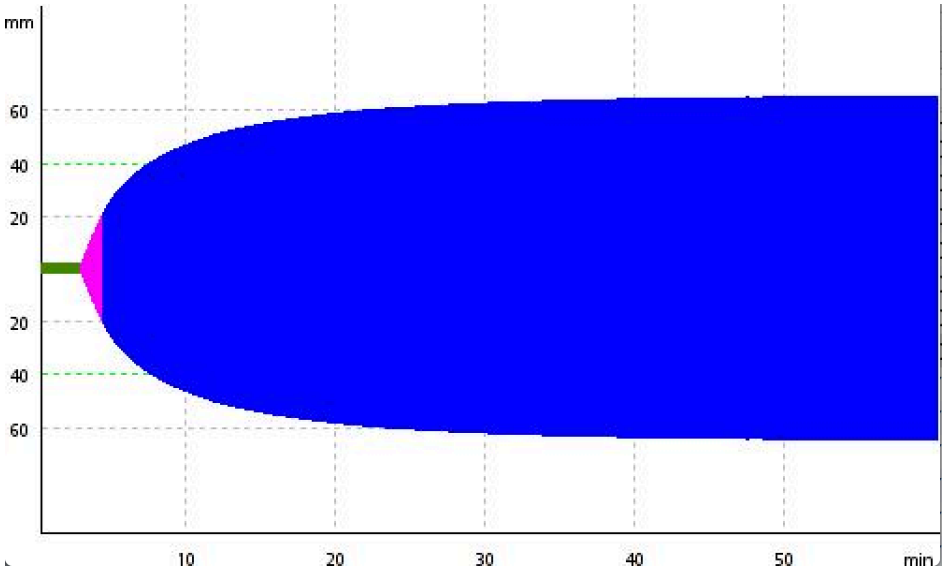
Figure 10b: comparison between TEG and TEM tracing and nomenclature.

ROTEM parameters are indicated below.

The use of the specific reagents allows to separately investigate the different pathway of the hemostasis (intrinsic and extrinsic pathway), reduce the reaction time, inhibit certain factor (such as heparin) and differentiate between the contribution of fibrinogen and platelet to clot formation.

The different profiles performed by means ROTEM analysis are shown below.

- **in-TEM profile** (Figure 11)**:** This profile is obtained by recalcifying the sample with the start-TEM® reagent; the use of specific in-TEM®, containing ellagic acid, allows the activation of the intrinsic pathway of coagulation and allows to evaluate the contribution the clot formation. This test is influenced by deficiency of intrinsic plasmatic factors (FXII, FXI, FIX and FVIII), deficiency of FXIII, administration of anticoagulant drugs (e.g. heparin), platelet, fibrinogen, fibrin polymerization and hyperfibrinolysis. In-TEM profile has low sensibility for slight inhibition of plasmatic factors and for inhibitor of primary hemostasis (e.g aspirin) [48].



(a)

(b)

Figure 11: (a) example of normal in-TEM profile in adult healthy dog;

(b) example of in-TEM profile in a patient affected by deficiencies of factor of the intrinsic pathway. A marked prolongation of CT is observed.

- **ex-TEM profile** (Figure 12): ex-TEM profile evaluates the extrinsic pathway of coagulation and is obtained, after the recalcification, by adding a specific ex-TEM® reagent containing tissue factor (thromboplastin). Ex-TEM profile is affected by deficiency of plasmatic factors (FVII, FX and FV), platelets deficiency, alteration of fibrinogen and fibrin polymerization and hyperfibrinolysis [48].

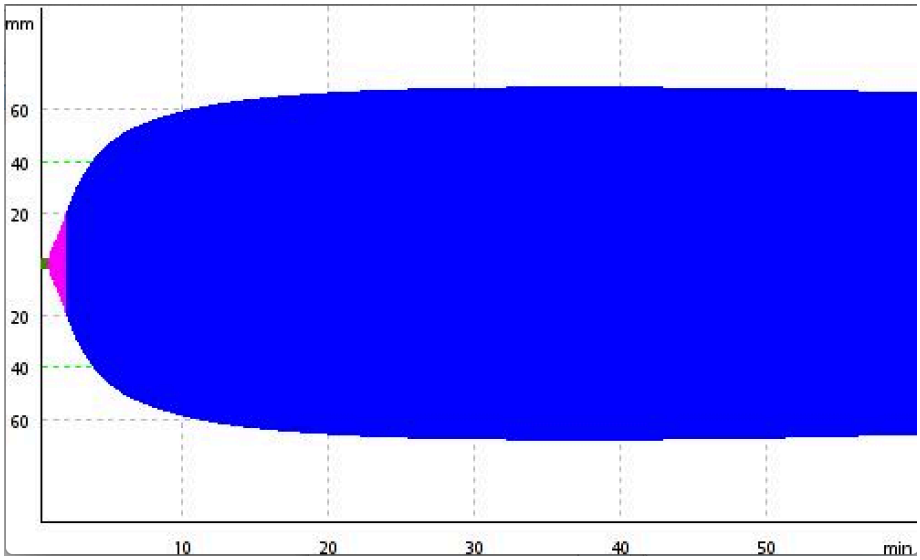


Figure 12: example of normal ex-TEM profile in adult healthy dog

- **fib-TEM profle** (figure 13): The fib-TEM profile allows to selectively evaluate the contribution of functional fibrinogen to clot formation. In this test coagulation is activated by the tissue factor (ex-TEM® reagent) and subsequently in the sample is added cytochalasin D (fib-TEM® reagent), a platelet inhibitor. Therefore the ROTEM tracing represent exclusively the fibrin component of the clot and its consistency is depends on fibrinogen concentration; defects in fibrin polymerization can determine a pathological value despite a normal fibrinogen concentration [48]. In this test the most important parameters is the MCF (maximum clot firmness); the difference in MCF between fib-TEM and ex-TEM provide an indirect measurement of platelet contribution to clot stability [48].

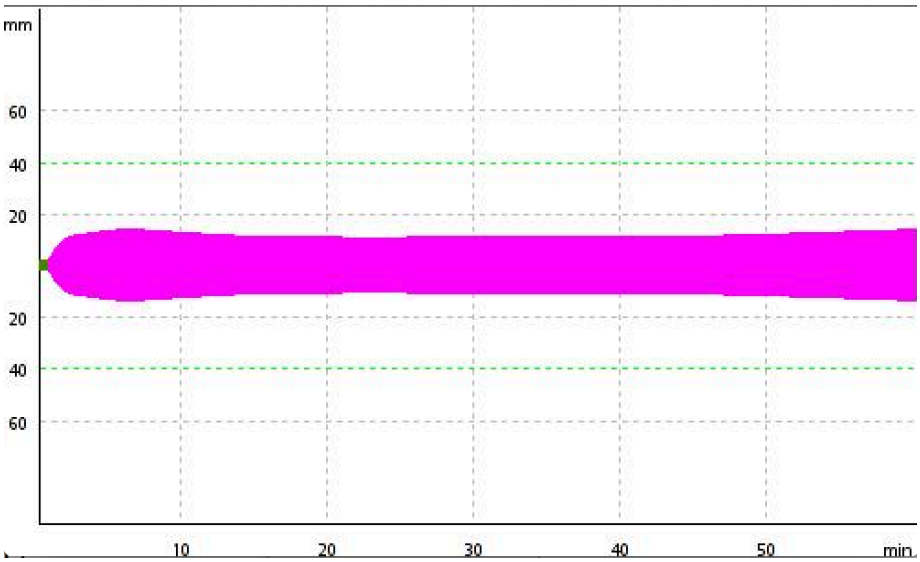
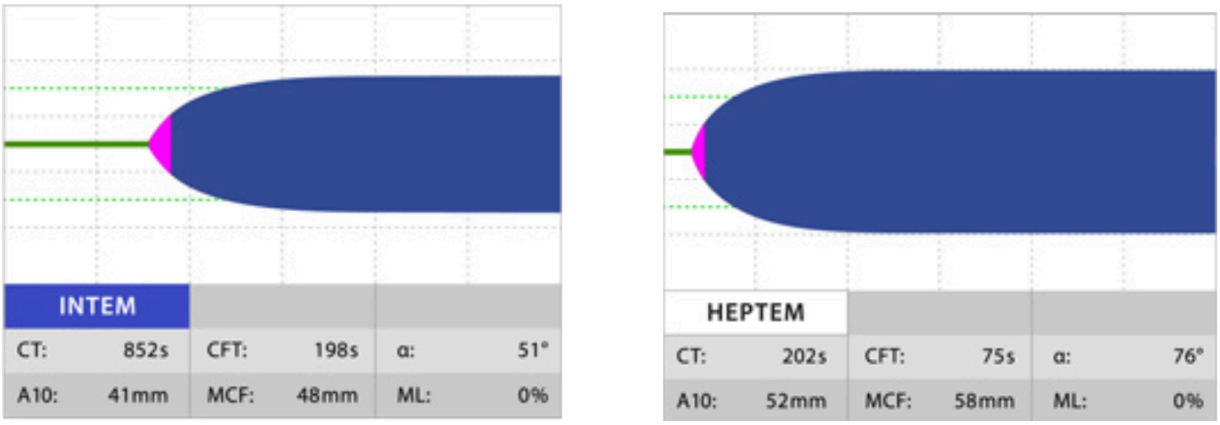


Figure 13: example of normal fib-TEM profile in adult healthy dog.

- **hep-TEM profile** (Figure 14): In hep-TEM profile coagulation is activated as in in-TEM profile with the addition of the specific reagent hep-TEM® containing the enzyme heparinise I, a heparin degrading enzyme. This profile allows to evaluate the coagulation without the influence of treatment with heparin, and in association with the in-TEM profile can assess the treatment effectiveness. If the patients has been treated with heparin and the difference between hep-TEM and in-TEM is very small, a deficiency of antithrombin III (ATIII) shoud be considered. The deficiency of ATIII is one of the most important factor of heparin resistence [48].



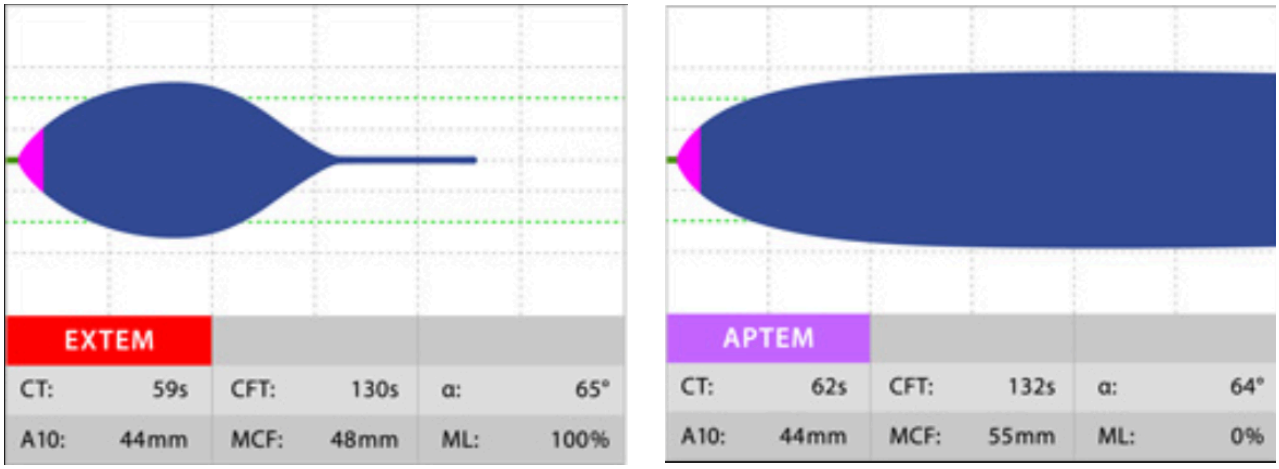
(a) **(b)**

Figure 14: (a) in-TEM profile with the influence of heparin;

(b) hep-TEM profile. The addition of the specific reagent hep-TEM containing the heparinise enzyme (enzyme degrading heparin) allows to evaluate the hemostatis in patients receiving anticoagulant therapy.

From: Results interpretation ROTEM delta and ROTEM sigma.

- **ap-TEM profile** (Figure 15): the ap-TEM assay consists in activating the extrinsic pathway of the hemostasis through the ex-TEM® reagent and the subsequent addition of the specific reagent ap-TEM® containing aprotinin, a fibrinolysis inhibitor. It is used as a screening test for detect the presence of hyperfibrinolysis [48].



(a) **(b)**

Figure 15: (a) ex-TEM profile in a patient affected by hyperfibrinolysis;
(b) ap-TEM profile.

PART II: COLLOIDAL SOLUTION

2.0 INTRODUCTION

Colloidal solutions are a kind of fluid widely used during fluidtheraphy in human and veterinary medicine. These solutions contain large molecules that cannot cross intact vascular barriers [50, 51]. The typical mean molecular weight of these compounds is greater than the capable of passing through capillary pores (60 to 80 kDa), and therefore, once infused, they largely remain within the vascular space and act by expanding and maintaining intravascular volume [50, 51].

Thanks to their properties, these solutions are primarily used in clinical practice for the management of hypovolemic shock, in order to increase the circulating blood volume, and during hypoalbuminemic states in order to increase and support the plasma colloid osmotic pressure [50, 51].

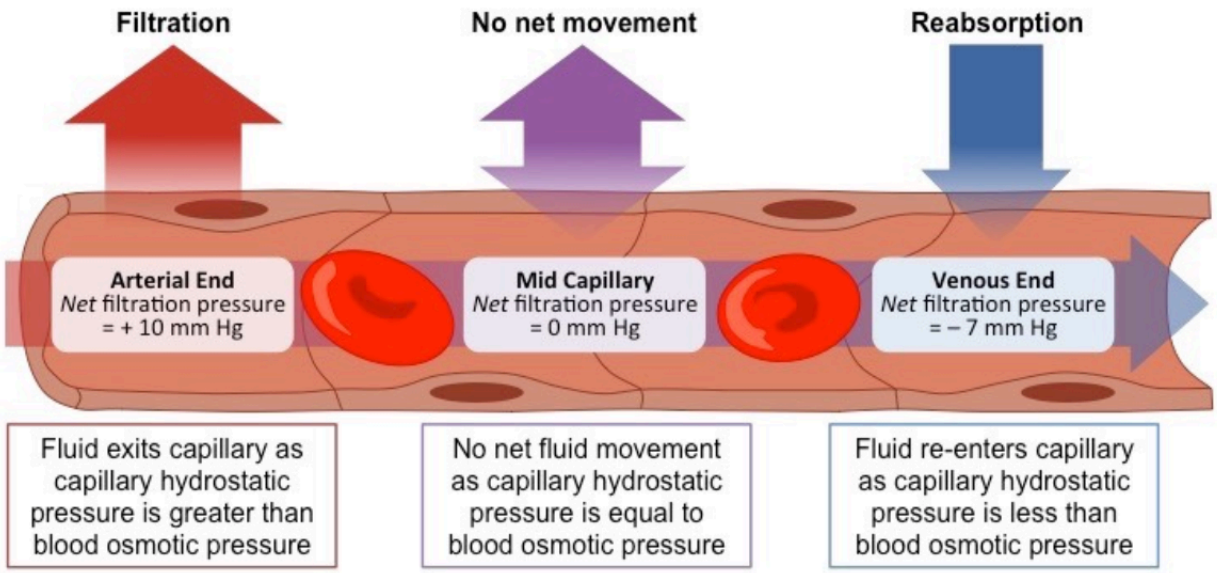
The fluid movement across a semipermeable membrane was first describe by Ernest Starling in 1896, and depend on both hydrostatic and oncotic forces acting on either side of a semipermeable membrane, such as a vascular endothelium, and were describe by Starling-Landis equation [52]. (Figure 15).

The plasma colloid osmotic pressure (COP: πc) is the forces that opposes fluid filtration out of the vascular space and largely depends on plasma albumin concentration and, to a lesser degree, on other plasmatic protein [53]. The plasma hydrostatic pressure (Pc) is the force responsible for fluid filtration out of the vascular space and depends on arterial blood pressure and vascular resistance [54]. The interstitial COP (πt), which contributes to fluid filtration out of the vessel, depends on interstitial albumin concentration.

Transvascular exchange depends on a balance between hydrostatic and oncotic pressure gradients. Fluid is filtered to the interstitial space under a dominant hydrostatic pressure gradient at the arteriolar portion of capillaries, and it was believed that it is absorbed back under a dominant COP gradient at the venular end.

Based on the Starling equation, a constant outward movement of fluid occurs on the arterial side of the capillary while a slight inward movement occurs on the venous side due to differences in hydrostatic pressure [55, 56].

Any imbalance between these forces or changes to the coefficient of filtration, such as occurs during hypoalbuminemia, capillary leak or fluid overload, results in fluid movement from the vascular space to the interstitium and/or in third space (pleural, peritoneum space), resulting in edema formation.



$$J_v = K_f([P_c - P_i] - \sigma[\pi_c - \pi_i])$$

Figure 15 : Modified Starling-Landies equation defining the driving forces for fluid movement across the normal continuous capillary membrane.

- J_v Net fluid flux
- K_f Filtration coefficient
- P_c Capillary hydrostatic pressure
- P_i Interstitial hydrostatic pressure
- σ Reflection coefficient
- π_c Capillary oncotic pressure
- π_i Interstitial oncotic pressure

Revision of Starling's equation

In recent years revisions of the Starling's law have been necessary due to some shortcomings [52]. In particular, in 2004, Adamson and colleagues showed that the effect of interstistial COP on transvascular fluid exchange is much less than predicted by the standard Staring equation, which therefore has to be revised [52, 57]. It is now established that non fenestrated capillaries normally filter fluid to the interstitial space through-out their length. Absorption through venous capillaries and venules does not occur [57]. Capillary COP opposes, but does not reverse, filtration. Most of the filtered fluid returns to the circulation as lymphs [57, 58, 59].

The discovery of a protein layer on the luminal surface of the endothelium has place a new focus on how the fluid movement occurs across the vascular wall [52, 55]. The glicocalyx is a web of negatively

charged membrane-bound glycoproteins and proteoglycans, which covers the entire endothelial surface [52, 55]. (Figure 16-17). Outwardly shifting proteins are bound in this filter-like structure, which becomes loaded with plasma proteins [52, 55]. This layer, called as endothelial glicocayx layer (EGL), separate plasma from a “protected region” of the subglicocalix space, which is almost protein free. Subglycocalix COP replace the interstitial COP as a determinant of trancapillary flow. Therefore it is the COP difference across the glycocalix that opposes fluid exit and maintains vascular integrity [52].

The glycocalyx has a transmural vascular barrier function, anti-inflammatory property, and impedes blood cells and plasma elements to directly keep in touch with the endothelium [51]. Based on the experimental study, every condition that causes alteration of the endothelial glycocalix layer (such as inflammation, sepsis, ischemia/reperfusion, increased natriuretic peptide), mainly through shedding of glycosaminoglycans, results in platelet aggregation, leukocyte adhesion, increased vascular permeability and edema formation [52, 59].

One aspect of the endothelial glycocalyx relevant to fluidtheraphy is that iatrogenic hypervolemia leads to degradation of this layer. During acute hypervolemia, atrial natriuretic peptide is secreted which causes rapid shedding of the glycocalyx with subsequent increase in permeability. The understanding of the important role of this layer and the consequences of its destruction has imposed new consideration regarding fluidtheraphy and volume overload [52, 59].

