The Effect of Hydroxyethyl Starch 130/0.4 On Canine Platelet Function

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Chapters three and four are for publication in scientific journals in which publication are in conjunction with the principle supervisors and other co-authors. The majority of study designs, experimental research and writing for publication were undertaken by myself as primary author with guidance from the principle supervisor and other co-authors.

Chapter three has been published in the American Journal of Veterinary Research, and copyright for publication in this thesis has been granted. Chapter four has been submitted to a scientific journal, and this chapter is consistent with the most recent copy submitted at the time of thesis submission. As a result of this, abbreviations may differ to the remaining thesis, and spelling in these two chapters are in American English.

Ethical approval for the experimental research in chapter three and four in this thesis has been granted by Murdoch University Animal Ethics Committee with approval number R2324/10 and R2398/11 respectively.

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Abstract

Hydroxyethyl starch (HES) 130/0.4 is an artificial colloid solution commonly used to treat shock in dogs. Adverse effects from this solution have been reported in people, including platelet dysfunction. It was unknown if HES 130/0.4 causes platelet dysfunction in dogs. Therefore, we investigated the in vitro and in vivo effects of HES 130/0.4 on platelet function in dogs. We used closure time (CT) using the platelet function analyser-100 and adenosine diphosphate-collagen cartridges as a platelet agonist, to measure platelet function in this report. In the in vitro study, two solutions were compared: HES 130/0.4 and HES 200/0.5. Citrated blood from ten healthy dogs was diluted 1:9 and 1:3 with HES 130/0.4, HES 200/0.5 and 0.9% sodium chloride (NaCl). Closure time of the diluted blood was measured. Only HES 200/0.5 increased the CT beyond the dilutional effect at the 1:3 dilution, to a median CT of 125 seconds (interquartile range, 117.5 to 139.5 seconds), suggesting that HES 200/0.5, but not HES 130/0.4, caused platelet dysfunction at this dilution. No effect of HES or dilution on CT was identified at the 1:9 dilution. In the in vivo study, haemorrhagic shock was induced by removing 48ml/kg of blood from eleven greyhounds under general anaesthesia. Dogs were randomised to receive either 20mL/kg of HES 130/0.4 or 80mL/kg of 0.9% NaCl intravenously over 20 minutes. Both the HES 130/0.4 and 0.9% NaCl group had a significantly increased mean CT after fluid administration to 91.4 seconds (95% CI 69.3-113.4) and 95.5 seconds (95% CI 78.2-112.8), respectively. The magnitude of change was significantly greater for the 0.9% NaCl group than the HES 130/0.4 group, indicating the increase in CT with HES 130/0.4 is most likely due to a dilutional effect. In this study we also investigated the effect of shock on platelet function by measuring CT before and after inducing haemorrhagic shock. We found haemorrhagic shock did not significantly change CT. This report suggests HES 130/0.4 does not cause significant platelet dysfunction in dogs.

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List of Tables and Figures

1. Chapter 1: INTRODUCTION, OBJECTIVES AND HYPOTHESES

1.1. Introduction

The aim of this thesis was to investigate the effect of two commonly used hydroxyethyl starch solutions, HES 130/0.4 and HES 200/0.5, on canine platelet function. The first study compared the two solutions in vitro and the second study focused on the effect of HES 130/0.4 in vivo. The second study utilised a shock model, therefore, we also had to determine the effect of shock on platelet function. Our assessment of platelet function was based on closure time (CT), as measured by the Platelet Function Analyser 100 (PFA-100).

1.2. Objectives

1. To determine if HES 130/0.4 and HES 200/0.5 increases CT in vitro, beyond a dilutional effect (Chapter 3).
2. To determine if haemorrhagic shock in anaesthetised dogs alters CT (Chapter 4).
3. To determine if HES 130/0.4 increases CT, beyond any effect of shock or haemodilution (Chapter 4).

1.3. Hypothesis

We hypothesised that HES 130/0.4 would increase CT in vitro and in vivo, as measured by the PFA-100. We also hypothesised that haemorrhagic shock in dogs would alter CT.