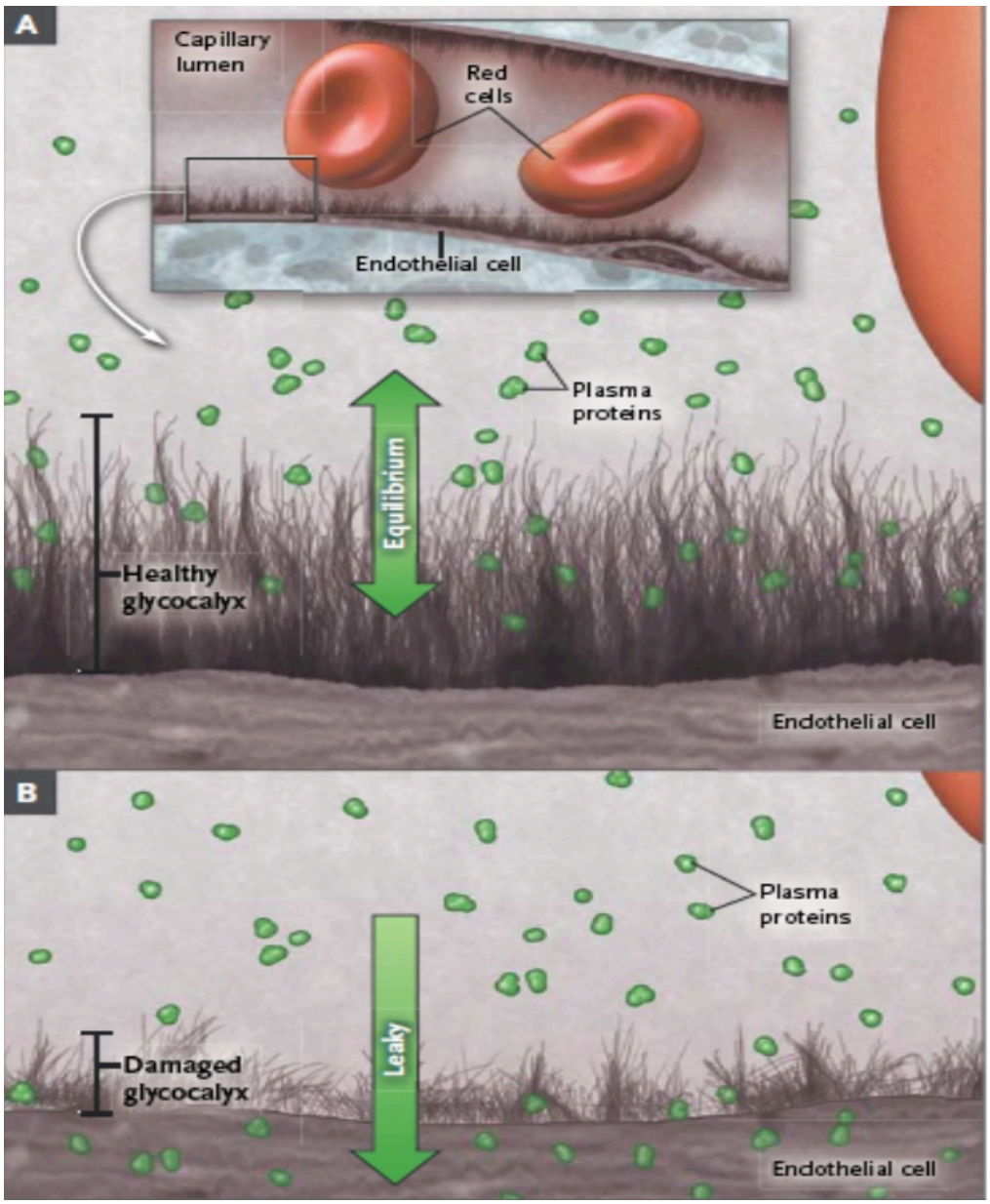


Figure 16: Schematic representation of the endothelial glycocalyx. Panel A shows a healthy maintaining a transcapillary equilibrium. Panel B shows a damage endothelial glycocalyx, movement of plasma protein into the interstitium and subsequent edema formation.
From: Myburgh JA, Mythen MG. Resuscitation fluids. N Engl J med 2013; 369(13): 1243-1251.

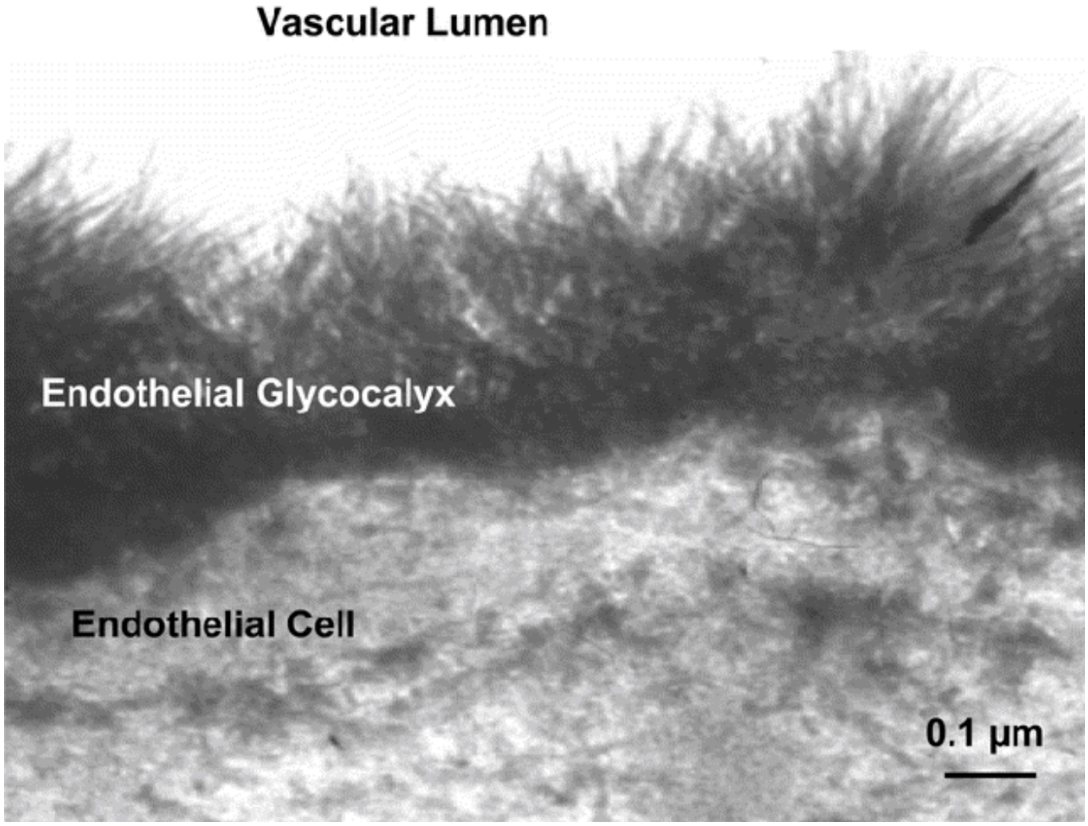


Figure 17: Representation by electronic microscope of glycocalix.
From: biofundation.org/tag/glycocalyx.

TYPE OF COLLOIDAL SOLUTION

Colloidal solutions used during fluidtheraphy are subdivided into natural and synthetic colloids; natural colloids include human albumin and blood products (, while synthetic colloids include hydroxyethyl starch, dextran and gelatins.

2.1 SYNTHETIC COLLOIDS

Artifcial colloids were developed as an alternate resuscitation fluid to albumin [50]. Hydroxyethyl starch (HES) solutions were first introduced in 1970s and are the most common synthetic colloid currently utilized in human and veterinary medicine. The remaining 2 classes of synthetic colloids, gelatins and dextrans, are less frequently used. Hydroxyethyl starch solutions are readily available and inexpensive, provide rapid volume expansion, and have a volume of distribution limited to the intravascular space.

2.1.1 Hydroxyethyl starch

Hydroxyethyl starch comprises a group of synthetic polymers routinely used in veterinary anesthesia and critical care medicine to maintain blood volume, counteract anesthesia-induced hypotension, to

resuscitate patients with hypovolemic shock and to support intravascular COP during hypoalbuminemic states [50, 51]. HES can be administered as a bolus, in order to increase intravascular volume during hypovolemic shock, or as constant rate infusion (CRI), in order to increase and support plasma COP during hypoalbuminemic states. The recommended dosages in veterinary medicine are largely extrapolated by human literature and consist in administration of 20-50 ml/kg/die, when administered as bolus, or 1-2 ml/kg/h if administered as a CRI [50, 51, 60].
Hydroxyethyl starch are synthetized by amylopectin a natural starch derived from either waxy maize or potatoes, which is hydroxylated in order to prevent rapid degradation by circulating α amylase and thus increase the intravascular persistence [61]. This is achieved by substitution of the hydroxyl (-OH) groups on the glucose units with hydroxyethyl ($-OCH_2CH_2OH$) groups [50].

2.1.1.1 Chemical Characteristic of HES solution

Hydroxyethyl starch solutions are classified based on their raw material, concentration, mean molecolar weight (M_w), molar substitution (MS) and C2/C6 ratio [61].
Two different raw materials have been used to extract amylopectin. The first products manufactured were made from waxy maize, composed of 95% amylopectin and, since 1992, products were also manufactured from potato-based starch, composed of 80% amylopectin and 20% amylose [61]. Due to the differences in composition and fine structure with the regard to the degree of branching, C2/C6 ratio, intrinsic viscosity, and positioning of the hydroxyethyl groups, waxy maize and potato starch based HES solution are not bioequivalent and cannot be assumed to have identical properties [61].
The percentage concentration of HES solution mainly affects the initial immediate volume effect after administration. In addition to the concentration of HES solution, the patient intravascular volume status and COP will play a role in how much vascular volume expansion occur after intravascular infusion [50].
Morover, the oncotic effect actually depends on the number of oncotically active particles and not so much to the concentration per se. Thus, a product with molecules of low M_w is likely to exert grater COP at similar concentration than products with molecules of high M_w [50, 52, 61].
Hydroxyethyl starch solution can be represented by the number-average (M_n) or the weight average (M_w); the M_n represent the total weight of polymers divided by the number of molecules, whereas M_w determines what fraction of the total mass of the solution each molecular size contributes and is typically the number that is represented on the HES packaging [50]. Osmotic effectiveness of HES depends on the number of particles in solution per unit volume. These characteristics affect the ability of HES solution to maintain intravascular volume, the rapidity of excretion, the degree of tissue accumulation and their reported side effects [50, 51].
Hydroxyethyl starch molecules with a MW below the renal threshold (45-60 kDa) are rapidly excreted in the urine, thus reducing the circulating number of HES particles and decreasing the osmotic effect of the circulating HES. The higher M_w molecules (greater than the renal threshold) are progressively hydrolysed in the plasma by α-amylase into two or more smaller molecules. This process provides an

on-going supply of osmotically effective plasma molecules until the molecules are hydrolysed to a size below renal threshold.

Based on the M_w HES products are divided into first generation solution with $M_w >$ 400 kDa, second generation solution with M_w of 200 to 400 kDa and third generation solution with $M_w <$ 200 kDa [61].

Besides the concentration and the M_w, which account for many of the effect of HES, the degree of MS and the substitution pattern (C2/C6 ratio) play a key role in the pharmacokinetics of HES products [50, 61]. Natural amylopectin is chemically modified by hydroxyethylation at the glucose subunits C2, C3 and C6 in order to increase solubility and inhibit degradation by α amylase [61]. The average number of hydroxyethyl residues per glucose subunit defines the MS, with higher substitution associated with delayed enzymatic degradation and longer persistence in the intravascular space [50]. Based on the MS, HES solution are categorized as **heta**starch (MS=0.7), **hexa**starch (MS=0.6), **penta**starch (MS=0.5) and **tetra**starch (MS=0.4) [50].

Finally the pattern of substitution (C2/C6 ratio) describes the locations of hydroxyethyl residues on the glucose subunit. The C2 and C6 carbon atoms are the main target for substitution with a lesser amount attached onto the C3 atom (Figure 18). Hydroxyethyl groups positioned on the C2 atom inhibit the access of α amylase to the linking bonds more effectively the substitution on atom C6; therefore a high C2/c6 ratio will favour a slower breakdown of the HES molecules.

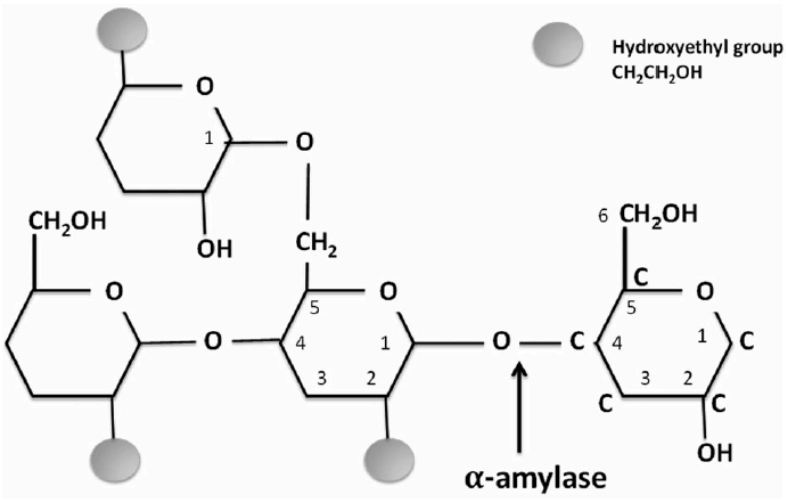


Figure 18: Structure of HES molecule. A segment of hetastarch is shown as amylopectin with hydroxyethyl groups substituted for hydroxyl groups at C2 and C6. Alpha-amylase, an endo amylase, hydrolyzes bounds within the molecular structure. The more substitution there are at the C2 position, the more difficult it is for the amylase to reach the bond.

From: From: Glover PA, Rudloff E, Kirby R. Hydroxyethyl starch: a review of pharmacokinetics, pharmacodynamics, current products an potential clinical risk, benefit and use. J Vet EM Crit Care 2014; 24(**6**): 642-661.

2.1.1.2 Distribution and clearance:

Has been demonstrated that the elimination half-life of HES is superior in healthy dogs compared to people. This difference has been attributed to the fact that dogs have a higher plasma α amylase concentration than humans being [50]. Renal excretion accounts for approximately 70% of the total HES elimination [50].

Secondary routes of HES elimination include extravasation and uptake with transient storage in the reticuloendothelial (RES) cell of the liver, spleen and lymph nodes. Hydroxyethylstach starch deposition in dogs has been demonstrated by histopathology in intravascular and interstitial space; hepatocytes; proximal renal tubular cell; in the RES of the liver, spleen and lymph nodes post infusion [62]. Whit time, HES molecules are catabolized by proteolytic enzymes in the RES cells or gradually redistributed into the circulation and excreted [50]. A third, minor route of elimination is through excretion in bile.

With increasing MS there is increased tissue storage of HES molecules; therefore repetitive administration of HES with MS >0.4 results in plasma e tissue HES accumulation. Newer HES generation (third HES generation) have been developed in order to reduce the persistence of HES molecules in the intravascular space, reduce the tissue storage and possibly the negative side effects of HES [61].

2.2 REPORTED SIDE EFFECTS

The most significant adverse effects reported with the use of synthetic colloids include acute kidney injury (AKI), coagulation disorders, anaphylactoid reaction, pruritus and increased mortality [63]. Specific patient population, particularly septic patient, appear to be at higher risk of adverse effects, and administration of artificial colloids to these patients may be associated with increased mortality.

Intracellular HES accumulation is implicated in the pathogenesis of major complication and organ dysfunction.

Significant debate surrounds the safety of HES solution with the most recent clinical data prompting the ban of their use in Europe and guidelines recommending against their use in certain patients [64].

2.2.1 Acute kidney injury

The pathophysiology of HES-associated AKI is not fully understood, and several mechanisms have been proposed to explain the tubular injury. Hyperoncotic – mediated ischemic injury, HES uptake by the renal interstitial reticuloendothelial system and osmotic nephrosis have been proposed pathophysiologies [50, 51, 65].

Hyperoncotic AKI is characterized by an increase in intravascular COP due to unfiltered, osmotically active colloid molecules coupled with low renal perfusion pressure in the glomerular arterioles is proposed to cause alteration of intraglomerular colloid osmotic forces, leading to the rduction or cessation of glomerular filtration [65]. Experimental models have demonstrated HES-induced renal

interstitial cell proliferation and macrophage infiltration and these changes are belived to contribute to the development of AKI. Lastly HES solution can induce osmotic nephrosis, a process of vacuolizatioin and swelling of proximal renal tubular cells. Preferential uptake of HES molecules occurs in proximal tubular luminal epithelial cells via pinocytosis, and intracellular lysosomal storage can lead to accumulation on intracellular water, cytoplasmatic swelling, and altered cellular integrity and function, culminating in tubular damage and the clinical syndrome of AKI [65, 66]. Distal tubules and collecting ducts are usually not affected [65].

2.2.2 Coagulopathy

Hypocoagulability and increased risk of bleeding after administration of HES in human patients is belived to be due not only to hemodilution, but also to direct effects of HES on the hemostatic system. Plasma accumulation of starch marcomolecules can lead to platelet dysfunction, decreased concentration of circulating vWF-FVIII, impaired factor XIII fibrin cross-linking and enhanced fibrinolysis resulting in a weaker and smaller clot [67].

The pathophysiology of HES-induced platelet dysfunction is multifactorial. Hydroxyethyl starch molecules induce cellular abnormalities that result in decreased agonist-induced expression of the glycoprotein GP IIb-IIIa receptor on platelet surface, culminating in inhibited platelet adhesion and aggregation [67]. There is additional evidence that HES molecules may bind to and coat the platelet surface, leading to further inhibition of activation, aggregation and adhesion to fibrinogen. Effects on platelet dysfunction are more pronounced with administration of early HES generation solutions [61].

Along with alteration in primary hemostasis, synthetic colloids, as previously mentioned, can also induce changes in secondary hemostasis. Acquired von Willebrand syndrome with decreased factor VIII activity is recognized consequence of administration of all classes of synthetic colloids. Data obtained from healthy human volunteers have shown up to 80% reduction in circulating factor VIII and vWF activity after HES administration. Although the precise pathophysiology remains incompletely understood, binding of colloids to factor VIII / vWF complexes is supposed to accelerate complex clereance from the plasma [67]. Furthermore, HES exerts a pro-fibrinolytic action as its incorporation in clots leads to accelerated conversion of fibrinogen to fibrin, resulting in a friable clot, as well as reduced clot firmness due to decreased interaction between activated factor XIII and fibrin [61, 67].

2.3 HES – suspended authorization and new warning

Results obtained from three large randomized controlled trials (RCTs), investigating the safety and efficacy of HES products, demonstrated that critically ill adult patients including patients with severe sepsis and those admitted to the intensive care unit (ICU) treated with HES were at a significantly greater risk of AKI requiring renal replace treatment (RRT) than those patients that received crystalloids for volume replacement [68-70]. Moreover two of the studies also documented a significantly higher risk of mortality with HES versus crystalloid theraphy (42,80). On Novembre 2012, these results prompted the German Medicine Agency, Federal Institute for Drugs and Medical Devices (BfArM), to call for a review of all HES infusion solution by the European Medicines Agency's Pharmacovigilance Risk Assessment Committee (PRAC), responsible for assessing all aspects of risk management regarding drug use in people. PRAC evaluated data submitted and on June 2013 concluded that in patients treated with HES, when compared to crystalloids, the risk of AKI requiring RRT and the risk of mortality was greater. PRAC determined that available data only showed a limited benefit of HES solution when used to treat hypovolemia and therefore a recommendation to suspend marketing authorizations for all HES-containing fluids was published on the website of the European Medicines Agency (EMA) on June 2013 [71]. Following an appeal by HES-manufacturing companies, e second EMA-PRAC review was carried out in September 2013. The European Commission's final decision letter, published on the EMA website in March 2014, stated that HES solution may be used to treat hypovolemia, where treatment with crystalloids alone is not sufficient, and that to minimize potential risk, HES solution should not be used for more than 24 hours and kidney function should be monitored.

PART III: RESEARCH PROJECTS:

HYDROXYETHYL STARCH SOLUTION (HES 130/0.4)

HUMAN MEDICINE

CLINICAL USE and APPLICATION:

- ✓ Replace intravascular volume circulating during hypovolemic shock (administration as a bolus)
- ✓ Increase and support plasma colloid osmotic pressure (COP) during hypoalbuminemic states (administration as a constant rate infusione [CRI])

SIDE EFFECTS

- ✓ **Acute Kidney Injury** (**AKI**): Increase requirement of renal replacement therapy
- ✓ **Coagulopathy:** Increase requirement of blood products transfusion

Especially in **critically ill** patients such as **septic** patients

September 2013. The use of colloidal solutions has been suspended

March 2014. EMA website: HES solution may be used to treat hypovolemia, where treatment with crystalloids alone is not sufficient and should not be used for more than 24 hours.

VETERINARY MEDICINE

CLINICAL USE and APPLICATION:

- ✓ Replace intravascular volume circulating during hypovolemic shock (administration as a bolus)
- ✓ Increase and support plasma colloid osmotic pressure (COP) during hypoalbuminemic states (administration as a constant rate infusione [CRI])

SIDE EFFECTS

Studies that demonstrate the presence of **renal** and **hemostatic** side effects, as it has been widely reported in human being, are **limited** in veterinary medicine.

- **The real impact of colloidal solution on hemostatic system ?**
- **The real efficacy of colloidal solution in increasing and support COP?**

CURRENTLY UNKNOWN

AIM of my PhD research:

Answer to some question that are still unsolved and make the use of these solutions controversial:

- **Third HES generation can be considered safe?**
- **Are they effective in increasing and maintaining COP during hypoalbuminemic states?**

In order to improve our knowdlege related to HES solutions

Three following research studies has been developed:

1. Thromboelastometric assessment of hemostasis following hydroxyethyl starch (130/0.4) administration as a constant rate infusion in hypoalbuminemic dogs.

2. Assessment of hemostasis in dogs with gastric dilatation-volvolus during resuscitation with hydroxyethyl starch (130/0.4) or hypertonic saline (7.5%).

3. Administration of hydroxyethyl starch (130/0.4) as a constant rate infusion in hypoalbuminemic dogs: evaluation of effects on plasma colloid osmotic pressure.

3.0 INTRODUCTION

In veterinary medicine current knowledge about the safety and the effectiveness of hydroxyethyl starch (HES) solution are limited and most of the available data are extrapolated from human medicine. However, in the recent years more attention has been focused on the use of colloidal solution, especially in order to assess whether the same side effects that have been describe in human medicine are also present in veterinary medicine.

During my PhD the attention has been focused on the possible alteration that these solutions may have on haemostasis, especially evaluating the effects of a tetrastarch, a third HES generation solution (HES 130/0.4).

As previously described the physical, chemical and pharmacological properties of colloidal solution depend on the molecular weight (Mw), degree of molar substitution (Ms) and C2/C6 ratio. Molecules with higher MS and C2/C6 ratio have a slower degradation rate, prolonged intravascular retention time and therefore more tissue accumulation and associated side effects. In order to improve safety and pharmacological properties, newer third-generation starch products (tetrastarch) with reduced Mw and MS have been developed. These products are characterized by shorter plasmatic half-life, improved pharmacokinetic and pharmacodynamics properties, and fewer side effects.

An hypocoagulable state and an increased risk of bleeding has been reported following HES administration in human patients; in particular impairment of coagulation is believed to be due not only to hemodilution effects, but also to direct effects that colloids exert on hemostatic system. In particular it has been observed that these solutions can cause platelet dysfunction, by compromising the mechanism of platelet adhesion and activation, decreased the plasma concentration of vWF-FVIII complex, compromise FXIII cross linking and enhanced fibrinolysis.

Studies available in veterinary medicine are currently limited, and an impairment of both primary and secondary hemostasis has been identified during in vitro and in vivo studies using both first (hetarstarch) and third (tetrastarch) HES generation.

However, in vitro studies have important limitations because are performed in closed system, the pharmacokinetics of the products is not taken into account (mechanism of tissue distribution, accumulation and elimination) and therefore can not be able to predict the in vivo effects of this solution.

Few in vivo studies are currently available and in most of them coagulation has been assessed following HES administration on healthy dogs, in dogs anesthetized for surgical procedures and hemodinamically stable and dogs with systematic inflammation experimentally induced.

Moreover, in most of these studies HES was administered as a bolus, in hemodinamically stable patients dogs and using, in some of them, high dosages (40 ml/kg over 30 minutes) that are not regularly used during clinical practice; therefore the results obtained could be significantly influenced by rate of administration, the dosages used and type of patients in which were administered.

Colloidal solutions are kind of fluid widely used during fluidtheraphy in human and veterinary medicine; can be administered as a bolus, in order to increase the circulating blood volume during

hypovolemic shock, or as a constant rate infusion (CRI) in order to increase and support plasma colloid osmotic pressure during hypoalbuminemic state.

The dosages used in veterinary medicine are extrapolated from human literature and consist in the administration of a total volume of 20-50 ml/kg/die, when administered as a bolus (divided in boluses of 10 ml/kg or 5 ml/kg over 20 minutes in dogs and cats respectively), or 1-2 ml/kg/h if administered as a CRI.

Similarly, studies evaluating the effectiveness of HES solution to increase and support plasma COP in dogs affected by hypoalbuminemia are limited.

The osmotic colloid pressure is by definition the pressure exerted by macromolecules across a semipermeable membrane; it is proportional to the number of molecules and not to their size (Hughes, 2000). Albumin is the main oncotic blood protein and contributes for 75% of normal COP. Plasma colloid oncotic pressure is the pressure exerts on vascular endothelium and helps to maintain vascular volume and prevent edema formation.

In patients affected by severe hypoalbuminemia fluidtheraphy is a challenge because the intravenous fluid administration could cause a further decrease of COP with subsequent increase of fluid movement from the intravascular space into the interstitium and increased risk of peripheral edema formation, free fluid accumulation in pleural and/or peritoneal space and pulmonary edema.

Colloidal solution are characterized by large mulecules with high molecular weight and can contribute to the maintenance of plasma COP. The increase and support plasma COP following the administration of HES as a CRI has been describe in veterinary medicine, but its effectiveness has never been evaluated. Studies currently available showing a transient increase of COP following HES administered as a bolus in both healthy and hypoalbuminemic dogs.

During the PhD period I've developed 3 research projects in order to deeply analyse the use HES solution and trying to answer to some question that are still unsolved and make the use of these solution controversial. In particular I evaluated the safety of the third HES generation (HES 130/0.4), assessing the effects of this solution on hemostasis, considering both methods of administration (CRI and bolus) in patient dogs admitted to the intensive care unit (ICU) and affected by naturally occurring disease. In addition to standard coagulation profile [protrombine time (PT), activated partial thromboplastine time (aPTT) and fibrinogen] hemostasis has been assess by means of thromboelastometer.

Patients admitted to the ICU are critically ill patients and therefore it is essential to know if the products used for their management during clinical practice can be considered safe or if their use could cause a worsening of the patient's clinical condition.

Finally I tried to answer to the question about the real effectiveness of this solution in increasing and support plasma COP when administered as a CRI during hypoalbuminemic states.

The three research projects have been report below and will be developed in the following section.

1. **Thromboelastometric assessment of hemostasis following hydroxyethyl starch (130/0.4) administration as a constant rate infusion in hypoalbuminemic dogs.**
   Published on **BMC Veterinary Research** on January 2018

2. **Assessment of hemostasis in dogs with gastric dilatation-volvolus during resuscitation with hydroxyethyl starch (130/0.4) or hypertonic saline (7.5%).**
   Submitted to the **Veterinary Research Communication** on March 2018.

3. **Administration of hydroxyethyl starch (130/0.4) as a constant rate infusion in hypoalbuminemic dogs: evaluation of effects on plasma colloid osmotic pressure.**
   Submitted to the **Veterinary Anaesthesia and Analgesia** on December 2017. Currently under revision.

3.1 THROMBOELASTOMERIC ASSESSMENT OF HEMOSTASIS FOLLOWING HYDROXYETHYL STARCH (130/0.4) ADMINISTRATION AS A CONSTANT RATE INFUSION IN HYPOALBUMINEMIC DOGS.

Published on **BMC Veterinary Research** on January 2018

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Abstract

Background: The primary aim was to evaluate by means of thromboelastometry (ROTEM) the effects of hydroxyethyl starch (HES) 130/0.4 administered as a constant rate infusion (CRI) on hemostasis in hypoalbuminemic dogs. The second aim was to use ROTEM analysis to detect whether all hypoalbuminemic dogs of our population were hypercoagulable.

Results: The study sample was 20 hypoalbuminemic dogs (albumin < 2 g/dl) with normal perfusion parameters and requiring intravenous fluid therapy. In order to support plasma colloid osmotic pressure, in addition to crystalloid, HES 130/0.4 was administered as a constant rate infusion at 1 ml/kg/h (group 1, n=11) or 2 ml/kg/h for 24 hours (group 2, n=9). Blood samples were collected at baseline (T0) and 24 hours postinfusion (T1); coagulation was assessed by standard coagulation profile (prothrombin time, activated partial thromboplastin time, and fibrinogen), and ROTEM analysis (in-TEM®, ex-TEM® and fib- TEM® profile).

No statistically significant differences in ROTEM values in group 1 were observed ($P > 0.05$), whereas in group 2 statistically significant differences ($P < 0.05$) were found at T1 in the in-TEM® profile [decrease in clot formation time ($P = 0.04$) and increase in α angle ($P = 0.02$)] and in the ex-TEM® profile [increase in maximum clot firmness ($P = 0.008$) and α angle ($P = 0.01$)]; no changes were identified in the fib-TEM® profile. In both groups, a statistically significant decrease ($P= 0.007$) in hematocrit was noted, whereas no statistically significant differences in platelet count and standard coagulation profile were found. In group 2, a statistically significant increase in TS values ($P = 0.03$) was noted at T1. ROTEM tracings indicating a hypercoagulable state were observed in 7/20 dogs at T0 (5/11 in group 1 and 2/9 in the group 2).

Conclusion: Our findings suggest that HES 130/0.4 administered as CRI does not cause hypocoagulability in hypoalbuminemic dogs. A trend toward hypercoagulability, probably related to the underlying diseases, was observed in group 2 at T1.
Although all dogs were hyoalbuminemic, only 7/20 were hypercoagulable at T0, confirming the lack of correlation between albumin level and prothrombotic state.

Keywords: dogs, hydroxyethyl starch, hypoalbuminemia, thromboelastometry.

Background

Hydroxyethyl starch (HES) comprises a group of synthetic polymers routinely used in veterinary anesthesia and critical care medicine to maintain blood volume, counteract anesthesia-induced hypotension, to resuscitate patients with hypovolemic shock or sepsis, and to support intravascular colloid osmotic pressure (COP) during hypoalbuminemic states [50, 51]. HES can be administered as a bolus or as a constant rate infusion (CRI) typically at a rate of 20-50 ml/Kg/day and 1-2 ml/kg/h, respectively [50, 51, 60], the doses are generally extrapolated from human literature [50, 51]. The physicochemical and pharmacological properties of HES, as well as its classification, depend on their mean molecular weight (MW), molar substitution (MS), and C2/C6 ratio [61]. The rate of degradation and elimination of HES depends on the MS and C2/C6 ratio, whit higher MS and C2/C6 ratio leading to a slower elimination and greater intravascular retention time and associated side effects [72]. To improve safety and pharmacological properties, newer third-generation starch products with reduced MW and MS have been developed [61] that have shorter half-life, improved pharmacokinetic and pharmacodynamic properties, and fewer side effects [73]. Nonetheless, controversy in human medicine and veterinary medicine persists concerning the safety of HES. Indeed, recent reports of side effects in people (e.g., acute kidney injury, tissue storage, and impaired primary and secondary hemostasis), led to a temporary suspension of HES products. Authorization was reinstated about a year later, together with new guidelines for contraindications in certain conditions (e.g., sepsis) [51].
Hypocoagulability and increased risk of bleeding after administration of HES in human patients is believed to be due to hemodilution and direct effects of HES on the hemostatic system. Plasma accumulation of starch macromolecules can lead to platelet dysfunction, decreasing expression and activation of the surface receptor GPIIb/IIIa, decreased concentration of circulating von Willebrand factor (vWf) and factor VIII, impaired factor XIII fibrin cross-linking, and enhanced fibrinolysis resulting in a weaker and smaller clot [61, 67].
In veterinary medicine, both *in vitro* [74-80] and *in vivo* [81-86] studies have demonstrated impairment of whole blood coagulation after the administration of HES. After blood dilution with both crystalloids and colloids, *in vitro* studies have found a dose-dependent decrease in platelet function, an increase in prothrombin time (PT) and partial activated thromboplastin time (aPTT), and changes in viscoelastic parameters toward hypocoagulability (thromboelastometry or thromboelastography), with

more severe impairment observed after dilution with HES [74-80]. Three *in vivo* studies have assessed only platelet function, whereas others studies have evaluated secondary hemostasis or whole blood coagulation following bolus administration of HES (doses ranging from 10 to 40 ml/kg in approximately 30 minutes), demonstrating hypocoagulability in healthy dogs or dogs affected by experimentally induced disease [81-86].

Viscoelastic techniques, such as thromboelastometric analysis (ROTEM), evaluate coagulation in whole blood samples. Since they take into account plasmatic and cellular elements, they better reflect the cell-based model of hemostasis, that describes coagulation as a dynamic process in which plasmatic coagulation factors interact with the cell surface [14, 42]. Rotational thromboelastometry use several reagents in different profiles to measure the kinetics of clot formation (time required for clot formation), the mechanical properties (clot strength), and the time required for clot dissolution (fibrinolysis) [40, 42]. It is the most useful tool to assess the presence of a hypercoagulable state, as compared with standard coagulation assays (PT, aPTT, fibrin degradation products and D-dimers) [40, 42].

To date, there are no published studies investigating the effects of HES 130/0.4 on whole blood hemostasis after its administration as a CRI in dogs with naturally occurring disease. Hypoalbuminemic dogs are a type of patients in which natural or synthetic colloids are frequently used to support the COP. These patients have also a high risk to form venous and arterial thrombi, and some studies have identified by means of ROTEM or thromboelastography (ROTEG) a hypercoagulable condition in dogs with protein losing enteropathy (PLE) or nephropathy (PLN) [87-90]. The hypothesized mechanism at baseline of this prothrombotic condition is a loss of proteins (from the kidney or the gut), which implies the loss of anticoagulant factors such as antithrombin [91-93]. In human medicine multiple mechanisms are involved in the development of hypercoagulability in case of hypoalbuminemia, including spontaneous platelet aggregation, increase in coagulation factors, hypofibrinolysis, decrease in antithrombin and protein C [94, 95].

The first aim of the present study was therefore to evaluate the effects of HES 130/0.4 administered as a CRI (at dose of 1 ml/Kg/h and 2 ml/Kg/h) on hemostasis in hypoalbuminemic dogs, by means of ROTEM. The hypothesis was that HES administration will cause a hypocoagulability state.

The second aim was to determine how many hypoalbuminemic dogs were hypercoagulable at presentation.

Methods

This randomized, clinical prospective trial was performed on client-owned dogs. The protocol was approved by the Bioethics Committee of the author's University. The dog owners were informed about the methods and aims of the study and gave their written informed consent.

Animals and study design

The dogs were selected from patients admitted to our Veterinary Teaching Hospital for hospitalization. The inclusion criteria were: hypoalbuminemia (albumin < 2 g/dl) regardless of

underlying disease, normal perfusion parameters, to require intravenous fluid therapy due to their underlying disease (e.g., increased losses, anorexia, dehydration). All dogs underwent complete physical examination and perfusion parameters were assessed by clinical evaluation and non-invasive measurement of arterial blood pressure using Doppler (Model 811-B, Parks Medical Electronics Inc., Oregon, USA). Exclusion criteria were pulmonary disease, cardiac and liver failure, renal azotemia, pre-existing hypocoagulability, and history of non-steroidal anti-inflammatory drugs, steroidal drugs, artificial colloid or blood products administration in the 4 weeks prior to the study. Additional exclusion criteria were abnormal perfusion parameters (heart rate >130 bpm, poor pulse quality, capillary refill time > 2 s or < 1 s, systolic blood pressure <90 mmHg and venous lactate >2 mmol/L) and serologic test positive to *Ehrlichia canis, Dirofilaria immitis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum, Anaplasma platys* and/or *Leishmania infantum*.

Blood samples were collected by atraumatic venipuncture of the jugular vein with a 20-gauge needle using minimum stasis. Samples that were difficult to obtain (e.g., venipuncture required numerous attempts, needle repositioning or interruption of blood flow into the tube) were discarded and blood collection was repeated from the contralateral jugular vein. After blood collection, cell blood count (CBC) (ADVIA® 120 Hematology, Siemens Healthcare Diagnostics, Tarrytown, NY, USA), serum concentration of albumin (ILAB 300 plus, Clinical Chemistry System, Instrumentation Laboratories, Milan, Italy), total solid and packed cell volume were measured according to the study protocol. Other specific analyses were performed to assess the cause of hypoalbuminemia, as needed on a case-by-case basis.

Coagulation was assessed by standard coagulation profile [prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen] (Coagulometer StART, Diagnostica Stago, Parsippany, NJ, USA), and by ROTEM analysis, (ROTEM®, TEM Innovations GmbH, Munich, Germany) for evaluating in-TEM®, ex-TEM®, and fib-TEM® profiles. Blood samples were divided into two tubes containing 3.2 % buffered sodium citrate (Vacumed 3.2% buffered sodium citrate, FL Medical, Torreglia, Italy).

Thromboelastometric analyses were performed according to PROVETS guidelines [96, 97], and the analyses run for 60 minutes. For each blood sample, three thromboelastometric profiles were performed: in-TEM®, ex-TEM®, and fib-TEM® assay to evaluate the intrinsic and the extrinsic pathway, respectively, and assess the functional fibrinogen contribution to clot formation. For the in-TEM® profile, the blood sample was recalcified using the start-TEM® reagent and coagulation was activated with the specific reagent containing ellagic acid, whereas the ex-TEM® profile was activated by adding thromboplastin after recalcification. To obtain the fib-TEM® profile, coagulation was activated using thromboplastin in addition to a specific reagent containing a platelet inhibitor (cytochalasin D). The following variables were assessed for each profile: clotting time ([CT], s); clot formation time [(CFT) s]; maximum clot firmness [(MCF)] mm]; and α angle (α,°). Clotting time describes the time in seconds from clot initiation until the fibrin polymers are produced and the amplitude reaches 2 mm; this parameter is dependent on the concentration and activity of plasma coagulation factors [40]. Clot

formation time, measured in seconds, is the time from initiation of clotting (2 mm) until an amplitude of 20 mm is reached [40]. Clot formation time and α angle provide information about the kinetics of clot formation and are predominantly affected by platelet count and function and fibrinogen concentration [40]. Maximum clot firmness represents the maximum amplitude reached by the clot, reflects maximal clot strength and stability, and is affected by fibrinogen, platelet count and function (except in the fib-TEM® profile where platelet are inhibited), thrombin, FXIII, and hematocrit (Hct) [40]. Additional calculated parameter is platelet contribution to maximum clot elasticity ($MCE_{platelet}$), which evaluates the platelet component to clot strength and is obtained as follows: $MCE_{platelet} = MCE_{extem} - MCE_{fibtem}$ [MCE=(MCF*100)/(100-MCF)]. [49]

A ROTEM tracing indicating hypocoagulability is characterized by an increase in CT and CFT and a decrease in MCF and α angle, whereas a ROTEM tracing indicating hypercoagulability is characterized by a decrease in CT and CFT and an increase in MCF and α angle. ROTEM tracings were considered abnormal when more than one ROTEM value was above (α angle, MCF) or below (CT, CFT) our reference ranges (Table 1).

Serological tests for *Ehrlichia canis*, *Dirofilaria immitis*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum, Anaplasma platys* (Snap 4DX, IDEXX Laboratories, Westbrook, ME) and *Leishmania infantum* (Snap Leishmania, IDEXX Laboratories, Westbrook, ME) were also carried out.