2. Chapter 2: LITERATURE REVIEW

2.1. Platelet Function

Haemostasis is a complex mechanism resulting in thrombus formation. Traditionally, haemostasis has been divided into separate stages, including primary haemostasis, secondary haemostasis and fibrinolysis. However, it has been found more recently to be a complex mechanism with all stages occurring synergistically, involving tissue factor bearing cells, platelets and the endothelium.[1] The new theory is referred to as the cell-based model of coagulation. Endothelial injury is the most common initiator of coagulation, caused by surgical incision or venepuncture, infectious disease processes, severe acidaemia, hypoxaemia, inflammation, hypotension and even during normal endothelial cell turn over.[2]

The first step in forming a thrombus is the adhesion of platelets to the site of injury.[3] Platelets are synthesised in bone marrow and smooth muscle cells, and, in small amounts, in the liver, spleen and kidneys.[2] Its production is activated by thrombopoietin, which is produced in the liver and kidneys. The average lifespan of platelets in circulation in the dog is approximately 6 days.[4] Platelets achieve primary haemostasis by forming a platelet plug in three interrelated steps. This includes adhesion of platelets to endothelial surfaces,[5] activation of platelets, and platelet aggregation.[6] The mechanism of platelet function can differ in high shear stress conditions within small and medium sized arteries compared to low shear stress conditions within large arteries and in veins.[5]

Endothelial injury initially exposes subendothelial collagen. As a result, platelets adhere to the extracellular matrix and surrounding endothelial cells, which is mediated by von Willebrand Factor (vWF), fibronectin, laminin and thrombospondin. Von Willebrand factor is an adhesive protein synthesised by platelet precursors, megakaryocytes. After synthesis, vWF is stored within alpha granules in platelets. In addition, they are also produced and stored in endothelial cells.[7] Von Willebrand factor release is stimulated by thrombin, fibrin, vasopressin, collagen, platelet-activating factor, epinephrine or histamine.[8] Once in circulation, it can bind to exposed collagen at the site of

endothelial injury. Platelets then adhere to the site of injury by binding to vWF, mediated by integrin $\alpha_{IIb}\beta_3$ receptors and glycoprotein (GP) Ibα, which is a component of the GP Ib-IX-V complex found on platelet surfaces.[5] During high shear stress conditions, binding of vWF on collagen and loose binding of vWF to GP Ibα receptors result in platelet rolling along endothelial surfaces, facilitated by P-selectin expression. During low shear stress conditions, fibronectin and laminin mediate adhesion. During the adhesion process, factor (F) VIII is released, and vWF forms a complex with FVIII to avoid rapid clearance from plasma.[9]

Once platelets are adhered to the subendothelial collagen, they are further activated by agonists such as collagen, vWF, thrombin, adenosine diphosphate (ADP), and thromboxane A_2 (TXA_2), as well as being activated by shear stress conditions.[10] Activation causes degranulation of α granules and dense bodies, increased cytosolic calcium concentration, conformational change of platelets, release of ADP, serotonin and epinephrine, and activation of protein kinase C.[6] Under high shear stress conditions, ADP is the primary promoter of platelet activation; while under low shear stress conditions, TXA_2 promotes platelet activation.[11] Thrombin plays a key role as it activates platelets under all shear conditions via the protease-activated receptors (PAR)1 and PAR4, as well as the GP Ib-V-IX complex .[5] Although activation of platelets usually follows adhesion, it can also occur independent of adhesion, for example when thrombin is generated during inflammation, which can promote pathologic thrombus formation.

Platelet activation allows platelet aggregation to occur which leads to the formation of platelet plugs. Aggregation occurs by binding of platelet surface receptors, primarily integrin $\alpha_{IIb}\beta_3$ as well as GP Ib-IX-V, mediated by vWF, fibrinogen, thrombin, ADP and TXA_2, in order to form a platelet plug.[6] Thromboxane A_2, produced by the arachidonic acid cascade in platelets, is one of the most important facilitators of aggregation by activating integrin $\alpha_{IIb}\beta_3$ receptors. In high shear stress conditions, vWF is the major ligand for platelet aggregation, while in low shear stress conditions fibrinogen is the major ligand.[12] Once a platelet plug is formed, a cross-linked fibrin meshwork can

then be formed to stabilise the platelet plug. The fibrin meshwork has been historically described as secondary haemostasis.

Concurrent to platelet plug formation, adjacent endothelium releases prostacyclin and nitrous oxide, which causes vasodilation and inhibits platelet aggregation. This prevents excessive thrombus formation.[13]

2.2. Platelet Function Analysis

There are several methods to assess platelet function in people and in dogs. Due to the complexity of primary haemostasis, and the importance of platelet interactions with cellular surfaces in vivo, no single platelet function test is without limitations. The different methods of platelet function analysis reviewed here are available for clinical and research use, and the understanding of the function and limitations of each test is important for interpretation of results. The PFA-100 is described in detail below, followed by other tests validated for use in veterinary medicine.

2.2.1. Platelet Function Analyser - 100

The PFA-100 is a point-of-care, bench top platelet analyser, which measures platelet function in high shear stress conditions in vitro. A small volume of citrated whole blood (0.8 mL) is placed in a reservoir in a disposable test cartridge, which is then aspirated by a vacuum through a stainless steel capillary. This mimics the high shear stress conditions that platelets encounter in small and medium sized arteries. The sample reaches a small aperture (150 μm diameter) with a membrane coated with collagen, and either ADP or epinephrine, depending on the type of cartridge selected. The high shear stress conditions combined with the platelet agonists result in platelet adhesion, activation and aggregation followed by platelet plug formation, which occludes the small aperture. The time from the onset of aspiration to the time of aperture closure is measured in seconds, and is recorded as the closure time (CT).[14]

The validation of the PFA-100 in dogs is based on several studies.[15-17] Reference intervals reported in these studies with the use of ADP and collagen cartridges were 53 – 98 seconds,[15] 47 - 81 seconds,[16] and 52 – 86 seconds.[17] Reference intervals reported in these studies with the use of epinephrine and collagen cartridges were 92 - >300 seconds,[15] 67 – 210 seconds,[16] and 97 - >300 seconds.[17] Closure times with epinephrine and collagen activating cartridges resulted in greater variability in results,[17] making this cartridge less reliable in dogs. The variability is thought to be due to the lack of ability for epinephrine to induce significant platelet aggregation in dogs compared with people.[18]

The PFA-100 has also been validated for use in greyhounds. This is important because greyhounds have different haematological reference intervals compared to non-greyhound breed dogs. A bleeding tendency during surgery has been reported in some greyhounds, which is thought to be due to increased fibrinolysis, although this mechanism is not completely understood. A study by Couto et al measured CT with ADP and collagen coated cartridges as well as epinephrine and collagen coated cartridges, which ranged from 63 to 92 seconds and 87 to 238 seconds respectively.[19] Following this study, the same investigators measured CT in greyhounds with clinical postoperative haemorrhage, which found no significant difference in CT between greyhounds with clinical evidence of haemorrhage and those without noticeable haemorrhage, indicating normal platelet function in greyhounds with bleeding tendencies.[20]

Several individual-related factors have shown to influence CT in dogs. Firstly a decrease in platelet count (< 100,000 platelets/µL[17]) can increase CT, due to the decreased availability of platelets. A decrease in haematocrit, or packed cell volume (PCV) (< 25[16] – 30%[15,17]) can also increase CT. A low haematocrit can affect platelet function due to changes in blood flow dynamics altering platelet interaction with the endothelium,[21] which may also hold true for contact with the PFA-100 aperture. Another proposed reason is due to decreased ADP release as a result of fewer available red blood cells.[22] Although a defined minimum PCV is difficult to specify, CT results should be interpreted in line with a concurrent PCV. Von Willebrand factor can also influence CT as it is essential in primary

haemostasis and platelet function.[15,17] Therefore, the PFA-100 has the clinical utility to diagnose von Willebrand Disease (vWD) in dogs,[17,23] and vWD should be considered if an increase in CT is detected in a clinical scenario.

As described above, the PFA-100 has been used for the diagnosis of vWD, with improved sensitivity and specificity compared with the traditionally used buccal mucosal bleeding time,[17] as well as contributing to the investigation of the mechanism of bleeding in greyhounds. It has also been used in dogs to monitor efficacy of aspirin therapy,[15] Scott's syndrome,[24] as well as to assess the effect of HES solutions on platelet function.[25,26] Unlike other platelet function tests such as flow cytometry, platelet aggregometry, and thromboelastography with platelet mapping, the PFA-100 has the added advantage of mimicking high shear stress conditions, which is more applicable to physiological conditions. However, as it is an in vitro test, it lacks platelet interaction with true endothelium and is limited by the lack of cellular release of factors and proteins which normally occurs during in vivo haemostasis. From our experience, although the use of this machine is technically simple, results can be variable at times, particularly if the CT is not measured immediately, venepuncture technique is poor, the sample is left to sit without adequate rocking, or the citrated blood sample is not kept at a standard temperature.