An intravenous catheter was inserted into the peripheral vein, and fluid therapy was started using lactated Ringer's solution (Ringer's lactate solution, Baxter S.p.A, Rome, Italy), to replace dehydration, maintenance, and ongoing losses, and HES 130/0.4 [Voluven, Fresenius Kabi Italia srl., Isola della Scala (VR), Italy] for COP support. Hydroxyethyl starch 130/0.4 was administered as a CRI for at least 24 hours. Colloid treatment [1 ml/kg/h (group 1) or 2 ml/kg/h (group 2)] was randomly assigned via a computer-generated program (Microsoft Excel, Redmond, WA, USA). The dogs were assessed for body temperature, respiratory rate and perfusion parameters every 4 hours. Blood samples were collected at baseline (T0), to assess all previously laboratory analysis described, and 24 hours after start of infusion (T1) CBC, albumin, packed cell volume, total solid (TS), and coagulation tests were repeated.

Statistical analysis

Data were collected and analyzed with the software Stata 14.2 (Stata Statistical Software, release 10, StataCorp LP, College Station, TX, USA). Normality of data was assessed using the Shapiro-Wilk test.

To evaluate changes in ROTEM parameters after administration of the two different doses of HES 130/0.4 (CRI of 1 and 2 ml/kg/h) at two time points (T0 vs. T1), a hierarchical linear mixed effects model was used, where the random effect is given by the individual subject. Bonferroni correction was applied to detect in which medium the difference was statistically significant. When the data did not meet the assumption of normality, the comparison was conducted with the sign test.

Analysis of the data showed that only some dogs were hypercoagulable and that many had been randomly assigned to group 1. Despite the small number of hypercoagulable animals, Friedman's test was carried out and the population divided (independent of HES dose administered) into two subgroups: hypercoagulable dogs (group H) and not hypercoagulable dogs (group NH). This was done to detect differences in ROTEM parameters at the two time points (T0 vs. T1).
A value of $P < 0.05$ was considered significant.

Results

Of the total of 25 adult dogs initially enrolled, 5 were excluded because of Leishmaniosis (n=2), liver failure (n=2), and hypocoagulability with an abnormal thromboelastometric tracing at T0 (n=1). The study sample comprised 20 dogs: 11 received HES 130/0.4 as a CRI at 1 ml/kg/h (group 1) and 9 at 2 ml/kg/h (group 2) for at least 24 hours. In group 1, the median age was 7 years (min 2-max 12) and the median body weight 27 kg (min 5-max 39); breeds included: Australian Shepherd (n=1), Border Collie (n=1), Rottweiler (n=1), German Shepherd (n=1), Beagle (n=1), and mixed breed (n=6). Seven were females (4 neutered and 3 intact) and 4 males (1 castrated and 3 intact). Dogs included were affected by acute protein losing enteropathy (n=4), chronic protein losing enteropathy (n=3), protein losing nephropathy (PLN) (n=1), or chylothorax (n=3).
In group 2, the median age was 7 years (min 2 – max 10) and the median body weight 17.8 kg (min 5 – max 44); breeds included: Rottweiler (n=1), English Bulldog (n=1), Pit bull (n=1), longhaired Dachshund (n=1), Labrador (n=1), Jack Russell (n=2), and mixed breed (n=2). Six were females (4 neutered and 2 intact) and 3 intact males. Dogs included were affected by chronic protein losing enteropathy (n=6), chylothorax (n=2) or hypoadrenocorticism (n=1).
There were no statistically significant differences in age, body weight, serum albumin concentration, ROTEM values, PT and fibrinogen concentration between the two groups at baseline (T0). The aPTT was slightly prolonged in group 2 at both T0 and T1 (β= 2.2, C.I. 95% and P=0.027, respectively), but still within the reference range.
Comparison between PT, aPTT, and fibrinogen concentration showed no statistically significant within-group differences between the two time points (T0 versus T1) (Table 1). While no statistically significant within-group differences in ROTEM values (T0 vs. T1) were found in group 1; statistically significant differences were noted in group 2 at T1: a decrease in CFT (P = 0.04) and an increase in the α angle (P = 0.02) in the in-TEM® profile and an increase in MCF (P = 0.008) and the α angle (P = 0.01) in the ex-TEM® profile (Table 1), whereas no changes was identified in the fib-TEM® profile and in the $MCE_{platelet}$. Nevertheless, there were no statistically significant differences in ROTEM parameters between the two groups at T1. In both groups, there was a statistically significant decrease in Hct (P = 0.007) at T1 (time-dependent reduction), but no statistically significant dose-dependent difference between the two dosages (Table 3). There was no statistically significant difference in platelet count at T1 in either group (Table 3). Finally, a statistically significant increase in

TS values (P = 0.03) in group 2 was noted at T1, but no statistically significant differences in albumin concentration in either group (Table 3).

ROTEM tracings indicating a hypercoagulable state were observed in 7/20 dogs at T0 (5/11 in group 1 and 2/9 in group 2). (Table 2) There were no statistically significant changes from T0 to T1 in either subgroup H or subgroup NH. Analysis of single animals classified as hypercoagulable (subgroup H) showed that the fibrinogen level was outside normal limits in 6/7 dogs (median 6.02, min 3.20 and max 8.53), whereas all the dogs classified as not hypercoagulable (subgroup NH) had levels within the normal range (median 3.26, min 2.23 and max 4.43) (Table 2). In addition, the $MCE_{platelet}$ was outside the normal range in all the hypercoagulable dogs (median 339, min 257 and max 404) and it was increased only in 3/13 classified as not hypercoagulable (median 205, min 142 and max 316) (Table 2).

Discussion

The main finding of the present study was that a hypocoagulable state was not observed by either 1 or 2 ml/kg/h of HES 130/0.4 in dogs with hypoalbuminemia. In contrast to our hypothesis, ROTEM analysis at T1 revealed statistically significant differences in some parameters consistent with a trend toward a hypercoagulability state after CRI at 2 ml/kg/h. In particular, in the in-TEM® profile a decrease in CFT and an increase in α angle, and in the ex-TEM® profile an increase in both α angle and MCF was found (Table 1). No statistical significant differences were shown comparing the ROTEM values of group 1 and group 2 at T1, then we can exclude that these variations have been induced by the different dose used in the group 2, and affirm that the change could be time dependent. As the dogs in this study presented with various different diseases, it is possible that an inflammatory state in the group 2 progressed during the 24 hours of observation from T0 to T1 and affected coagulation in a different way. Since there is a well-established link between inflammation and coagulation, it might be possible that the inflammatory processes impaired activation of pro- and anti-coagulant factors, fibrinolysis, and induced abnormalities in platelets and endothelial components, but in our study we have not looked into markers for inflammation and antithrombin level [98].

Other hematological factors are known to influence the ROTEM analysis and may have affected our results. The CFT, α angle, and MCF can be influenced by some sample features such as platelet count, fibrinogen concentration, and Hct [40]. Our results have not found any statistically significant difference in platelet count and fibrinogen concentration, whereas a statistically significant reduction in Hct, after 24 hours of infusion, was noted in both groups at T1. Hematocrit can affect ROTEM results, leading to a hypocoagulable tracing when Hct is increase or a hypercoagulable tracing when it is decreased [47, 99]. Smith et al. (2012) hypothesized that a whole blood sample with a decreased Hct has a greater concentration of coagulation factors (the plasma to erythrocyte ratio changes in the ROTEM cup), leading to an artifactual hypercoagulability tracing when the blood is analyzed with ROTEM, and vice versa in case of increased Hct [47, 99]. Although no statistically significant

difference in variation of Hct between the two groups was found from T0 to T1, the trend toward hypercoagulability identified in group 2 could be partially explained by the lower Hct values reached in this group at T1 (T1: Group 1, 39% and Group 2, 35% of Hct).

Although the platelet count remained unchanged between T0 and T1 (group 2), authors hypothesized that an increase in platelet activity could have been present. Indeed, no alterations were identified in the fib-TEM® profile, where platelet activity was inhibited. ROTEM is not a specific tool to evaluate platelet function, but $MCE_{platelet}$ could be used to assess the contribution of platelets activity to clot elasticity, thus eliminating the influence of fibrinogen. However, no difference was identified between T0 and T1 in $MCE_{platelet}$, and then a platelet contribution to hypercoagulable trend could be excluded.

Our findings differ from those reported in previous *in vitro* and *in vivo* studies that evaluated the effects of HES 130/0.4 on hemostasis by means of viscoelastic techniques (ROTEM and ROTEG). In two *in vitro* studies, when whole blood samples were diluted with HES 130/0.4 (1:22, 1:9, 1:4), a hypocoagulable ROTEM tracing was found only at the highest dilution (1:4, mimicking *in vivo* administration of 30 ml/kg) [77, 80]. Whereas, an impairment of secondary hemostasis has been identified after 1:5.5 dilution (comparable to a fluid dose of 20 ml/Kg) using ROTEG analysis [78]. The results of *in vitro* studies cannot be directly extrapolated to predict *in vivo* results, as the effect that a HES solution may have on hemostasis is largely determined by its *in vivo* pharmacokinetics [100].

Only three *in vivo* studies evaluating changes in hemostasis following HES 130/0.4 administration, are currently available in literature. One evaluated platelet function, whereas the other two assessed viscoelastic properties of whole blood [83, 84, 86]. Gauthier et al. (2015) found a significant prolongation of aPTT and a hypocoagulable ROTEG after administration of HES 130/0.4 (bolus of 40 ml/kg over 30 minutes) in healthy dogs and dogs with induced systemic inflammatory response syndrome [83]. Reutler et al. (2017) detected by means of ROTEM an impairment of whole blood coagulation after administration of a single bolus of HES (15 ml/kg over 30-40 min) in dogs undergoing general anesthesia for arthroscopy or imaging studies [86]. Comparison between our study and those mentioned above is difficult, because of the differences in study populations, rate and volume infused. The dogs in the present study were affected by naturally occurring diseases that cause hypoalbuminemia, whereas the sample populations in the other studies were healthy, anesthetized dogs or dogs with experimentally induced disease. In addition, the chosen method of HES administration, implied different infusion rate; during bolus the total amount of dose (15 or 40 ml/kg) is infused over a short period of time, resulting in an increase in intravascular volume (especially in hemodynamically stable dogs) and a greater hemodilution as compared with the administration as a CRI, where the total volume (24 or 48 ml/Kg) is equally divided over 24 hours. Moreover, different rates are associated with a diverse degree of tissue accumulation and elimination [72].

At the time of inclusion in this study, only 7/20 dogs had a ROTEM tracing indicating hypercoagulability, and defined as a ROTEM profile with more than one parameter outside the institutional reference range. The fibrinogen level was normal at T0 and the $MCE_{platelet}$ altered in 3

patients in the NH group. Differently in the H group, the fibrinogen level and the $MCE_{platelet}$ were outside the upper range in 6/7 and 7/7 animals, respectively (Table 2). These results indicate an influence of the fibrinogen amount on the ROTEM tracing and an increase in the platelet aggregation, which can partially explain the hypercoagulbility detected at T0. In literature has been reported that an increase in platelet activity is associated with hypoalbuminemia in dogs with PLN [93, 101, 102]. Since there were no statistical significant differences from T0 to T1 in group H and group NH, in ROTEM results and $MCE_{platelet}$, we can excluded the possibility that the hypercoagulability trend, seen at T1 in the group 2, was affected by the hypercoagulable patients.

Using ROTEG analysis, recent studies in dogs affected by PLE and PLN have identified hypercoagulability in the majority of them [88-90]. Due to the differences in the studies populations and the criteria for assessing hypercoagulability, a comparison with our study is not possible [88-90]. Until today, no standardized definition of hypercoagulability by ROTEM analysis in dogs has been established, although the PROVETS guidelines were issued in the attempt to achieve conformity across studies using viscoelastic techniques [103]. As there is insufficient evidence to recommend a definition of hypercoagulability in companion animals, the definition is left to the authors' discretion [103].

An increased risk of thromboembolic events has been reported for dogs affected by PLN or PLE. The underlying cause of hypercoagulability remains incompletely understood; potentially involved mechanisms are loss of antithrombin and increased platelet aggregation [93, 101, 102]. Since we did not measure antithrombin activity, we are unable to determine its influence on our ROTEM results. Although multiple mechanisms have been involved in hypercoagulable state [94, 95], the incidence of thromboembolism dramatically increases when serum albumin concentration is less than 2.0-2.5 g/dL, in human patients with PLN [94, 95]. In contrast, veterinary studies have shown that hypercoagulability identified with ROTEM analysis does not appear to be correlated with hypoalbuminemia [88-90], and only a weak correlation between serum albumin concentration and antithrombin activity has been identified in hypercoagulable dogs, indicating that albumin level cannot reliably predict it [88-90, 104, 105]. These findings highlight that the prothrombotic state in dogs is not related only to hypoalbuminemia but to other abnormalities as well.

Refractometric evaluation was used to measure the TS concentration. Our results showed a statistically significant increase in TS after 24 hours (T1) of HES 130/0.4 as a CRI at 2 ml/kg/h (Group 2). Bumpus et al. (1998) reported that after the addition of a large volume of hetastarch (after an *in vitro* dilution of 1:4 corresponding to *in vivo* 22 ml/kg) the refractometer reading of TS increased [106]. Although the in vitro dosage administered in the aforementioned study differs from dosages used in the study herein, the higher dose of HES used in the group 2 may have interfered with the refractometric reading. Furthermore, the refractometer reading of HES 130/0.4 is 4.5 mg/dl, the same as that of the products used in the study by Bumpus et al [106].

This study has several limitations. The small sample size, limits the ability to generalize the results obtained. Moreover, the clinicians were not blinded to the HES doses administered, which could have

introduced a bias. The study population included dogs with diverse diseases, which might have several degrees of inflammation and different effect on hemostasis. In addition, we did not have a control group without treatment or treated only with a CRI of isotonic crystalloids, to determine whether the changes in hemostasis could be consequent to the progression of disease/inflammation or also related to the crystalloid infusion. In this regard, because the total amount of 24-hour administration of crystalloids solution was not recorded, the influence of this variable could not be assessed.

Further studies on larger samples of dogs with naturally occurring disease causing hypoalbuminemia are needed to create disease categories and take into account the amount of infused crystalloid.

The assessment of HES 130/0.4 pharmacokinetics in dog could allow understanding the amount of its accumulation and the rate of elimination, especially when the colloid is administered as a CRI. In this context it would be important to evaluate the risks and benefits associated with HES therapy and its efficacy in providing oncotic support.

Another important field of research is to investigate the prevalence of hypercoagulability in hypoalbuminemic dogs, to better understand the impact of the underlying disease on the prothrombotic state and to correctly identify which hypoalbuminemic dogs could really benefit from anticoagulant therapy. Finally, further research for investigate the contribution of platelet function to hypercoagulable state, by means specific tools such as the PFA-100 or platelet aggregometer, would be interesting.

Conclusion

Our findings suggest that CRI of HES 130/0.4 (1-2 ml/kg/h over 24 h) does not cause hypocoagulability in hypoalbuminemic dogs. A trend toward hypercoagulability, probably related to the underlying disease and associated degree of inflammation, was noted in group 2 at T1. Although all dogs were hyoalbuminemic, only 7/20 were hypercoagulable at T0, confirming the lack of correlation between albumin level and prothrombotic state.

List of abbreviations:

aPTT: activated partial thromboplastin time; CFT: clot formation time; COP: colloid osmotic pressure; CRI: constant rate infusion; CT: clotting time; HES: hydroxyethyl starch; MCF: maximum clot firmness; MCE: maximum clot elasticity; MCEplatelet: platelets contribution to clot elasticity; PLE: protein losing enteropathy; PLN: protein losing nephropathy; PT: prothrombin time; ROTEG: thromboelastography; ROTEM: thromboelastometery.

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Ethics approval and consent to participate

The protocol was approved by the Bioethics Committee of the Turin University. The dog owners were informed about the methods and purpose of the study and gave their written informed consent.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AB and BB contributed to the conception and design of the study. ABotto and BB acquired the data. AB interpreted the data. AB, ABotto and BB drafted the manuscript. CM performed the statistical analysis, AT, FR and GM contributed to the conception of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Table 1: ROTEM and standard coagulation profiles results, before and 24 hours after hydroxyethyl starch 130/0.4 infusion.

	GROUP 1 (1 ml / kg / h) N=11		**GROUP 2** (2 ml / kg / h) N=9		**Institutional reference ranges**
ROTEM	**T0**	**T1**	**T0**	**T1**	
In-TEM®					
CT (s)	180 (132-268)	166 (61-209)	188 (112-279)	141 (108-240)	126-363 s
CFT (s)	63 (45-132)	59 (41-113)	73 (41-86)	58 * (38-81)	47-224 s
MCF (mm)	72 (56-80)	72 (58-88)	68 (62-77)	72 (62-78)	50-75 mm
α angle (°)	77 (65-81)	79 (69-82)	76 (73-82)	78 * (74-82)	55-81 °
Ex-TEM®					
CT (s)	44 (27-61)	45 (20-77)	46 (30-89)	40 (33-80)	29-92 s
CFT (s)	67 (38-159)	78 (49-132)	81 (51-96)	62 (49-92)	54-275 s
MCF (mm)	77 (60-82)	70 (64-83)	70 (66-81)	72 * (67-86)	36-73 mm
α angle (°)	76 (61-83)	76 (65-82)	74 (71-80)	77 * (73-81)	47-79 °
Fib-TEM®					
CT (s)	47 (31-107)	39 (21-57)	45 (29-92)	39 (32-80)	14-102 s
MCF (mm)	25 (7-38)	16 (8-46)	15 (9-30)	20 (14-78)	6-26 mm
α angle (°)	77,5 (70-86)	76 (30-86)	76,5 (67-81)	77 (71-84)	40-78 °
$MCE_{platelet}$	294 (142-404)	211 (169-441)	206 (175-383)	235 (183-571)	50-235

Stadard coagulation					
aPTT (s)	12.5 (9.6-15.4)	12.3 (8.7-16)	13.9 (12.1-16)	14.5 (11.7-16)	12-16 s
PT (s)	7.5 (6.6-9.6)	7.15 (6.6-9.9)	7.4 (6.2-10)	7.3 (6.3-10)	8-10 s
Fibrinogen (g/L)	3.5 (2.5-8.5)	3.6 (2.4-7.9)	3.4 (2.2-7.4)	4.1 (2.2-7.1)	1.5-4.50 (g/L)

Values of institutional reference ranges for ROTEM parameters are expressed as 95% confidence intervals.[77]
In-TEM®, intrinsic thromboelastometry pathway; **Ex-TEM®**, extrinsic thromboelastometry pathway; **Fib-TEM®**, functional fibrinogen;
CT, clotting time; **CFT**, clot formation time; **MCF** maximum clot firmness;
PT: prothrombin time; **aPTT**: activated partial thromboplastin time.
* Indicates statistically significant differences between T0 and T1 ($P < 0.05$).

Table 2: ROTEM results at baseline (N=20).
Values reported in bold indicate the hypercoagulable dogs at T0 (N=7) and are outside the institutional reference ranges.