There are limited studies on the use of the PFA-100 for detecting increased platelet reactivity or evaluating the risk of bleeding. One study in people found that CT was associated with increased perioperative red blood cell transfusion requirements in patients with aortic valve stenosis,[27] however there are no other studies which specifically address the association between CT and risk of haemorrhage. One study measured CT and platelet aggregometry immediately following trauma, which found increased platelet reactivity measured by aggregometry and this correlated with a decreased CT. Further studies are warranted to investigate the significance of a decreased CT and the use of CT to predict clinical bleeding.[28]

2.2.2. Other platelet function tests

2.2.2.1. Buccal mucosal bleeding time

Buccal mucosal bleeding time (BMBT) is a simple in vivo method for diagnosing primary haemostatic disorders, which can differentiate from secondary haemostatic disorders.[29] A small incision is made on the mucosa of the upper lip with a standardized instrument, while the lip is being reflected dorsally with a gauze bandage tied over the muzzle. While gently removing blood from the mucosa with a filter paper, the time from when the incision is made until the time taken for bleeding to cease is measured in seconds.[30] Two studies have reported normal BMBT in dogs with mean and standard deviations of 2.73 +/- 0.77 minutes (range 1.83 – 4.75 minutes),[32] and 2.62 +/- 0.49 minutes (range 1.68 – 4.14 minutes).[30] A prolonged BMBT was found in dogs with vWD, thrombocytopenia, thrombocytopathia, severe uraemia, and with aspirin therapy.[30] Buccal mucosal bleeding time in greyhounds has also been reported with a mean reference interval of 2.15 minutes +/- 0.73 minutes (range 0.88 – 4.12 minutes), which is different to non-greyhound breeds.[32] This study described one of the major limitations which are inter-observer and intra-observer variability which can both range up to approximately 2 minutes within the same dog. Another limitation of BMBT is that it does not predict surgical bleeding in dogs.[30]

2.2.2.2. Platelet Aggregometry

Platelet aggregometry is considered the gold standard method for detecting platelet dysfunction. There are two methods of platelet aggregometry, light transmission aggregometry using platelet rich plasma or electrical impedance aggregometry using whole blood, with both methods being validated for use in dogs.[33-35] Platelet aggregometery measures platelet aggregation in response to various agonists added to platelet rich plasma or whole blood samples, depending on the method utilised. Platelet agonists that have been used include ADP, collagen, arachadonic acid, thombin and epinephrine.[36] With light transmission aggregometry, the sample becomes more translucent over time as platelet aggregate, and therefore light transmission through the sample increases.[33] Light

transmission is recorded graphically, which can evaluate the time to maximum aggregation and overall rate of aggregation.[36] The more recently developed method of electrical impedance has the advantage of being able to use whole blood samples, which eliminates the process of producing platelet rich plasma for analysis. With this method platinum electrodes are placed in the whole blood sample, and the blood is gently stirred.[34-35] Once the agonist is added, platelets form a monolayer over the electrodes, which impede electrical current between the two electrodes. The transmitted current is then traced into a graph similar to light transmission aggregometry.

One of the major benefits of platelet aggregometry is its ability to quantitatively measure platelet function with the addition of varied platelet agonists. This technology has been used to measure platelet function in dogs after being given various therapies that may affect platelet function. Platelet aggregometry showed no significant change in platelet aggregation after HES 670/0.75 administration to dogs, despite a significant decrease in platelet count.[37] However it should be noted that the major limitation of this study is the small sample size. Various studies have also utilised this technology to investigate the effect of pimobendan,[38] lipid emulsions,[39] non-steroidal anti-inflammatory drugs,[40] aspirin,[41] and clopidogrel on platelet function in dogs.[41] Platelet aggregation has also been used to demonstrate decreased platelet function in dogs with infections,[42] and increased platelet function in dogs with malignancies,[43] which also demonstrates its benefits in being able to measure platelet hyper-reactivity. Experimentally, this technology has also been used to analyse platelet function in frozen canine platelet concentrates.[44] In people this technology has been used for diagnosing vWD,[45] although it has not been used in dogs for this purpose. Future scope for this technology for clinical use to diagnose primary haemostatic disorders, including vWD and to monitor drug therapy, are promising but its use is currently limited by availability.

Limitations to light transmission platelet aggregometry include the requirements of sample preparation in order to produce platelet rich plasma. It can be time consuming and require expertise, the centrifugation process can activate platelets, and the temperature of blood and the

time elapsed between sample collection and processing can also affect results.[46] Other limitations of aggregometry are that it does not mimic physiological conditions such as shear stress, and with light transmission aggregometry, the lack of red blood cells may also alter results.

2.2.2.3. Flow cytometry

Flow cytometry is a platelet function test validated for use in dogs,[47,48] which has the ability to assess platelet membrane glycoproteins, ligands, and platelet derived microparticles in platelets before and after being stimulated by platelet agonists including ADP, collagen, thrombin and epinephrine.[36] Whole blood, platelet rich plasma or washed platelet samples can be used. Platelets are fluorescently labelled and light scatter is detected to quantify the type and number of receptors, and platelet surface antibodies expressed on platelet surfaces. One of the benefits of this platelet function test is that it can be used to investigate the mechanism of platelet dysfunction by labelling platelet receptors,[49,50] and has been crucial in the development of our understanding of the mechanism of effects of different drugs on platelet function.[51] In dogs, flow cytometry has been used to investigate the function of platelets during inflammation,[52,53] and in dogs with immune mediated haemolytic anaemia.[54,55] It has also been used to investigate the effect of different therapeutics, such as aspirin[56] and cyclosporine,[51] on platelet function. More recent investigations have used flow cytometry to assess the viability of platelets in canine platelet transfusion products.[57,58] One of the major limitations of flow cytometry is that it is a bench top platelet function test limited to laboratory use, and in addition does not mimic in vivo platelet function under shear stress conditions.

2.2.2.4. Thromboelastography

Thromboelastography has been employed to assess hypercoagulability and hypocoagulability in dogs. However, thromboelastography alone is a global coagulation function test and, therefore, does not specifically assess platelet function. More recently however, thromboelastography with platelet

mapping has become available, which measures the influence of platelet function on thromboelastography.

Thromboelastography involves adding a small amount of whole blood to a small cup with a pin suspended in the middle. Depending on the machine utilised, either the cup rotates (thromboelastography) or the pin rotates (thromboelastometry). As only thromboelastography has been used for platelet mapping, the following discussion will be based on thromboelastography alone. With thromboelastography platelets can be activated by different activators, such as tissue factor, activator F, kaolin, arachidonic acid and ADP. Torque is created by rotation of the pin, which creates a graph reflecting generation of a clot. Several measurements can be made from the graph including reaction time (R), which is the time to initiate fibrin formation; K-time (K), which is the time to form a clot strength of 20 mm; alpha-angle (α), which describes the speed of fibrin build up and cross-linkage; maximum amplitude (MA), which is the maximum clot strength; and clot lysis after 30 minutes (LY30) and 60 minutes (LY60) after MA.

Thromboelastography with platelet mapping requires three samples run concurrently. The first sample is citrated blood with kaolin activation ($MA_{thrombin}$), which measures the maximum clot strength for global coagulation. The second sample is heparinised blood activated with activator F (MA_{fibrin}), which isolates the fibrin contribution to the clot. The third sample is the MA_{fibrin} sample with either ADP (MA_{ADP}) or arachidonic acid (MA_{AA}) as an additional agonist. The formula ($MA_{ADP/AA} - MA_{Fibrin}$)/($MA_{Thrombin} - MA_{Fibrin}$) x 100 is used to determine the platelet inhibition response.

Thromboelastography with platelet mapping has shown to be associated with platelet aggregation in people.[59] Thromboelastography has been used in dogs to assess the effect of clopidogrel[41] and non-steroidal anti-inflammatory drugs on platelet aggregation[60] which also showed association with platelet aggregometry. Thromboelastography has also been utilised in the assessment for platelet function in dogs with hyperadrenocorticism.[61] Thromboelastography has the advantage of being able to assess the global formation of a clot, including hypercoagulability and hypocoagulability, and now

with platelet mapping, includes specific assessment of platelet function. However one of the major disadvantages of thromboelastography with platelet mapping is the expense of requiring two thromboelastography machines to run concurrently, and currently there is minimal literature validating its use in dogs.

2.2.2.5. Sonoclot

Sonoclot is a global coagulation test that also has capability to measure platelet function. It has been validated for use in dogs[40] and has been shown to be useful in monitoring unfractionated heparin doses in dogs.[62] It has a probe with a sensor that detects torque as blood clots within a cuvette. The sensor detects the change in torque during clot formation which is displayed in a graph. The graph represents sonoclot activated clotting time (ACT), which is the time in seconds till change in torque is detected; clot rate, which is the rate of fibrin formation; and platelet function (PF), which is calculated by the software from the peak strength of the clot and the time to peak clot formation. Despite the name, PF, this calculated value also involves fibrin formation, therefore is not specific to platelet function alone. Compared to platelet aggregometry, this test is limited to thrombin-stimulated platelet activation, therefore Sonoclot cannot be used to determine the effect of certain platelet inhibitors such as clopidogrel and aspirin.[63]

2.2.2.6. Cone and plate(let) analyser

The cone and plate(let) analyser is another bed-side platelet function test that uses whole blood in high shear stress conditions like the PFA-100, and has the advantage of being able to measure increase and decrease in platelet function. However this test in only validated for research use in people, and has not been validated for use in dogs.[36]

2.2.3. Summary

The role of platelet function in haemostasis is complicated by multiple factors contributing to its activation, adhesion to endothelium, and aggregation resulting in final clot formation. Therefore,

despite the multiple methods available in order to investigate platelet function, there is no single test which provides all the information required to assess platelet function. An ideal test would be readily accessible, easy to use with minimal inter-observer and intra-observer variability, have the ability to measure variable components of platelet function, and in addition, mimic physiological conditions. The PFA-100 fulfils some of these criteria as it is easy to perform, produces a measurement which is easily compared, has been validated for use in experimental research and is a bed-side test which mimics physiological shear stress conditions. However using multiple methods of platelet function analysis would be able to provide more information on the mechanisms of platelet function.