T0	**In-TEM®**				**Ex-TEM®**				**Fib-TEM®**					
G 1 (N=11)	CT	CFT	MCF	α angle	CT	CFT	MCF	α angle	CT	MCF	α angle	MCE platelet	Fib	Disease
Dog 1	216	85	72	73	43	56	**80**	**80**	107	**38**	73	**339**	3.2	APLE
Dog 2	132	104	75	73	43	159	64	61	56	119	25	144	3.5	APLE
Dog 3	142	54	70	79	39	67	69	76	45	11	70	210	3.2	CPLE
Dog 4	144	52	70	80	27	74	67	75	31	13	73	188	2.7	CPLE
Dog 5	180	132	56	65	61	143	60	62	51	7	ND	142	3.5	APLE
Dog 6	180	45	75	81	41	**38**	**79**	**83**	38	**34**	**86**	**325**	**6.2**	PLN
Dog 7	156	47	**80**	80	53	66	**81**	79	50	**28**	**83**	**387**	**6**	CPLE
Dog 8	163	63	75	77	44	83	77	75	41	16	78	**316**	4.4	CH
Dog 9	241	50	**79**	80	61	**47**	**82**	**81**	54	**34**	**79**	**404**	**8.5**	CH
Dog 10	268	103	62	69	48	107	68	74	47	14	77	196	3.3	CPLE
Dog 11	184	66	70	76	46	60	**77**	78	37	**29**	**80**	**294**	**5.3**	APLE
G 2 (N=9)														
Dog 12	241	52	**77**	79	46	**51**	**82**	**80**	45	**30**	**80**	**383**	**7.4**	CPLE
Dog 13	279	86	68	73	48	82	69	73	46	14	67	206	2.2	CPLE
Dog 14	198	79	67	74	40	96	67	71	36	22	79	175	4.2	CPLE
Dog 15	196	51	71	79	43	60	73	78	46	15	76	**253**	2.7	CPLE
Dog 16	**119**	**41**	72	**82**	30	57	75	79	29	**30**	**81**	**257**	**4.6**	CH
Dog 17	188	73	67	75	59	89	69	73	60	15	75	205	3.5	CH
Dog 18	151	75	64	76	38	72	70	75	38	22	77	205	2.8	CPLE
Dog 19	112	57	73	78	56	81	73	74	31	9	ND	**260**	2.4	AD
Dog 20	133	80	62	74	89	86	66	73	92	14	69	178	3.4	CPLE

G 1, Group 1 (1 ml/kg/h); **G 2**, Group 2 (2 ml/kg /h); **In-TEM®**, intrinsic thromboelastometry pathway; **Ex-TEM®**, extrinsic thromboelastometry pathway; **Fib-TEM®**, functional fibrinogen; **CT**, clotting time (s); **CFT**, clot formation time (s); **MCF**, maximum clot firmness (mm); **$MCE_{platelet}$**, platelets contribution to clot elasticity; **Fib**, fibrinogen level (g/L); **AD**, hypoadrenocorticism; **APLE**, acute protein losing enteropathy; **CH**, chylothorax; **PLN**, protein losing nephropathy; **CPLE**, protein losing enteropathy; **ND**, not determined.

Institutional reference ranges: In-TEM® (CT, 126-363 s; CFT, 47-224 s; MCF, 50-75 mm; α angle, 55-81°), Ex-TEM® (CT, 29-92 s; CFT, 54-275s; MCF, 36-73; α angle, 47-79°), Fib-TEM® (CT, 14-102 s; MCF, 6-26; α angle, 40-78°)[77] and fibrinogen level (1.5-4.50 g/L).

Table 3: Results of laboratory parameters, before and 24 hours after hydroxyethyl starch 130/0.4 infusion.

	GROUP 1 (1 ml/kg/h) N=11		**GROUP 2** (2 ml/kg/h) N=9	
	T0	**T1**	**T0**	**T1**
Hematocrit (%)	40 (29-54)	39 * (25-53)	38 (25-50)	35 * (25-55)
Platelet count (x 10E09 cell/L)	366 (156-853)	340 (145-800)	397 (125-641)	422 (134-731)
Total Solid (g/L)	0.36 (0.3-0.55)	0.36 (0.25-0.54)	0.32 (0.25-0.5)	0.35 * (0.27-0.5)
Albumin (g/L)	0.16 (0.12-0.19)	0.16 (0.11-0.24)	0.15 (0.13-0.18)	0.14 (0.11-0.22)

Values are expressed as median (minimum-maximum).

* Indicates statistically significant differences between T0 and T1 ($P < 0.05$).

3.2 ASSESSMENT OF HEMOSTASIS IN DOGS WITH GASTRIC DILATATION-VOLVULUS, DURING RESUSCITATION WITH HYDROXYETHYL STARCH (130/0.4) OR HYPERTONIC SALINE (7.5%).

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Abstract

The study's aim of this study was to evaluate, by means of thromboelastometry (ROTEM), the impact on hemostasis of the administration of an intravenous bolus of hydroxyethyl starch 130/0.4 (HES) or hypertonic saline 7.5% (HS) in dogs resuscitated for gastric dilation volvulus. The dogs were randomly assigned to receive HES at 10 ml/Kg or HS at 4 ml/Kg per 15 minutes. Blood samples were collected at baseline (T0), and at the end of bolus (T1).

The study included 13 dogs in HES group and 10 dogs in the HS group. Statistically significant differences between T0 and T1 were: an increase of CFT (P = 0.046), a decrease in MCF (P = 0.002) in ex-TEM profile, and a decrease of MCF (P=0.0117) in fib-TEM profile, for HES group; an increase of CT (P=0.048) and CFT (0.0019), and a decrease of MCF (P=0.031) and α angle (P=0.036) in ex-TEM profile, a decrease in α angle (P=0.036) in in-TEM profile, a decrease in MCF (P=0.017) in fib-TEM profile, and a decrease of MCE_{plt} (P=0.021), for HS group.

After bolus administration, a hypocoagulable trend was observed in the ex-TEM profile for the HS-treated group, whereas only minimal changes in ROTEM analysis were noted for the HES-treated group,; mean values of ROTEM variables remained within the reference intervals in both groups. In dogs with normal ROTEM profile, no relevant clinical signs associated to hemostatic alterations were reported. Further studies are needed to confirm the dose related effect of HES or HS administration on coagulation.

Key words: coagulation, thromboelastometry, hydroxyethyl starch, hypertonic saline

Abbreviations:

aPTT activated partial thromboplastin time

CT clotting time

CFT clot formation time

GDV gastric dilation and volvulus

HES hydroxyethyl starch

HS hypertonic saline

DIC disseminated intravascular coagulation

MCF maximum clot firmness

MCE_{PLT} platelet contribution to maximum clot elasticity

PT prothrombin time

ROTEM thromboelastometer

ROTEG thromboelastography

Introduction

Intravenous (IV) fluid therapy for resuscitation from shock differs by type of fluid, dosage, side effects, and indications. The two major categories are represented by crystalloid and colloid solutions [107].

Hydroxyethyl starches (HES) are artificial colloid with widespread use in veterinary medicine for intravascular volume expansion [61]. Side effects reported after HES administration in humans include, coagulopathies, kidney injury, and tissue storage, which seem to be positively related to mean molecular weight, molar substitution, and C2/C6 ratio [61, 108, 109]. Hemostatic alteration more likely result from hemodilution than a direct action of HES macromolecules, which cause a platelet dysfunction with decreased expression of integrin $\alpha_{IIb}\beta_{3,}$ a reduction in clotting factor activities (e.g., factor VIII and von Willebrand factor), decrease fibrinogen polymerization, and impair fibrinolysis [67]. Both *in vitro* and *in vivo* veterinary investigating hemostatic alterations in dogs after blood dilution with HES or after administration of these synthetic colloids, have used HES at different molecular weight, degree of substitution, and dosages. Though most studies found a decrease in platelet aggregation and hypocoagulability, often dependent on HES dosage or type, none to date have reported clinical bleeding [75-78, 80-85].

Hypertonic saline (HS), a type of crystalloid solution with high osmolality, is mainly indicated for *small volume* fluid resuscitation in patients with head trauma or hypovolemic shock [107, 110]. HS administration is associated with benefits and side effects as well: while it may help to reduce endothelial swelling, improve cardiac output, and modulate inflammation, its rapid administration (1 ml/kg/min) may cause bradycardia, hypotension and vomiting [111-114]. Altered coagulation in human has also been reported [115, 116]. Three recent veterinary studies conducted in dog, have shown a decrease in platelet function and hypocoagulability after HS dilution [80, 117, 118].

Gastric dilation and volvulus (GDV) generally affects large-breed dogs. This syndrome causes cardiovascular compromise and systemic alterations that require fluid resuscitation for the initial stabilization phase [119]. In such dogs, the cause of shock is multifactorial: compression of caudal vena cava decreases pre-load, fluids are lost in the gastrointestinal tract, and cardiac arrhythmias, ischemia-reperfusion injury and systemic inflammatory response syndrome could developed [120, 121]. In dogs with GDV, hemostatic abnormalities and disseminated intravascular coagulation (DIC) have been reported in prospective and retrospective studies that used a standard coagulation profile; however, the use of thromboelastography has been described only in one abstract to date [119-125]. Thromboelastometry/thromboelastography better represent the cell-based model of coagulation, because asses hemostasis using whole blood, taking into account both the plasma and cellular components. Using this technique, clinicians are better able to early identify hypocoagulability and hypercoagulability early [14, 40, 42].

The aim of this study was to evaluate, by means of thromboelastometry, the impact on hemostasis of the administration of an intravenous bolus of HES (130/0.4) or HS (7.5%), in dogs resuscitated for gastric dilation volvulus.

Materials and Methods

Study Animals

The study protocol was approved by the Bioethical Committee of the University of Turin (protocol number 47077) and Bologna (DL 26/2014, Project 581). This prospective, randomized, multicenter investigation involved client-owned dogs. The owner gave their written, informed consent for participation.

All dogs enrolled were patients admitted to the Veterinary Teaching Hospital (University of Turin or University of Bologna) for suspected GDV syndrome based on clinical signs. Inclusion criteria were: diagnosis of GDV based on history, clinical signs, abdominal radiographs, and surgical exploration, and evidence of shock (heart rate >130 bpm, poor pulse quality, capillary refill time > 2 s or < 1 s, systolic blood pressure <90 mmHg and venous lactate >2 mmol/L). Exclusion criteria were: administration of non-steroidal anti-inflammatory drugs, corticosteroid, and artificial colloid or blood products in the 4 weeks prior to enrolment in the study, and a history of cardiac, pulmonary, renal or liver failure.

At presentation, clinical data were collected, including recent history and a complete physical examination. Whole blood samples were collected to perform laboratory analysis. For each dog the APPLE fast scoring system was calculated on admission, to stratify illness severity as previously described [126].

After application of a catheter in each cephalic vein, blood samples (T0) were collected for CBC (ADVIA 120 Hematology, Siemens Healthcare Diagnostics, USA), biochemical evaluation (ILAB 300 plus, Clinical Chemistry System, Instrumentation Laboratories, Italy), venous blood gas analysis

(including electrolyte) (ABL 800 Flex; A. de Mori S.p.A., Italy), packed cell volume, total solids, standard coagulation profile (Coagulometer StART, Diagnostica Stago, USA) and thromboelastometric analysis (ROTEM) (ROTEM, Tem International GmbH, Germany). Intravenously fluid therapy with crystalloids solution (15 ml/Kg per 15 minutes of Ringer lactated) was then administered together with methadone 0.2 mg/Kg IV, and thoracic and abdominal radiographs were obtained.

After the radiographic confirmation of GDV, the dogs were randomly assigned to receive HES 130/0.4 at 10 ml/Kg or HS 7.5% at 4 ml/Kg per 15 minutes. If necessary, percutaneous decompression of the stomach was performed during the bolus. On completion of the bolus, whole blood was collected again (T1) for blood gas analysis, packed cell volume, total solids, albumin (ALB), standard coagulation profile and ROTEM analysis. Respiratory rate, heart rate (associated with a constant electrocardiogram monitoring), capillary refill time, metatarsal pulse quality, systolic blood pressure, and rectal temperature were evaluated during all treatment phases.

To estimate the percent change in blood volume obtained after the bolus, the formula previously reported by Silverstein et al. (2005) was applied: [(T0 Hct/T1 Hct)– 1] x 100; where T0 Hct is the PCV before fluid administration and T1 Hct is the PCV at the end of the bolus [127].

After the protocol, at the discretion of the attending physician, boli of fluids were administered until the dog was stable to be anesthetized and perform gastric decompression, gastric lavage and surgery.

Assessment of Hemostasis

Whole blood samples for the coagulation profile were collected by jugular venipuncture (20-gauge needle) and placed into two tubes containing 3.2% trisodium citrate (1 part citrate: 9 parts blood). Samples that were difficult to obtain (e.g., repeated venipuncture attempts, needle repositioning or interruption of blood flow into the tube) were discarded and blood draws were made from the contralateral jugular vein.

Secondary hemostasis was evaluated by means of standard plasma-based assays (PT, aPTT and fibrinogen). Thromboelastometric analyses were performed according to PROVETS guidelines and the analyses run for 30 minutes [96, 97]. Viscoelastic techniques like ROTEM analysis measure clot formation kinetics, clot firmness, and rate of dissolution (fibrinolysis) [40, 42]. For each sample, in-TEM, ex-TEM and fib-TEM profiles were performed to evaluate the intrinsic pathway (activation by ellagic acid), the extrinsic pathway (tissue factor activation), and functional fibrinogen (platelets inactivated with cytochalasin D), respectively. The following parameters were assessed for each profile: clotting time ([CT], s); clot formation time ([CFT], s); maximum clot firmness ([MCF], mm); α angle (α, °); profiles are represented as reaction curves (Fig. 1). CT represents the first phase of fibrin formation, from activation of the test to a clot amplitude of 2 mm; this parameter is mainly affected by the concentration of plasma coagulation factors and coagulation inhibitors (e.g., antithrombin or drugs). [40, 42]. CFT expresses the velocity of clot formation and is affected predominantly by platelet count and function and by fibrinogen activity. MCF, the maximum firmness the clot reaches, is

determined by both platelet count and function and fibrin formation in the presence of factor XIII [40, 42]. The α angle corresponds to the slope of the tangent on the elasticity curve; it describes the kinetics of clot formation and is affected predominantly by platelet count and function and fibrinogen [40, 42]. The reference ranges for these ROTEM parameters were previously established at our institution in 45 healthy dogs [77]

An additional calculated parameter is MCE_{plt} (platelet contribution to maximum clot elasticity), which evaluates platelet contribution to clot elasticity, and is obtained as follows: $MCE_{plt} = MCE_{extem} - MCE_{fibtem}$ [MCE=(MCF*100)/(100-MCF)] [49].

Abnormal ROTEM analysis was defined as more than one ROTEM parameter outside of the maximum or minimum values of our reference interval, in a single profile (Table 1). Changes in parameters that characterize a hypercoagulable trend are a decrease of CT or CFT and increase MCF or α angle, whereas an increase in CT or CFT, and a decrease in MCF or α angle indicate a trend toward hypocoagulable.

Statistical Analysis

Data were entered in an ad hoc database, analyzed with Stata 14.2 (Stata Statistical Software: Release 11. StataCorp LP, USA), and tested for Normality by Shapiro-Wilk test. To verify the absence of differences between the two groups, t-Student test was performed when data resulted Normally distributed, otherwise the Wilcoxon rank-sum test was used. To assess the differences between time T0 and time T1, ANOVA for paired data was used when data were normally distributed, otherwise the Wilcoxon matched-pairs signed-ranks test was used. ANOVA or Friedman were also used to assess the differences between the two groups and the two consecutive times.

A value of $P < 0.05$ was considered significant.

Results

Twenty six dogs were included in the study: 13 in the HES-treated group and 13 in the HS-treated group. Three patients in the HS group were excluded: 2 for technical reasons (ROTEM malfunction), and another 1 that died before the end of protocol. The HES-treated group was composed of 7 females (2 entire and 5 spayed) and 6 males (5 intact and 1 neutered), the median age was 10 years (min 1-max 13) and the median body weight was 35 kg (min 17-max 55); breeds included were: Bloodhound (n=1), Boxer (n=1), Chow chow (n=1), Hound dog (n=1), Italian Mastiff (n=1), Pyrenean Mountain Dog (n=1), Dobermann (n=2), Mixed breed (n=2) and German shepherd (n=3). The HS-treated group included 4 females (1 entire and 3 spayed) and 6 males (5 intact and 1 neutered), the median age was 10.5 years (min 2-max 14) and the median body weight was 37 kg (min 20-max 61); breeds comprised were: Bull Mastiff (n=1), Great Dane (n=1), Leonberger (n=1), Pit bull (n=1), German shepherd (n=2) and Mixed breed (n=4).

Results at baseline (T0)

ROTEM values and laboratory results parameters of interest are presented in tables 1 and 2, respectively. At baseline (T0), the HES-treated group was characterized by: 1/13 dogs was anemic (PCV < 37%), 2/13 were thrombocytopenic (platelets <128x10^9/L), 7/13 had albumin level outside the lower reference range (ALB < 3 g/L), and 5/13 had lactated > 6 mmol/L. None had PT or aPTT outside of the upper reference range, fibrinogen level was low in1/13 (< 1.45 g/L) and increased in 1/13 (> 3.85 g/L). (Table1) The median value of APPLE fast score was 24 (min 18- max 41), 4/13 dogs had gastric necrosis, 3/13 underwent gastrectomy, and 1/13 was euthanized for economic reasons.

At T0, the HS-treated group was characterized by: none anemic dog (PCV < 37%), 1/10 was thrombocytopenic (platelets <128x10^9/L), 5/10 had an albumin level outside the lower reference range (ALB < 3 g/L), and 5/10 had lactated > 6 mmol/L. None had PT or aPTT outside the upper reference range; the fibrinogen level was low in 1/10 (< 1.45 g/L). (Table1) The median value of APPLE fast score was 22.5 (min 10- max 40), 1/10 dogs had gastric necrosis, 1/10 underwent gastrectomy, and 4/10 were euthanized for economic reasons.

There were no statistically significant differences at baseline (T0) between the two groups in age, body weight, PCV, TS, serum albumin concentration, PT, aPTT, fribinogen level, PLT number, lactate concentration, Na, Cl, APPLE score and ROTEM values.

At T0, all dogs except 3 dogs in the HES-treated group had ROTEM tracings classified as normal. Hypercoagulability was detected in 1/13 dogs and hypocoagulability in 2/13 dogs, both hypocoagulable of which had normal PT and aPTT and low platelet count, while one had low fibrinogen level (Table 3).

Comparison between T0 and T1

Table 1presents ROTEM values, standard coagulation profile, and platelet count obtained at the two time points (T0 vs. T1) and results of comparisons. Statistically significant differences between T0 and T1 in the HES-treated group were: an increase in CFT (P = 0.046), a decrease in MCF (P = 0.002) in the ex-TEM profile, and a decrease in MCF (P=0.0117) in the fib-TEM profile. No difference was found between PT and aPTT concentration, either at baseline that after the bolus, whereas a statistically significant decrease in fibrinogen level was observed (P=0.0005).

Statistically significant differences between T0 and T1 in the HE-treated group were: increased CT (P=0.048) and CFT (0.0019), and decreased MCF (P=0.031) and α angle (P=0.036) in the ex-TEM profile; a decrease in α angle (P=0.036) in the in-TEM profile; a decrease in MCF (P=0.017) in the fib-TEM profile, and a decrease in MCE_{plt} (P=0.021). No difference was found in aPTT before and after the bolus administration, whereas there was a statistically significant increase in PT (P=0.0039) and a statistically significant decrease in fibrinogen concentration (P=0.027).

After HES bolus, a statistically significant decrease was found in PCV (P=0.003), TS (P=0.0005) and albumin (P=0.0002); whereas a statistically significant increase was shown in Cl (P=0.0005). (Table2)

After HS bolus, statistically significant decrease was found in PCV (P=0.0001), TS (P=0.0028), and ALB (P=0.0044); whereas a statistically significant increase was shown in Cl (P=0.0003), Na (P=0.0008). (Table2)

After bolus (T1), no difference was found between HES and HS-treated groups in the delta percent change of blood volume, PCV, TS and ALB, whereas there was a statistical significant difference in ex-TEM MCF (P=0.0014), that increase in HS group.

ROTEM tracings for the two hypocoagulable dogs at T1 showed a continuous hypocoagulable state after HES administration, with a further decrease in fibrinogen level in dog 4 and an increase in PT and aPTT outside the reference range in dog 7 (Table 3). In these dogs, a tendency to bleed was observed during or after surgery. Postsurgical abdominal bleeding was noted in dog 4 and the hemorrhage, hemodynamic instability, and coagulopathy were resolved with transfusion of fresh frozen plasma. Dog 7 experienced bleeding during surgery, followed by epistaxis and hemodynamic instability during recovery from anesthesia. The owners refused other treatment and opted for euthanasia.

Discussion

The present study evaluated the possible negative effects on coagulation of two infusion solutions (HES and HS) administered as a bolus during the resuscitation phase in dogs affected by GDV.