2.3. Hydroxyethyl Starch Solutions

2.3.1. Properties of Hydroxyethyl Starch solutions

Hydroxyethyl starch (HES) solutions are artificial colloid solutions that are used to treat shock by expanding blood volume. The colloid molecules are made by modification of natural polymers of amylopectin made from waxy maize starch.[64] There are a variety of HES solutions available, which are characterised by their physical and chemical characteristics including mean molecular weight (MW), degree of substitution (MS), and hydroxyethylation ratio at carbon position C2 and C6 (C2/C6 ratio). These properties influence the rate of degradation and separate the solutions into slowly degradable solutions such as HES 670/0.75, rapidly degradable solutions such as HES 130/0.4, or solutions having a medium rate of degradation such as HES 200/0.5. Slowly degradable products tend to have greater adverse effects. Other important properties include the carrier solution type in which the starch molecules are suspended in, such as 0.9% NaCl or a balanced crystalloid solution, and the concentration of starch molecules within the solution, most commonly 6% or 10%.[64] These properties are discussed in more detail in this chapter.

The MW of HES solutions describe the mean MW of a polydispersed solution, meaning, there are a broad range of HES molecules with different MWs in one solution.[64] The MW is often described as being high (> 400 kDa), medium (200 – 400 kDa), or low (< 200 kDa). The MW can influence the rate of degradation of HES solutions; with higher MW solutions having a slower rate of degradation, and lower MW solutions having a faster rate of degradation. The mean MW can be calculated by two different methods; the number average MW and the weight average (or mass average) MW.[65] If there are larger molecules in a solution, the weight average MW is influenced to a greater degree than the number average MW. The ratio of weight average MW to the number average MW will give an index of the degree of polydispersity of the solution.[65]

Lederer et al reported that previous methods of MW determination for currently labelled HES solutions are inaccurate.[66] Using size exclusion chromatography coupled with low-angle laser light scattering techniques, HES labelled 450/0.7 was determined to have a MW of 670 KDa and HES 200/0.5, a MW of 240 KDa.[66] However HES 130/0.4 was found to have an accurate mean MW of 130 KDa as determined by low angle laser light scattering technique.[67] However, despite further accuracy on size, the in vitro molecular weight described by the manufacturer differs to the eventual in vivo MW. This is because when HES solutions are administered intravascularly, the small molecules (< 50 kDa) are rapidly excreted by the kidneys.[68] The larger molecules are hydrolysed by amylase into smaller molecules, as discussed in the pharmacokinetics chapter, resulting in an increase in the number of molecules in vivo. As a result the in vivo MW is lower than the in vitro MW.[64] Metabolism of the molecules actually improves the colloid osmotic pressure, as it is the number of molecules, not absolute MW, that determines colloid osmotic pressure.

The MS describes the average number of substitutions of hydroxyl groups with hydroxyethyl residuals at carbon position 2 (C2) and carbon position 6 (C6) on each glucose subunit within the hydroxyethyl starch polymer.[64] Solutions can be highly substituted (0.62 – 0.75), have a medium substitution (0.5) or low substitution (0.4). The MS can be calculated by the formula, MS = W/(1-W) x

162/44; where W is the weight fraction of hydroxyethyl groups in the polymer, 162 is the mass of the starch, and 44 is the mass of the hydroxyethyl group.[65] The MS determines the ability of amylase to cleave HES molecules in vivo into smaller molecules, resulting in a smaller in vivo MW.[67] Higher MS solutions are more difficult to cleave by amylase, resulting in reduced enzymatic degradation and prolongation of intravascular retention.[65]

The C2/C6 ratio is the ratio of hydroxyethylation at the C2 position compared to the C6 position. This property is also important as there is greater difficulty in cleaving HES molecules at C2 compared to C6. Hence, HES products with a higher C2/C6 ratio have a slower rate of degradation.[69]

Another important characteristic of HES solutions is the solvent in which HES molecules are suspended in. Commonly used solvents include 0.9% NaCl or balanced crystalloid solutions. The characteristics of the solvents can influence acid-base and electrolyte balance. It has been shown that 0.9% NaCl as a solvent causes a significant decrease in blood pH, and an increase in chloride concentration and base deficit, compared to a balanced crystalloid solution.[12] The concentration of hydroxyethyl starch molecules in the solvent is commonly 6% or 10%, depending on the product. The concentration of the solution affects the resulting plasma concentration,[70] altering the rate of excretion and contributing to adverse effects, as well as the volume expansion effects.