Bolus administration has produced only minimal changes in ROTEM parameters in the HES-treated group and a trend toward hypocoagulability in the ex-TEM profile for the HS-treated group. The clinical relevance of these results is questionable, since the mean values obtained at T1 remained within the reference range. Standard coagulation profile assessment showed no changes in PT and aPTT in the HES-treated group, a statistically significant increase in PT in the HS-treated group (within the reference interval), and a statistically significant decrease in fibrinogen level in both groups (within the reference interval). Similar results on standard coagulation profile were observed by Seshia et al. (2018) after administration of 5 ml/Kg of HS over 15 min and 20 ml/Kg of HES over 20 minutes, in healthy dogs [128].

ROTEM results have shown a statistically significant increase in CFT and a decrease in MCF in the ex-TEM profile and a decrease in MCF in the fib-TEM profile, in the HES-treated group. These alterations, observed between T0 and T1, indicate a decrease in clot firmness and could be related to a decrease in fibrinogen concentration and platelet function. In ROTEM analysis, CFT, α angle, and MCF parameters in particular can be influenced by some sample features such as platelet count, fibrinogen concentration, and hematocrit [40] A decrease in MCF in both ex-TEM and fib-TEM profiles (in the latter, platelets are inhibited by cytochalasin D) and no change in MCE_{plt}, could implied that fibrinogen impairment is the major determinant of these ROTEM changes.

Only three *in vivo* studies have evaluated changes in hemostasis following HES 130/0.4 administration [83, 86, 128]. Reutler et al. (2017), after a single bolus administration of HES (15 ml/kg over 30-40 min) in dogs undergoing general anesthesia, detected ROTEM results similar to ours

(significant increase in in-TEM CFT, and a significant decrease in ex-TEM, in-TEM, and fib-TEM MCF) [86]. Also, Gauthier et al. (2015) found a hypocoagulable trend with ROTEG (increase in K, and decrease in MA and α angle), after bolus administration of 40 ml/kg over 30 min in dogs with induced systemic inflammatory response syndrome [83]. Finally, Seshia et al. (2018) administered 20 ml/Kg of HES over 20 min in healthy dogs and observed a decrease in MCF in the in-TEM and ex-TEM profiles [128]. However, the ROTEM analysis was performed 1 hour after completion of bolus.

In the HS-treated group, several ROTEM parameters were different between T0 and T1: a statistically significant increase in α angle in the in-TEM profile, a decrease in MCF in the fib-TEM profile, and an ex-TEM profile indicative of hypocoagulability tendency were observed. Moreover, a decrease in MCE_{plt} was indicative of reduced platelet activity.

The potential effects of HS administration on hemostasis could be related to hyperosmolarity, which reduce coagulation efficiency, interferes with platelet function and whole blood coagulation, and impairs clotting factors activity, fibrin formation and cloth strength [129-131]. In human patients undergoing elective craniotomy or suffering from traumatic brain injury *in vivo* administration of HS have caused only minimal changes in ROTEM profiles (increased CFT in the ex-TEM and decreased CT in the in-TEM profiles) or no changes at all, respectively [132, 133]. The effect of bolus administration of HS on hemostasis is dose and osmolality dependent. The results of previous studies could be affected by the lower osmolality of the hypertonic solution used (HS 3%) and the different dose administered (1-3 mg/Kg Vs. 4 ml/Kg) [132, 133]. Recent *in vitro* and *in vivo* veterinary studies have demonstrated a negative dose-dependent HS effect on canine hemostasis [80,117, 118].

In vitro studies have found impairment of CFT and MCF in ex-TEM profile of ROTEM analysis, after whole blood dilution with HS (using HS at two different osmolality, 3 and 7.2%), but the *in vitro* studies only partially reflect the effects induced by an *in vivo* condition, because they evaluate a close and static system [80, 117]. A recent *in vivo* study has assessed whole blood coagulation by means of ROTEM and platelet function using PFA-100 in dogs with intracranial hypertension treated with HS (7.2%) or mannitol [118]. The authors have reported that HS decreases platelet function, but only a decrease in CT in the fib-TEM profile was observed in the ROTEM analysis. The discrepancy between their and our results could be explained by the different amount of isotonic crystalloid administered and the differences in dog population. Indeed, our dogs were in shock and had hypovolemia, hypoperfusion, and most had acidosis. After the HS bolus administration, an increase in sodium and a decrease in pH were recorded (not statistically significant but values were below the reference interval), with worsening of acidosis and increase in osmolality, all factors that might have affected hemostasis in these patients.

Regarding the other laboratory parameters evaluated, a statistically significant decrease in PCV, TS and ALB were noted in both groups at T1, indicating a potential hemodilution effect consequent to both HES and HS administration. The amount of hemodilution appeared similar in the two groups, because PCV, PT, ALB and the delta percent change of blood volume were not different at T1.

Despite the significant changes observed in several ROTEM parameters in HS-treated group, and the few changes found in the HES-treated group, comparison between the two groups at T1 has shown only a statistically significant increase in ex-TEM MCF in the HS-treated group. This finding could indicate that HS has a greater effect the than HES on coagulation, but further studies with a larger sample size are needed to confirm this result.

Unlike PT and aPTT, ROTEM analysis has identified two dogs as hypocoagulable at baseline. After bolus administration of HES, the ROTEM values worsened and clinical bleeding developed in both dogs, during or after surgery. Studies evaluating coagulation in dogs with GDV have reported multiple hemostatic abnormalities at hospital presentation, mainly indicative of hypocoagulability, due to consumption of clotting factors and platelets caused by DIC [122, 124]. In these previous studies, coagulation was investigated by means of standard coagulation profile, platelet count, antithrombin, D-dimers and fibrin degradation products. Only one abstract published to date has described the use of a viscoelastic technique (ROTEG) in canine patients with GDV, reporting that dogs with baseline ROTEG values outside the reference range had higher mortality than dogs without abnormalities [125].

The present study has several limitations. The small sample size limits the ability to generalize the results obtained. In addition, there was no control group treated only with isotonic crystalloids, to determine the amount of changes in hemostasis due to hemodilution versus a direct effect of HES or HS. It would have been useful to determine the platelet count also at T1, to identify a decrease in platelet number that could influence ROTEM parameters as like CFT, MCF, and α angle, although the MCE_{PLT} assessment allowed for evaluation of platelet contribution. Finally, authors have evaluated hemostatic changes after a bolus administration of HES or HS, and we don't know what happens to coagulation at the end of resuscitation.

In conclusion, the clinical relevance of hemostatic impairments detected by ROTEM analysis after bolus administration of HES or HS in dogs with GDV remains to be defined. Further studies are needed to better understand the dose-related effects of HES or HS administration on dog coagulation.

Table 1: ROTEM analysis and standard coagulation profiles.

	HES GROUP N=13		**HS GROUP** N=10		**Institutional reference intervals**
ROTEM	**T0**	**T1**	**T0**	**T1**	
In-TEM					
CT (s)	162 (127-365)	151 (113-223)	170 (134-220)	190 (155-240)	126-363 s
CFT (s)	115 (40-368)	120 (47-465)	88 (58-160)	104 (57-191)	47-224 s
MCF (mm)	58 (41-73)	58 (39-71)	62 (50-72)	57 (44-70)	50-75 mm
α angle (°)	68 (41-82)	68 (36-81)	74 (62-79)	71* (60-78)	55-81 °
Ex-TEM					
CT (s)	47 (30-169)	46 (26-110)	40 (30-70)	42* (37-85)	29-92 s
CFT (s)	102 (44-365)	130* (51-463)	85 (56-152)	119* (62-148)	54-275 s
MCF (mm)	62 (39-89)	58* (36-76)	65 (54-81)	58* (52-86)	36-73 mm
α angle (°)	73 (33-83)	65 (38-83)	75 (60-82)	70* (62-79)	47-79 °
Fib-TEM					
CT (s)	51 (28-59)	44 (27-473)	39 (32-73)	44 (29-78)	14-102 s
MCF (mm)	12 (5-33)	10* (4-23)	14 (10-24)	11* (7-25)	6-26 mm
$MCE_{platelet}$	156 (59-760)	128 (52-287)	154 (100-409)	121* (101-261)	50-235
Standard coagulation					
aPTT (s)	12.4 (12-14.2)	12.4 (12.1-19.8)	11.3 (9-15.2)	10.9 (9.8-15)	12-16 s
PT (s)	7.8 (6.1-9.5)	7.9 (6.4-11.4)	6.9 (6.3-9)	7.8* (6.4-9.5)	8-10 s
Fibrinogen (g/L)	2.4 (1.3-4)	2.1* (0.9-2.7)	1.9 (0.5-2.8)	1.5* (1.1-2.1)	1.5-4.50 (g/L)

Values are expressed as median (minimum-maximum).

In-TEM, intrinsic thromboelastometry pathway; **Ex-TEM**, extrinsic thromboelastometry pathway; **Fib-TEM**, functional fibrinogen;

CT, clotting time; **CFT**, clot formation time; **MCF** maximum clot firmness; **PT**, prothrombin time; **aPTT**, activated partial thromboplastin time;

T0, blood sample collected before bolus; **T1**, blood sample collected after bolus;

HES group; dogs that received a bolus of hydroxyethyl starch 130/0.4; **HS** group, dogs that received a bolus of hypertonic saline 7.5%.

Institutional reference interval for ROTEM parameters are expressed as 95% confidence intervals [77]

* Indicates statistically significant differences between T0 and T1 (P< 0.05).

Table 2: Laboratory parameters of interest.

	HES GROUP N=13		**HS GROUP** N=10		**Institutional reference intervals**
	T0	**T1**	**T0**	**T1**	
PCV (%)	50 (30-55)	40* (28-48)	43.5 (39-51)	37* (28-42)	37.5-58.3 %
Platelet count (x 10E09 cell/L)	168 (88-624)		239.5 (104-456)		128-543 x10E09 cell/L
Total Solid (g/L)	0.65 (0.58-0.92)	0.55* (0.4-0.76)	0.74 (0.52-0.89)	0.6* (0.4-0.75)	0.55-0.72 g/L
Albumin (g/L)	0.29 (0.24-0.39)	0.22* (0.13-0.32)	0.3 (0.19-0.34)	0.25* (0.16-0.3)	0.3-0.39 g/L
Chloride (mmol/L)	114 (82-119)	115* (90-122)	116 (107-130)	129* (109-139)	109-120 mmol/L
Sodium (mmol/L)	146 (134-154)	145 (134-151)	147 (134-153)	154* (139-161)	140-150 mmol/L
pH	7.33 (7.22-7.39)	7.35 (7.16-7.4)	7.31 (7.11-7.39)	7.28 (7.15-7.32)	7.33-7.37

Values are expressed as median (minimum-maximum). PCV, microhematocrit, T0, blood sample collected before bolus; T1, blood sample collected after bolus; HES group; dogs that received a bolus of hydroxyethyl starch 130/0.4; HS group, dogs that received a bolus of hypertonic saline 7.5%.

* Indicates statistically significant differences between T0 and T1 (P < 0.05)

Table 3: Altered ROTEM tracings in 3 dogs, before and after bolus administration of hydroxyethyl starch 130/0.4 (HES group).

	Dog 4		**Dog 7**		**Dog 8**		**Institutional reference intervals**
ROTEM	**T0**	**T1**	**T0**	**T1**	**T0**	**T1**	
In-TEM							
CT (s)	140	127	182	223	141	113	126-363 s
CFT (s)	206	**390**	**368**	**465**	**40**	47	47-224 s
MCF (mm)	50	**41**	**41**	**39**	73	71	50-75 mm
α angle (°)	59	**42**	**41**	**36**	**82**	81	55-81 °
Ex-TEM							
CT (s)	**118**	**104**	**169**	**110**	40	34	29-92 s
CFT (s)	**295**	**463**	**365**	**455**	**44**	51	54-275 s
MCF (mm)	45	36	39	37	89	76	36-73 mm
α angle (°)	53	**38**	**41**	**42**	**81**	**81**	47-79 °
Fib-TEM							
CT (s)	59	85	57	**473**	37	27	14-102 s
MCF (mm)	**5**	**4**	**5**	**4**	**33**	23	6-26 mm
$MCE_{platelet}$	77	52	59	55	**760**	287	50-235
Standard coagulation							
aPTT (s)	12	12.5	13.5	**19.8**	11.2	11.8	12-16 s
PT (s)	8.5	9.4	9.4	**11.4**	8	8.6	8-10 s
Fibrinogen (g/L)	**1.29**	**0.88**	1.73	1.82	4.04	2.67	1.5-4.50 (g/L)
Platelet count (x 10E09 cell/L)	**101**		**88**		624		128-543 x10E09 cell/L

In-TEM, intrinsic thromboelastometry pathway; **Ex-TEM,** extrinsic thromboelastometry pathway; **Fib-TEM**, functional fibrinogen;

CT, clotting time; **CFT**, clot formation time; **MCF** maximum clot firmness;

PT, prothrombin time; **aPTT**, activated partial thromboplastin time.

Bold values are outside the reference interval. Institutional reference interval for ROTEM parameters are expressed as 95% confidence intervals[77]

3.3 ADMINISTRATION OF HYDROXYETHYL STARCH (130/0.4) AS A CONSTANT RATE INFUSION IN HYPOALBUMINEIC DOGS: EVALUATION OF EFFECTS ON PLASMA COLLOID OSMOTIC PRESSURE.

Submitted on December to the **Veterinary Anaesthesia and Analgesia**. Currently under revision.

Abstract

Objective – Evaluate the effects of two constant rate infusion of hydroxyethyl starch (HES) 130/0.4 on plasma colloid osmotic pressure (COP), in hypoalbuminemic dogs.

Study design – Cohort study.

Animals – 24 client-owned dogs.

Methods – Hypoalbuminemic dogs (albumin < 2 g/dl) with normal perfusion parameters requiring intravenous fluid therapy, were enrolled. In addition to crystalloid, HES 130/0.4 was administered as a constant rate infusion over 24 hours at 1 ml/kg/h (group 1, n=15) or at 2 ml/kg/h (group 2, n=9), in order to support plasma COP. Before infusion, a blood sample was collected to perform cell blood count, serum electrophoresis and serologic tests for some infective diseases. Plasma COP, albumin, packed cell volume and total solids were evaluated serially at baseline (T0) and then at 6, 12 and 24 hours after the start of infusion.

Results –Twenty-four dogs were included. No statistically significant differences in COP were found between the two groups; however a high level of variability has been identified within the single individual: each dog responds to the infusion of HES in an unpredictable way. Among the other laboratory analysis, packed cell volume was significantly decreased in group 1 at T12 and T24 compared with T0 ($p<0.001$ in both cases) and total solids were significantly increased in group 2 at T12 and T24 compared with T0 ($p<0.008$).

Conclusion and clinical relevance - Infusion of HES 130/0.4 at dose of 1 ml/Kg/h and 2 ml/Kg/h per 24 hours, administered in hypoalbuminemic dogs didn't change the plasma COP.

Introduction

Colloid osmotic pressure (COP) is the pressure exerted by macromolecules across a semipermeable membrane, and it is proportional to the number of molecules present, irrespective of their size [134]. Albumin is the most plentiful protein in the body and accounts for about 60 to 70% of plasma COP, whereas globulins and fibrinogen have a limited effect [51, 135]. Conventionally, the Starling equation is used to describe the distribution of fluids from the capillary into the interstitial space as the result of the equilibrium between hydrostatic pressure and plasma COP [134, 136].

Accordingly, any condition that increases the hydrostatic pressure or decreases the COP could cause fluid movement into the interstitial space [given a normal filtration coefficient (K_f) and reflection

coefficient (σ)]. Some 50 years ago, a protein layer on the luminal surface of the endothelium was discovered: the endothelial glycocalix layer is a web of glycoprotein and proteoglycans that covers the luminal side of endothelial cells, separating plasma and erythrocytes from the subglycocalyx space [58, 59]. The introduction of the concept of subglycocalyx COP, in addition to plasmatic COP, has led to a revision of the Starling equation [58, 59].

The diseases associated with a decrease in COP are primarily those that cause a reduction in plasma proteins, particularly albumin. Several equations have been derived from plasma protein concentration to monitor the plasma COP, but because a poor correlation exists between calculated and measured COP, especially in critically ill patients, COP needs to be determined by direct measurement [137, 138]. In patients with a decrease in intravascular COP fluid therapy poses challenges, because of the risk of further decreasing COP and increasing fluid filtration into the interstitial space, with formation of peripheral edema and effusion. Moreover, the glycocalyx is semipermeable to certain macromolecules such as albumin, and increases of fluid movement to the interstitium may increase the loss of these proteins [139, 140].

Synthetic colloids are fluids characterized by large molecular size. In normal conditions colloid molecules are retained within the vessels longer than crystalloids, resulting in longer-lasting plasma volume expansion, and should contribute to support plasma COP [61, 141, 142]. Hydroxyethyl starches (HES) are the most widely used synthetic colloids, and their pharmacological properties (oncotic effect, excretion and half-life) depend on their mean molecular weight, molar substitution, and C2/C6 ratio [143]. The intravascular retention time of the molecules and the oncotic effect, are related to molar substitution and the C2/C6 ratio, which regulate the rate of HES degradation by plasma α-amylase [61, 144]. In human studies, HES administration has been associated with some side effects such as tissue accumulation, anaphylactic reaction, hypocoagulability and acute kidney injury [50, 51].

Few in vivo studies have assessed COP after HES administration in canine patients [82, 145-147]. Two, in particular, have evaluated the effectiveness of a bolus of HES to increase in COP and time lasting, in hypoalbuminemic dogs. Administration of a single bolus of HES has resulted in a transient increase in COP, indicating that multiple doses or continuous administration could be necessary to maintain the rise in COP, although these previous studies have used different doses of HES and no relationship was found between dose and effects [145, 146]. Other recent studies have obtained contrasting results: Gauthier et al. (2014) have observed an increase in COP after the administration of a bolus of HES, in both healthy dogs and dogs with induced systemic inflammatory response syndrome (SIRS), whereas Chohan et al. (2011) found no similar increase in healthy anesthetized dogs [82, 147].

In veterinary medicine, the administration of HES as a CRI, at a rate of 1-2 ml/Kg/h (0.45-0.9 ml/lb/h) has been reported, but no studies to date have evaluated the efficacy of this protocol to increase COP [50, 51]. The aim of the present study was to evaluate the effect of HES 130/0.4 administered as a CRI on plasma COP in hypoalbuminemic dogs.

Materials and Methods

This randomized, clinical prospective study involved client-owned dogs. The protocol was approved by the Bioethics Committee of the author's University, and the owners of all dogs recruited for participation in the study were informed about the study protocol and gave their written consent.

Animals

Adult dogs (>1 year) with hypoalbuminemia [albumin <2 g/dl (20 g/L)]were recruited among patients admitted to our Veterinary Teaching Hospital for hospitalization. Enrolled patients required intravenous fluid therapy to restore ongoing fluid losses and/or treat dehydration due to their underlying disease (e.g., increased losses, anorexia, and dehydration). Exclusion criteria were the presence of cardiac, pulmonary, renal or liver failure, abnormal perfusion parameters (e.g. heart rate >130 bpm, poor pulse quality, capillary refill time > 2 s or < 1 s, systolic blood pressure <90 mmHg and venous lactate >2 mmol/L), the suspicion of multiple myeloma and positive serological for *Ehrlichia canis* and/or *Leishmania infantum*.

Study Design

After placement of a catheter in a peripheral vein, fluid therapy with crystalloids solution (Ringer lactated) was administered and the amount calculated considering the percentage of dehydration, ongoing losses and maintenance daily requirement. To support colloid osmotic pressure, HES was added as a constant rate infusion (CRI) and the dogs were randomly assigned to receive 1 ml/Kg/h (0.45 ml/lb/h) or 2 ml/Kg/h (0.9 ml/lb/h) of HES for at least 24 hours [50,51], by a computer-generated program (Microsoft Excel, Redmond, WA, USA). The pharmacological treatment begun at T0 remained unchanged during colloid infusion; if necessary for the wellness of the patient, drugs were added, and the dog was removed from the study.

Respiratory rate, heart rate, capillary refill time, systolic blood pressure (using Doppler), metatarsal pulse quality and hydration status were evaluated every 4 hours; body weight was obtained twice daily.

Samples Collection and Analysis

Before infusion (T0), a blood sample was collected to perform CBC (ADVIA 120 Hematology, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) analysis with blood smear evaluation, serum elettrophoresis, creatinine, urea, glucose, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase (ILAB 300 plus, Clinical Chemistry System, Instrumentation Laboratories, Milan, Italy) and serological tests for Ehrlichia canis, Borrelia burgdorferi, Anaplasma phagocytophilum, Anaplasma platys (Snap 4 Dx, IDEXX Laboratories, Westbrook, Maine, USA) and Leishmania infantum (Snap Leishmania Test, IDEXX Laboratories, Westbrook, Maine, USA). Plasma COP (Osmomat 050, Gonotec, GmBH, Berlin, Germany), albumin,

venous blood gas analysis and electrolytes (ABL 800 Flex, A. DE MORI S.p.A., Milano, Italy), packed cell volume (PCV) and total solids were evaluated serially at T0, 6, 12 and 24 hours after initiation of infusion (T6, T12, T24, respectively). For COP evaluation, whole blood was collected in tubes with lyophilized heparin, and plasma was separated by centrifugation. The plasma COP was measured by means of Osmomat 050, according to manual of instructions. The instrument delivers two consecutive measurements for each sample analyzed; the comparison of both values allows the evaluation of the quality of measurement result.

Determination of reference intervals

The institutional reference values for plasma COP were determined in adult dogs admitted to the hospital for routine neutering or blood donation. Inclusion criteria were healthy status, based on complete history and physical exam, and updated prophylaxis for ectoparasites and *Dirofilaria immites*. Blood samples were collected to evaluate CBC, renal and hepatic profile, albumin, serum elettrophoresis, plasma COP, packed cell volume and total solid.

As previously described, the plasma for COP analysis was obtained from the centrifugation of the heparinized whole blood and the COP determination was assessed by the Osmomat 050.

Statistical analysis

Data were collected and entered in an *ad hoc* database.

As the Osmomat 050 returns two measurements of the same sample, the intraclass correlation coefficient (ICC) was calculated to estimate the reliability of the two measurements. A test for Normality based on skewness, one based on kurtosis and another combining the two tests into an overall test statistic were performed. Levene's robust test statistic was applied to verify the equality of variances. To verify the homogeneity of the two groups at T0, Student's T-test for normally distributed data was performed; otherwise the Wilcoxon rank-sum test was used.

To compare the results obtained from samples collected at the four time points (T0, T6, T12, T24), a multilevel linear mixed model was used and adjusted for repeated measures, where the random effect was given by the individual and the fixed effect by the time (T0, T6, T12, T24). A similar model was performed to adjust for the disease. Bonferroni adjustment was applied as needed. The Residual Intraclass Correlation for pairs of responses at the individual level of the model was also calculated to verify variability among individuals. The Wilcoxon matched-pairs signed-ranks test was applied to not normally distributed parameters.

All statistics were performed using Stata 14.1 (Stata Corp 14.1, Special Edition College Station, Texas, USA). The level of significance was set at $p<0.05$.

Results

Dogs

A total of 29 dogs were enrolled to evaluate changes in plasma COP during a constant rate infusion of HES at two dosages: 15 in group 1 (1 ml/kg/h) and 9 in group 2 (2 ml/kg/h). Dogs excluded were affected by cardiovascular disease (n=2), by hepatic failure (n=1) and by leishmaniosis (n=2). Group 1 was composed of 6 intact males and 1 castrated, 6 intact females and 2 spayed, with a median age of 7 years (min 1–max 12); median body weight was 26.7 Kg (min 5–max 39) [58.8 lb (min 11–max 85.9)] and the breeds included mixed breeds (n=8), German Shepherd (n=2), Pinscher (n=1) and one each of Rottweiler, Australian shepherd, Boxer and Border collie.

Group 2 was composed of 3 intact males, 4 intact females and 2 spayed females, with a median age of 7 years (min 2–max 10); median body weight was 17.8 Kg (min 5–max 44) [39.2 lb (min 11–max 97)] and the breeds included Jack Russell (n=2) and one each of Rottweiler, mixed breed, Labrador retriever, Dachshund, Hound dog, Pit bull and English bulldog.

In group 1, 12/15 dogs were affected by diarrhea (5/12 with acute diarrhea and 7/12 with chronic diarrhea), 2/15 by chylotorax and 1/15 by protein losing nephropathy (PLN). In group 2, 6/9 dogs were affected by diarrhea (1/9 with acute diarrhea and 5/9 with chronic diarrhea), 2/9 by septic peritonitis and 1/9 by hypoadrenocorticism. Peripheral edema or effusion (excluding a specific cause of effusion such as chylotorax or peritonitis) was noted in 3/15 in group 1 (with abdominal effusion) and 2/9 in group 2 (with abdominal effusion).

Laboratory analysis

The correlation between the two measurements delivered by the Osmomat 050 was very high: ICC=0.995 (CI95% 0.99-1). Table 1 presents the laboratory results for variables of interest and COP values measured at T0, T6, T12, and T24. There were no statistically significant differences in any of the parameters evaluated at T0 between the two groups, and the COP values were below the lower reference ranges in all dogs (from 17 to 26 mmHg). No statistically significant differences in the COP results were pointed out by the hierarchical model nor between the times, neither between the two different doses (table 2). But a high level of variability has been identified within the single individual: each dog responded to the HES infusion in a different way from the others (figure 1). No statistically significant differences between sampling times were observed even after correction by disease.

For the other laboratory values (Table 1), a significant decrease in PCV was noted in the group 1 (dogs treated with 1 ml/Kg/h of HES) at T12 and T24 over baseline (T0) ($p<0.001$ in both cases); a significant increase in total solids was shown in the group 2 (dogs treated with 2 ml/Kg/h of HES) at T12 and T24 over baseline (T0) ($p<0.008$).

Reference interval

The reference intervals for plasma COP were derived from samples from 64 dogs: 26 were intact males and 4 castrated males, 22 were intact females and 12 spayed females, with a median age of 3 years (min 1 –max 8) and a median body weight of 22.3 Kg (min 5–max 45) [49.2 lb (min 11–max 99.2)]. Clinical parameters were normal and laboratory evaluations were all within our institutional reference range. The breeds included were: mixed breeds (n=31), Golden Retriever (n=7), Border collie (n=6), Labrador retriever (n=3), Cocker Spaniel (n=3), Kurzhaar (n=2), Australian shepherd (n=2), English setter (n=2) Pittbull (n=2), Poodle (n=2) and one each of Epagneul Breton, German shepherd, Dogo Argentino and Czechoslovakian Wolfdog. The institutional reference interval for plasma COP ranged from 17 to 26 mmHg.

Discussion

The reference interval for plasma COP obtained in our institution, is similar to that reported by Rudloff et al. (2000), although different instruments were used [Osmomat 050 (COP: 17 to 26 mmHg) and Wescor 4400 (COP: 21 to 25 mmHg), respectively] [148].

In this study, no changes in plasma COP were identified after infusion of HES 130/0.4 at a dosage of 1 ml/Kg/h (0.45 ml/lb/h) and 2 ml/Kg/h (0.9 ml/lb/h) over 24 hours, administered in hypoalbuminemic dogs. No statistically significant differences in the COP results were pointed out between the sampling times or doses, but high level of variability has been identified within the single individual and each dog responds to the infusion of HES in an unpredictable way. This trend is represented more clearly in the figure 1.

Regarding the other results obtained in this study, a decrease in PCV was observed only in group 1 at T12 and T24. This result could indicate a certain degree of haemodilution, but the concentration of albumin and total solids was unchanged and no haemodilution was seen in group 2 which received a double dose of HES. Unfortunately, authors cannot explain this result.

In group 1, no change in the values of total solids as measured by refractometry was found at any time point , whereas in group 2 an increase was seen only at T12 and T24, though it has been reported that this measurement could be affected by the colloid solutions [106]. Since the refractive index of tetrastarch 130/0.4 is 42 g/L (4.2 g/dl), dilution of blood with colloids could change the refractive index of plasma, but a high dose of infused volume is probably needed to interfere with the refractometer reading [106, 147].

Revision of Starling equation has questioned the clinical utility of measuring plasma COP, because it seems that the main factor responsible for fluids exchange could be the subglycocalix COP and the integrity of the glycocalyx [57- 59, 149]. In the first decade of 2000, it was discovered that Starling's equation overestimated the effect of interstitial fluid COP on fluid exchange between the intravascular and the interstitial space [57, 58]. Above the normal capillary pressure of 20 mmHg, an infusion of colloid solution should increase capillary pressure, raise the volume of filtration to the interstice, but preserve plasma COP; whereas, an infusion of crystalloids should decrease COP and raise the

filtration volume more than colloid solutions [150]. In our study, although COP did not differ before and after HES administration in our population of hypoalbuminemic dogs, colloid infusion may probably have helped to maintain the fluid in the intravascular space, thus decreasing the rate and amount of fluids lost in the interstitial space. This statement remains a hypothesis, since there was no control group that received only crystalloid infusion in the present study. Moreover, the introduction of the concept of glycocalyx raises the question of the need to restore normal COP value in hypoalbuminemic dogs. Indeed, glycocalyx integrity could be more important than increasing plasma COP and products like pooled albumin, plasma and plasma substituted could contribute to capillary sealing, rather than acting on the plasma COP [52, 151].

Previous studies evaluating the changes in COP after HES administration in dogs obtained different results. Direct comparison with our data would be challenging because of the differences in pathological conditions, generation of colloid solutions, and doses and rate of administration [82,145-147]. Two studies have evaluated the effects of a HES bolus in hypoalbuminemic dogs. Smiley et al (1994) have administered HES (not specifying which type) as a bolus at a dose ranging from 9 to 26 ml/Kg (4-11.8 ml/lb) over 6 to 8 hours [145]. They noted a significant increase in mean COP, but no relationship between dose and magnitude of increase [145]. Furthermore, it was reported an improvement in edema or effusion after HES administration. It is difficult to relate this result with an increase intravascular COP, because the revised Starling law states that, since no absorption occur by the capillaries, filtered fluid returns to the intravascular compartment by the lymphatic vessels [58, 59]. Moore et al. (1996) have measured the duration of action of a single dose of HES 450/0.7 [dose ranged from 7.7 to 43.9 ml/Kg (3.5-19.9 ml/lb)] administered over approximately 6 hours, and have found a significant increase in mean COP after HES administration in all dogs, but the effect disappeared within 12 hours after administration [146]. In that study was also observed that the increase in COP was not significant in the dogs with acute gastrointestinal protein loss, whereas in our study the disease it did not appear to influence the COP.

One study have compared the effects of an equal dose of synthetic colloid (HES 130/0.4; 40 ml/Kg (18.1 ml/lb) or saline, administered over a period of 30 minutes, in healthy dogs and dogs with induced SIRS. An increase in COP was observed in both groups treated with HES, with a major increase in the healthy dogs [147]. Interestingly, the rise in COP was greater in the healthy dogs and lasted for 1 to 4 hours, as compared to the 1 to 2 hours noted for the ill dogs. We can hypothesize that, in light of the revised Starling equation, this difference might have been related to an acquired alteration in the glycocalix in the ill dogs, leading to an increase in capillary flow towards the interstitium [52, 59].

Chohan et al. (2011) have obtained different results after evaluating the administration of HES 600/0.75 or lactated Ringer's (both fluids at a dose of 10 ml/Kg, for 20 minutes) in healthy dogs. At 1 hour post-infusion, a significant decrease in COP was observed in both groups, with significantly lower COP in the lactated Ringer's group than in the HES group [82]. The explanation could be that

the dogs were evaluated during anaesthesia and a study of Dismukes et al. (2010) has shown that COP decreases by an average of 5 mmHg in healthy dogs undergoing general anaesthesia [152].

The different results obtained in aforementioned studies could be related to the dose (bolus) and type of colloid (old generation HES with high molecular weight and high grade of substitution) administered. In our study, another reason for the unchanged COP is that we used a new generation HES (130/0.4) administered as a CRI. It would be interesting to evaluate whether HES 130/0.4 administered as a bolus affects the COP and what happen when a CRI follows the bolus. Although, the administration of HES as a CRI, rather than a bolus, might be more indicated in normovolemic and hypoalbuminemic dogs, because the increase in capillary pressure above the normal value increases the volume of transendothelial flow (of fluids and proteins) with a loss of albumin in the interstitial space [150, 153, 154].

At authors knowledge, this is the first study to evaluate the effects of HES 130/0.4, administered as a CRI, on plasma COP in hypoalbuminemic dogs, but some limits could be affected the generalization of these results. One limitation is the lack of a control group treated with an equal dose of crystalloid to evaluate the trend of COP and confirm whether or not it decreases during this type of infusion. Another limitation is the small sample size that could introduce a Type II error. Since the total amount of crystalloid solution, administered for 24 hours, was not recorded, the influence of this variable on our results was not assessed.

Other studies are needed to evaluate the real benefits of HES administration in hypoalbuminemic dogs and to assess a method to determine its, possibly relating the effects to the dosages. Also the possible side effects (e.g., haemostatic and renal effects) that might be associated with the administration of colloid solution need to be investigated and identify whether the negative effects related to HES administration could outweigh the positive ones.

Table 1 Laboratory analysis for variables of interest and COP values measured at T0, T6, T12 and T24 (Group 1 N=15; Group 2 N=9).

Group 1 [1 ml/kg/h (0.45 ml/lb/h)]

Variable	T0	T6	T12	T24
COP (mmHg)	9.2 (6.3-14.3)	9.3 (7.3-14.7)	9.3 (6.9-11.8)	8.9 (6.7-14.7)
Packed cell volume (%)	40 (29-54)	39 (28-53)	37 (28-49)*	39 (25-42)*
Total solids (g/dl)	3.3 (2.8-5.5) [33 (28-55) g/L]	3.5 (2.4-5.4) [35 (24-54) g/L]	3.7 (2.3-5.4) [37 (23-54) g/L]	4.2 (2.4-5.6) [42 (24-56) g/L]
Albumin (g/dl)	1.6 (1.2-1.9) [16 (12-19) g/L]	1.6 (1.1-2.2) [16 (11-22) g/L]	1.6 (1-2) [16 (10-20) g/L]	1.6 (1.1-2.4) [16 (11-24) g/L]
Fibrinogen (mg/dl)	443 (232-1174) [4.43 (2.32-1.17)g/L]			

Group 2 [2 ml/kg/h (0.9 ml/lb/h)]

Variable	T0	T6	T12	T24
COP1 (mmHg)	9.5 (6.1-14.9)	8.4 (6.6-15.3)	9.7 (5.9-14.4)	8.6 (5.9-14.9)
Packed cell volume (%)	38 (25-50)	34 (24-55)	35 (25-55)	34 (24-55)
Total solids (g/dl)	3.2 (2.5-5) [32 (25-50) g/L]	3.5 (2.5-5) [35 (25-50) g/L]	3.6 (2.4-5)* [36 (24-50) g/L]	3.5 (2.7-5)* [35 (27-50) g/L]
Albumin (g/dl)	1.5 (1.3-1.8) [15 (13-18) g/L]	1.5 (1.1-2.1) [15 (13-18) g/L]	1.5 (1.2-2.2) [15 (12-22) g/L]	1.4 (1.1-2.2) [14 (11-22) g/L]
Fibrinogen (mg/dl)	318 (223-741) [3.18 (2.23-7.41)g/L]			

Data are reported as median (minimum-maximum). COP, colloid osmotic pressure.

The institutional reference interval for COP ranges from 17 to 26 mmHg.

* statistically significant difference between T0 and T12 and between T0 and T24, $p<0.05$

Table 2 Mixed model results.

The random effects parameters and the ICC both indicate a great variability among the individuals. No differences between sampling the times, or between the two groups were found.

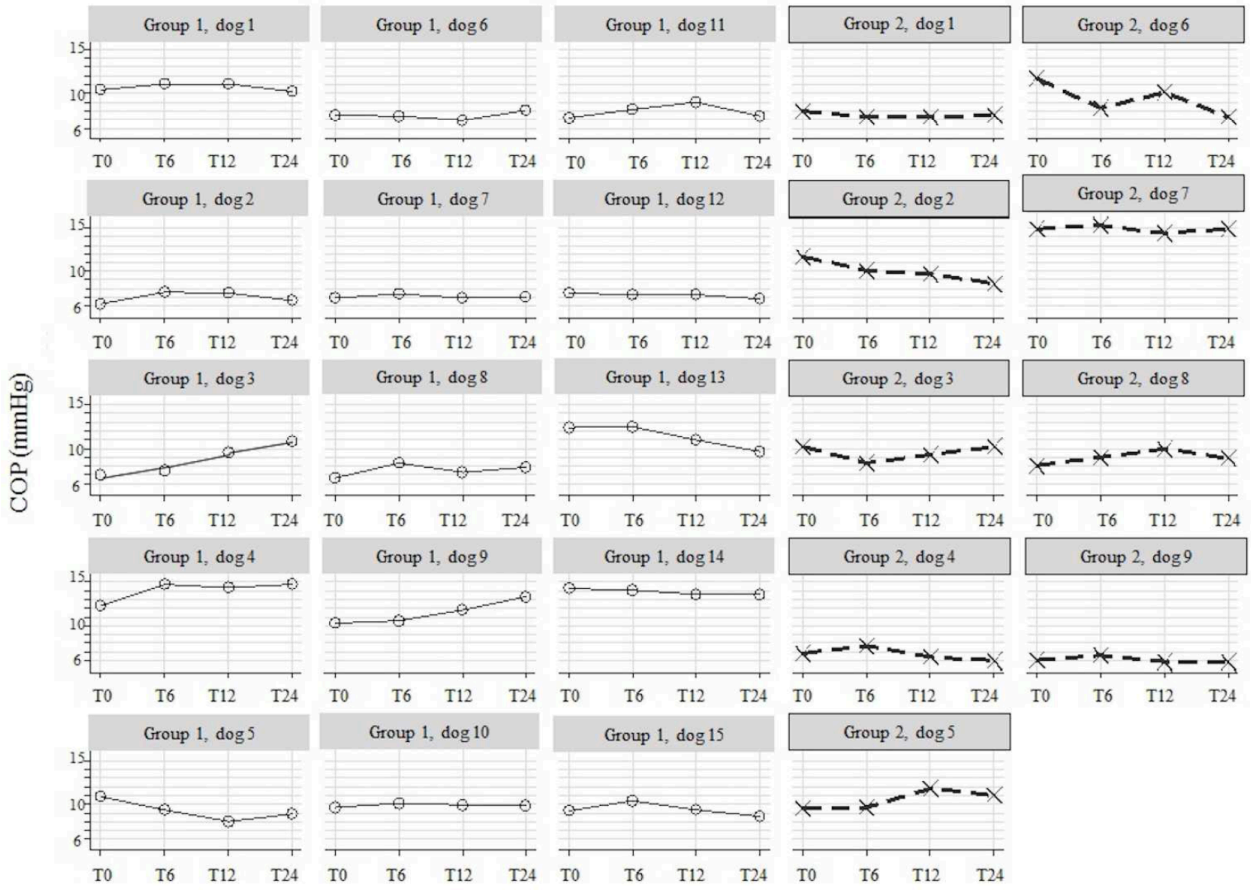
	β	95% Confidence Interval		P>z
Group 1				
T6	0.533333	-0.11315	1.179814	0.106
T12	0.346667	-0.29981	0.993147	0.293
T24	0.34	-0.30648	0.986481	0.303
Group 2				
T0	0.422222	-1.60658	2.451028	0.683
T6	-0.08889	-2.1177	1.939917	0.932
T12	0.2	-1.82881	2.228806	0.847
T24	-0.31111	-2.33992	1.717695	0.764
Reference	9.233333	7.990949	10.47572	0

Random-effects Parameters	Estimate	95% Confidence Interval	
Animal reference	5.211111	2.894211	9.382756
ICC	0.86	0.76	0.93

The reference is the T0 of Group 1. Group 1, CRI at 1 ml/kg/h (0.45 ml/lb/h); Group 2, CRI at 2 ml/kg/h (0.9 ml/lb/h); T0, baseline; T6, 6 hour after the start of CRI; T12, 12 hour after the start of CRI; T24, 24 hour after the start of CRI; ICC, residual intraclass correlation.