The two solutions most commonly used in people are 10% HES 200/0.5 and 6% HES 130/0.4. The use of high MW HES 670/0.75 has been associated with significant adverse effects and its use has fallen out of favour. Hydroxyethyl starch 200/0.5 has a medium mean MW of 200 kDa, a medium MS of 0.5, and a C2/C6 ratio of 5:1, and is commonly described as a pentastarch solution. Due to these properties, HES 200/0.5 has a medium rate of degradation when compared to other HES solutions.[70] Hydroxyethyl starch 130/0.4 has a low mean MW of 130 kDa, a low MS of 0.4 and a high C2:C6 ratio, and is commonly described as a tetrastarch solution. Despite the high C2/C6 ratio, the low MW and MS have a greater influence on the behaviour of this solution, resulting in a rapid rate of degradation.[65] The HES 130/0.4 solution under investigation described in this thesis has a

concentration of 6% suspended in a 0.9% NaCl solvent, although HES 130/0.4 in 10% solutions and balanced electrolyte solvents are also available.

2.3.2. Pharmacokinetics

Once polydispersed HES solutions are administered intravenously, HES molecules with a MW lower than the renal threshold (40 - 60 KDa) are excreted by the kidneys.[67,72] Larger molecules are cleaved by amylase, increasing the number of molecules contributing to colloid osmotic pressure.[67,72] There is also a small increase in MS in vivo as the more highly substituted molecules resist cleavage. A small proportion of HES molecules are stored in the tissues and are excreted following redistribution.[67]

Because 6% HES 130/0.4 has a low MW and low MS, it has the highest plasma clearance rate (31.4 mL/min) and shorter half-life in people (12.1 hours), compared to the higher MW higher MS HES solutions, after a single dose administration of 500mLs.[70] The maximum plasma concentration achieved was 3.7mg/mL. By 24 hours after administration, plasma concentrations decreased to less than 0.5 mg/ml. Compared to this, 6% HES 670/0.75 (which has a high MW of 670 KDa and a high MS of 0.75) and 10% HES 200/0.5 had a much slower rate of plasma clearance of 0.98 mL/min and 9.24 mL/min respectively; a longer half-life of 46.4 hours and 30.6 hours respectively; and higher maximum plasma concentration of 13mg/mL and 8mg/mL respectively.[67]

2.3.3. Pharmacodynamics

The pharmacodynamic property of interest for HES solutions is the effect of these solutions on blood volume expansion. A study by Silverstein et al[73] showed that administration of 20mL/kg of HES 670/0.75 immediately increased blood volume by 40% and was able to maintain an increase of 30% for 240 minutes. In comparison, 80ml/kg of 0.9% NaCl was able to increase blood volume by 76%, however rapidly decreased to 20% blood volume expansion, despite the larger volume administered. This effect can be explained by Starling's principle, which states that net fluid flow across a vessel

wall is dependent on the filtration coefficient, reflection coefficient, the balance between capillary and interstitial hydrostatic pressure and the balance between the capillary and interstitial colloid osmotic pressure. This can be described by the following equation:

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

where J_v is the net fluid flow across a vessel wall, P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, π_c is the capillary oncotic pressure, π_i is the interstitial oncotic pressure, K_f is the filtration coefficient, and σ is the reflection coefficient.[74,75] Therefore, administration of HES solutions, which have a higher MW and therefore higher oncotic pressure compared to crystalloid solutions, will result in increased intravascular oncotic pressure, resulting in prevention of extravasation of fluid, compared to a crystalloid solution.[76-78] However it is also important to remember that artificial colloids such as HES will also cause an increase in hydrostatic pressure, resulting in some degree of extravasation of fluid into the interstitial space. The degree of extravasation of fluid, and hence an HES solution's ability to expand blood volume, is also altered by patient factors such as albumin balance between the capillary and interstitium and capillary permeability, which can both be altered in diseased states.