Figure 1 Trend of COP values measured at sampling times in each dog.

COP, colloid osmotic pressure; Group 1, CRI at 1 ml/kg/h (0.45 ml/lb/h); Group 2, CRI at 2 ml/kg/h (0.9 ml/lb/h); T0, baseline; T6, 6 hour after the start of CRI; T12, 12 hour after the start of CRI; T24, 24 hour the after start of CRI.



ADDITIONAL RESEARCH PROJECT

4.0 THROMBOELASTOMETRIC ASSESSMENT OF HEMOSTASIS IN NEWBORN PIEMONTESE CALVES.

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Abstract.

We investigated possible age-related differences in coagulation profiles in bovine species by means of rotational thromboelastometric (ROTEM) analysis. We evaluated hemostasis by ROTEM in newborn Piemontese calves at birth (T0), 8 d (T8), and 15 d (T15) of age and compared the ROTEM results obtained in 16 newborn calves with 28 adult Piemontese cattle. Hemostasis was evaluated using standard coagulation tests and ROTEM analysis, obtaining in-TEM, ex-TEM, and fib-TEM profiles. Statistically significant differences in the ROTEM profiles of newborn calves were found between T0 and T8 and between T0 and T15 ($p < 0.05$) but not between T8 and T15. Differences between ROTEM profiles of calves and adults were statistically significant at T0 ($p < 0.05$) but no differences were found at T15 ($p < 0.05$). Hence, ROTEM reference intervals for adult cattle can be used to evaluate profiles in Piemontese calves ≥8 d of age.

Key words: Italy; Piemontese cattle; thromboelastometry; veal calves.

Introduction

The rate of development of the hemostatic system differs among mammals. At birth, hemostasis is immature in the lamb, intermediate in the foal and the pig, and relatively mature in the rabbit. In human infants, the coagulation profile takes several weeks to reach adult values [155-160]. In newborn

calves, activated partial thromboplastin time (aPTT) and prothrombin time (PT) values are within the normal adult range in most studies [156,158, 160-162]. The coagulation system of newborn calves appears to be generally efficient, and the small differences from adults do not affect hemostatic function. For example, the plasma activities of factors VII, VIII:C, IX, X, XI, and fibrinogen concentration are at the lower end of the normal adult range, although the levels of the anticoagulant proteins α2-macroglobulin and antithrombin are markedly lower in calves than in adult cattle [156,158, 160-162].

Conventional coagulation tests rely on technologies that evaluate single steps of coagulation or single plasma factors. Because hemostasis is a dynamic process that also involves cellular elements, viscoelastic techniques, such as rotational thromboelastometry (ROTEM), may better represent hemostasis in vivo [14]. Such techniques require a whole blood sample for analysis and are able to measure clot formation kinetics (the time needed to make a clot), evaluate the mechanical properties of clot (clot firmness), and determine the rate of clot dissolution (fibrinolysis) [40,42]. Blood samples

are processed by adding specific reagents to generate ROTEM profiles: the in-TEM profile for the intrinsic pathway; the ex-TEM profile for the extrinsic pathway; and the fib-TEM profile correlated to functional fibrinogen levels. Thromboelastography and ROTEM analysis have been applied to evaluate hemostasis in numerous species; however, few studies in cattle have been reported to date [40, 42,157,159,163]. The analytical performance of ROTEM and normal reference intervals have been defined in adult cattle and calves in a study population of 48 adult cattle (15 Piemontese females
and 33 males of different beef breeds) and 14 Holstein–Friesian calves (6 mo old).5 The bovine thromboelastogram presented several features distinct from equine and canine tracings, and ROTEM was an accurate assay for the evaluation of hemostasis in cattle. In a later study, the same authors applied ROTEM to investigate the hemostatic effect of lowdose dexamethasone treatment in Friesian calves [157]. Finally, a 2014 study established reference intervals for thromboelastography in dairy cows in different lactation periods (≤30 d postcalving, 31–99 d postcalving, and ≥100 d postcalving) and tested sample stability up to 100 h [163].

Given the increasing application of viscoelastic techniques in cattle, we thought it useful to determine whether there are differences in ROTEM assay results in relation to animal age, breed, or sex, as reported for other biochemical parameters. We therefore evaluated hemostasis by ROTEM
in newborn Piemontese calves at birth and 8 and 15 d of age, and compared the ROTEM results between the calves and adult Piemontese cattle.

Materials and methods

The study protocol was approved by the local Ethical and Animal Welfare Committee of the Department of Veterinary Science, University of Turin. Calves were judged healthy on physical examination, complete blood count (CBC), and standard coagulation profile (PT, aPTT, and fibrinogen).Calves were excluded if born premature, by caesarean section or dystocia, had received pharmacological treatment, or were noted to have clinical signs of hypocoagulability.

ROTEM analysis was performed in adult Piemontese cattle judged healthy on physical examination, CBC, basic biochemical profile, and standard coagulation profile. Animals were excluded if they had received pharmacological treatments within 1 mo before the start of the study, were noted to
have clinical signs of hypocoagulability, were in the final month of pregnancy, or in the lactation period. Whole blood samples were collected by jugular venipuncture (20-ga needle) once from the adults and from the calves within 24 h of birth (T0) and then at 8 d (T8) and 15 d (T15) of age. Samples that were difficult to obtain (e.g.,repeated venipuncture attempts, needle repositioning, or
interruption of blood flow into the tube) were discarded, and blood draws were taken from the contralateral jugular vein. Whole blood was divided into 3 test tubes: 2 containing 3.2% trisodium citratea for analysis of hemostasis, and 1 containing K3 -EDTAb for CBC and analysis of a fresh blood smear. An additional tube was used to obtain serumd for biochemical analysise in the adult

cattle. Secondary hemostasis was evaluated by a standard coagulation profile (PT, aPTT, and fibrinogen) using plasma. For ROTEM analysis,g whole blood samples were stored at room temperature in 3.2% trisodium citrate tubes and analyzed 30 min after collection following the PROVETS guideline [14].

Analyses were performed according to the manufacturer's instructions, using the automated pipette included with the instrument kit, which dispenses 300 µL of blood and 20 µL of activator or inhibitor in a cup for each test run of 30 min [96].

For each sample, in-TEM, ex-TEM, and fib-TEM profiles were obtained to evaluate the intrinsic pathway (with activation by ellagic acid), the extrinsic pathway (with tissue factor activation), and fibrinogen function (platelets inactivated with cytochalasin D), respectively.

The following parameters were recorded for each profile: clotting time (CT; in s); clot formation time (CFT; in s); maximum clot firmness (MCF; in mm); and alpha angle (α; in degrees [°]). The profiles are presented as reaction curves (Fig. 1). CT represents the first phase of fibrin formation, from activation of the test to a clot amplitude of 2 mm; this parameter is mainly affected by the concentration of plasma coagulation factors and coagulation inhibitors (e.g., antithrombin or drugs) [40,42]. CFT expresses the velocity of clot formation and corresponds to the time necessary for the clot to increase from 2 to 20 mm; it is affected predominantly by platelet count and function and by fibrinogen activity. MCF, the maximum firmness the clot reaches and maximum amplitude reached during the test, is determined by both platelet count and function and fibrin formation in the presence of factor XIII [40,42]. The α angle corresponds to the slope of the tangent on the elasticity curve; it describes the kinetics of clot formation and is affected predominantly by platelet count and function and fibrinogen.

The data were entered into an ad hoc database. The normal distribution of data was verified by means of a test for normality based on skewness and on kurtosis. To verify the presence of differences among the 3 groups of calves, when the assumption of normality was respected, an analysis of variance (ANOVA) for repeated measures was performed;

otherwise, the Friedman nonparametric 2-way ANOVA was used. In the case of comparison between 2 groups, the Mann–Whitney 2-sample statistic was performed. Significance was set at $p < 0.05$.

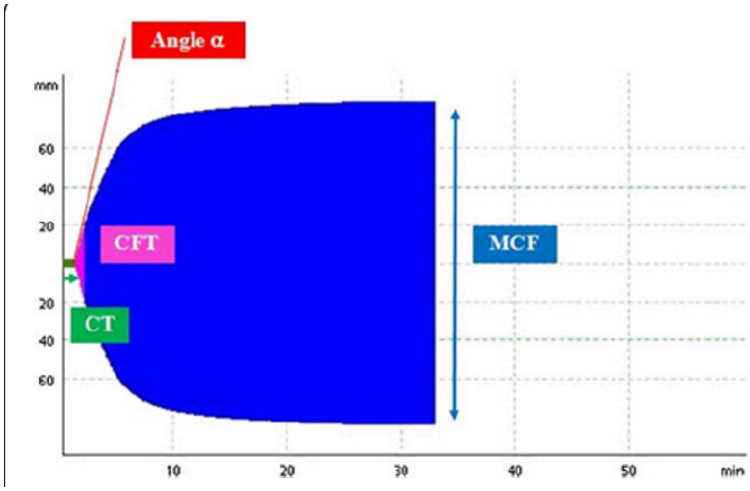

Figure 1: Example of a TEMogram

CT: clotting time**; CFT** clot formation time**; MCF** maximum clot firmness

Results

Sixteen Piemontese calves born on the farm of the Department of Veterinary Science, University of Turin were enrolled. One calf showed an alteration on the standard coagulation profile (increased PT and aPTT) and was excluded. The final study sample was 15 calves (7 male and 8 female). There were no differences in ROTEM profiles between female and male calves (Table 1). There were statistically significant differences in the 3 profiles of the samples obtained at the 3 times.

In the in-TEM profile, MCF was significantly increased at T0 versus T8 ($p = 0.01$); there were no statistically significant differences between T0 and T15 or between T8 and T15. In the ex-TEM profile, CFT was significantly prolonged at T0 versus T8 ($p = 0.001$) and T15 ($p = 0.005$), and the α angle was significantly smaller at T0 than at T8 ($p = 0.001$) and at T15 ($p =0.004$); there were no statistically significant differences between T8 and T15 for any of the parameters. In the fib-TEM profile, only the MCF value was significantly lower at T0 than at T8 ($p = 0.001$) and at T15 ($p = 0.02$); there were no statistically significant differences between T8 and T15.

The aPTT was prolonged at T0 versus T8 ($p = 0.0001$) and T15 ($p = 0.0001$) but there were no statistically significant differences between T8 and T15; there were no significant differences in PT between any of the 3 times (Table 2).

Fibrinogen concentration was significantly lower at T0 than at T8 ($p = 0.009$). Statistically significant differences in CBC values were noted (Table 3). Hematocrit (HCT) was significantly higher at T0 than at T8 ($p = 0.002$) and at T15 ($p = 0.002$) and at T8 than at T15 ($p = 0.005$). Hemoglobin (Hb) was significantly higher at T0 than at T8 ($p = 0.032$) and at T15 ($p = 0.004$) and at T8 than at T15 ($p=0.026$). Platelet count was significantly lower at T0 than at T8 ($p = 0.035$); there were no statistically significant differences in platelet count between T0 and T15 or between T8 and T15.

The total number of adult Piemontese cattle was 28 animals (15 female and 13 male, median age 19 mo, range 18– 78). Statistically significant differences in ROTEM results were observed between adults and calves at T0 (Table 4): MCF on the in-TEM profile was significantly decreased (p =0.009), CFT on the ex-TEM profile was significantly prolonged (p = 0.029), and the α angle was significantly smaller in the calves than in the adults (p = 0.01); CT and CFT on the fib-TEM profile were significantly prolonged (p = 0.004 and p = 0.003, respectively); and MCF and the α angle were significantly smaller (p = 0.008 and p = 0.034, respectively) in the calves. There were no statistically significant differences in ROTEM results between the adults and the calves at T15
(Table 4).

Discussion

The ROTEM tracings at birth were similar to those at 8 and 15 d of age in newborn Piemontese calves. Significant differences in the in-TEM, ex-TEM, and fib-TEM profiles were observed only at T0 versus T8 and versus T15. MCF in the in-TEM profile was lower at T0 than at T8. The ex-TEM profile showed prolonged CFT and a smaller α angle at T0 versus T8 and T15. Because CFT, MCF, and α angle are predominantly affected by platelet number and function, and by fibrinogen, the results from samples obtained at T0 were probably influenced by the low platelet number and fibrinogen level present at T0 [40,42].

Furthermore, MCF in the fib-TEM profile was lower at T0 than at T8 and at T15. Because platelet function was inhibited, only the fibrinogen contributed to MCF: the fibrinogen concentration was low at T0 and increased over the next 15 d [40,42]. Analysis of CBC results showed higher HCT and Hb levels and a lower platelet count at birth than at 8 and at 15 d of age. This observation is consistent with findings from a previous study [161,164]. Erythrocytes are less fragile and larger in fetal calves than in adult cattle [164]. After birth, the mean corpuscular volume of erythrocytes progressively decreases from fetal values of 97 fL to 37 fL during the first 8–12 wk of age, and then declines again until ~2 y of age [164]. Differences in hematological parameters measured at the 3 times could partly account for the differences in the ROTEM results at T0. Indeed, a study using canine whole blood samples showed that ROTEM results were influenced by coagulation factors, platelet and fibrinogen concentrations, as well as by red blood cell mass [47]. Platelet, fibrinogen, erythrocyte, and Hb concentration were significantly correlated with CFT, α angle, and MCF [47]. The relationship between HCT and coagulability, as measured via ROTEM, has been suggested as an artefact of the plasma volume loaded into the tool [47]. Samples with a high HCT provide a lower mass of coagulation factors participating in the ROTEM reaction given a decrease in the liquid component of the blood, thus causing apparent hypocoagulability [96]. Our finding of a low platelet number at birth is shared by others in their study on Holstein dairy calves, which reported that platelet number increased markedly from birth to day 14 and then rose slightly up to day 84 of life [161].

Comparison of the standard coagulation profiles showed statistically significant differences in aPTT and fibrinogen. Compared with subsequent measurements, aPTT at birth was prolonged, but the final value fell within the normal adult interval for our laboratory. This result is in line with published data that report a standard coagulation profile within the adult range for newborn calves [158,160,162]. The fibrinogen level was low at birth, increased significantly at 8 d, and then decreased at T15 (differences not statistically significant), as found by others, who reported a below normal adult range for fibrinogen at birth, followed by a marked increase during the first 7 d of life and a decrease at day 30 [160].

Comparison of ROTEM results of calves at T0 with the results of adult cattle showed differences similar to comparing results of calves at T0 and T8 or T0 and T15; no differences were found between ROTEM results in calves at T15 and adults. The low platelet count, fibrinogen level, and HCT concentrations at birth might have contributed to the differences in the ROTEM tracings between the calves and the adults. Our findings indicate that reference ROTEM intervals for newborn calves should be used at birth, whereas reference intervals of adult cattle can be used to evaluate a ROTEM analysis in calves ≥8 d of age.

The limitations of our study were that the sample size was too small to derive reference intervals and the population included only Piemontese cattle. Further studies are needed to determine whether there are breed-related differences in ROTEM analysis.

TABLE 1: Results of ROTEM analysis in Piedmontese calves (N=15).

In-TEM	**T0**	**T8**	**T15**
CT (s)	304 (203-424)	290 (178-472)	240 (162-440)
CFT (s)	85 (60-291)	59 (36-219)	52 (37-166)
MCF (mm)	73 (54-80)	81 (62-91)*	78 (53-85)
Alpha (°)	73 (44-78)	79 (56-83)	79 (33-82)
Ex-TEM	**T0**	**T8**	**T15**
CT (s)	88 (61-111)	83 (66-101)	75 (65-116)
CFT (s)	120 (89-159)	79 (60-127) *	92 (35-142) #
MCF (mm)	73 (55-83)	79 (68-85)	79 (58-85)
Alpha (°)	71 (66-74)	76 (60-79)*	74 (35-83) #
Fib-TEM	**T0**	**T8**	**T15**
CT (s)	87 (65-128)	85 (70-165)	78 (60-137)
CFT (s)	249 (72-431)	101 (60-426)	168 (48-347)
MCF (mm)	24 (19-39)	36 (24-57) *	32 (23-38) #
Alpha (°)	71.5 (64-78)	77.5 (65-81)	75 (63-81)

Values are expressed as median (minimum-maximum);

CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; α, α angle; °, degrees.

* statistically significant differences between ROTEM values in newborn calves at T0 and T8 ($p \leq 0.05$).

statistically significant differences between ROTEM values in newborn calves at T0 and T15 ($p \leq 0.05$).

TABLE 2: Standard coagulation profile in Piedmontese calves at T0, T8, and T15 (N=15).

	T0	T8	T15	Normal range
PT (s)	29.12 ± 5.63	25.85 ± 2.7	26.11 ± 4.49	18-38
aPTT (s)	42.62 ± 5.39 *, #	34.82 ± 5.16	32.26 ± 4.33	28- 50
Fibrinogen (mg/dl)	295.06 ± 92.02*	394.73 ± 120.97	326.23 ± 111.89	200-500

Values are expressed as mean ± standard deviation; aPTT, activated partial thromboplastin time; PT, prothrombin time.

* statistically significant differences between standard coagulation profile values in newborn calves at T0 and T8 ($p \leq 0.05$).

statistically significant differences between standard coagulation profile values in newborn calves at T0 and T15 ($p \leq 0.05$).

TABLE 3: Complete blood count (CBC) in Piedmontese calves with statistically significant differences between T0, T8, and T15 (N=15).

	T0	T8	T15	Normal range
Hct (%)	34.14 ± 5.75 *, #	30.35 ±5.52§	29.70 ± 5.41	25.8 – 40.1
Hb (g/dl)	11.02 ± 1.88*, #	9.99 ± 1.85§	9.86 ± 1.57	9.8 – 15.3
PLT (x 10^3 cells/μl)	427 ± 105*, #	811 ± 286	805 ± 246	412 – 1003

Values are expressed as mean ± standard deviation;

Hct, hematocrit; Hb, hemoglobin; PLT, platelet.

* statistically significant differences between CBC in newborn calves at T0 and T8 ($p \leq 0.05$).

statistically significant differences between CBC in newborn calves at T0 and T15 ($p \leq 0.05$).

§ statistically significant differences between CBC in newborn calves at T8 and T15 ($p \leq 0.05$).

TABLE 4: ROTEM analysis in newborn calves and adult Piedmontese cattle.

	Newborn calves (N=15)		**Adult cattle** (N=28)
In-TEM	**T0**	**T15**	**In-TEM**
CT (s)	304 (203-424)	240 (162-440)	358 (141-496)
CFT (s)	85 (60-291)	52 (37-166)	73 (45-134)
MCF (mm)	73 (54-80)*	78 (53-85)	78.5 (64-87)
Alpha (°)	73 (44-78)	79 (33-82)	76 (64-81)
Ex-TEM	**T0**	**T15**	**Ex-TEM**
CT (s)	88 (61-111)	75 (65-116)	78 (62-169)
CFT (s)	120 (89-159)*	92 (35-142)	97 (38-183)
MCF (mm)	73 (55-83)	79 (58-85)	76 (69-85)
Alpha (°)	71 (66-74)*	74 (35-83)	74 (61-82)
Fib-TEM	**T0**	**T15**	**Fib-TEM**
CT (s)	87 (65-128)*	78 (60-137)	74.5 (17-96)
CFT (s)	249 (72-431)*	168 (48-347)	105 (46-381)
MCF (mm)	24 (19-39)*	32 (23-38)	31 (19-46)
Alpha (°)	71.5 (64-78)*	75 (63-81)	74 (55-81)

Values of ROTEM analysis in newborn calves are expressed as median (minimum-maximum);
Values of ROTEM analysis in adult Piedmontese cattle are expressed as median (minimum-maximum);
CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; α, α angle; °, degrees.
* statistically significant differences between ROTEM values in newborn calves at T0 and adult cattle ($p \leq 0.05$).

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