Although there are no studies on the pharmacodynamics of 6% HES 130/0.4 or 10% HES 200/0.5 in dogs, several studies have assessed the pharmacodynamics of these solutions in people. Administration of 500mL of HES 130/0.4 over 15 minutes resulted in a plasma volume increase of 380mls over 4 to 6 hours, which returned to baseline within 8 to 24 hours. Despite the lower plasma concentration and shorter half-life of HES 130/0.4, there was no difference in plasma volume effect compared to 10% HES 200/0.5 or 6% HES 670/0.75.[79,80] Also, there was no blood volume expansion effect 24 hours after a single dose despite the persistence of HES solution in plasma.[79,81]

2.4. Indications for use of hydroxyethyl starch solutions

Hydroxyethyl starch solutions are used in the treatment of shock to rapidly increase and maintain intravascular blood volume. There are several advantages of artificial colloid solutions compared to crystalloid solutions. One advantage is that it can minimise development of interstitial oedema by maintaining colloid osmotic pressure.[82,83] As less of the solution redistributes outside of the vascular space, it provides longer intravascular volume expansion effects compared with administration of crystalloid solutions,[73] therefore requiring smaller volumes.[84] Additional benefits may include the ability to reduce capillary permeability,[85,86] and anti-inflammatory effects.[87-89]

2.4.1 Use of hydroxyethyl starch solutions in the treatment of shock

Shock can be defined as inadequate oxygen delivery (DO_2) to tissues resulting in failure to meet oxygen demands, impairing oxidative metabolism.[90] Failure of the cardiovascular system to deliver oxygen in order to meet oxygen demands is defined as circulatory shock.[90] To maintain organ function, the cardiovascular system distributes oxygen to tissues to facilitate energy production, which is generated within mitochondria as adenosine triphosphate (ATP).[91] Adenosine triphosphate is hydrolysed to ADP for normal cellular function. Adenosine triphosphate production also occurs during glycolysis, where 2 ATP are produced per glucose molecule, an anaerobic process. When there is adequate oxygen available, pyruvate, which is an end product of glycolysis, enters the Kreb's cycle resulting in oxidative phosphorylation and production of an additional 36 ATP per glucose molecule. During shock, aerobic metabolism can no longer occur and pyruvate converts to lactate instead of entering the Kreb's cycle. This produces nicotinamide adenine dinucleotide, a reducing substrate, which is used to feed back into glycolysis and continue the small production of 2 ATP per glucose molecule. This eventually results in inadequate energy production for cellular function resulting in cellular and organ dysfunction.

The primary indication for the use of HES solutions is for rapid blood volume expansion in patients with circulatory shock. Some clinicians prefer using HES solutions for fluid resuscitation instead of crystalloid solutions, as colloid solutions have the benefit of maintaining a longer blood volume

expansion effect following administration.[73] Although natural colloids, such as human albumin, are preferable in people due to potential risks associated with HES solutions, HES solutions are still widely utilised in dogs due to the limited availability of canine albumin, the risks of human albumin use in dogs, in addition to the costs associated with natural products.[92] There is also some risk to rapidly infusing any natural product due to potential immunological incompatibility.

Silverstein et al demonstrated that the blood volume expansion effects of 80mL/kg of crystalloid solution was no different to 20mL/kg of an artificial colloid solution.[73] Four dogs weighing 20kg were administered different resuscitative fluids one week apart, including an isotonic crystalloid solution of 0.9% NaCl, a hypertonic crystalloid solution of 7.5% NaCl, and two artificial colloid solutions Dextran-70 and 6% HES 670/0.75, as well as a control group of no resuscitative fluid administration. In-line haematocrit monitoring was used to determine real-time changes in blood volume during and after fluid administration for up to 240 minutes. For each fluid type, the degree of blood volume expansion was related to the volume of fluid administered. Immediately after the completion of crystalloid administration, the peak blood volume rapidly declined, while immediately after colloid administration the blood volume continued to increase, indicating intravascular fluid shift from other compartments. The cumulative blood volume expansion was greatest with colloids. However, this study did not specifically investigate more modern HES solutions, 6% HES 130/0.4 or 10% HES 200/0.5.

Gandhi et al compared volume efficacy of 6% HES 130/0.4 with 6% HES 670/0.75 in a controlled double blinded multicentre trial in people.[81] Efficacy of HES solutions was measured by the volume required for intraoperative volume replacement, as guided by central venous pressure and arterial blood pressure. They found that the volume required for intraoperative volume replacement was not significantly different between the two solutions. Gallandat et al compared volume efficacy between 6% HES 130/0.4 and 10% HES 200/0.5 in a prospective randomised double-blinded multicentre study in people.[93] Fifty nine people undergoing coronary artery bypass grafting were

enrolled in the study. The efficacy as a volume expander was measured by the required volumes of fluid, hemodynamic parameters (heart rate, mean arterial pressure, central venous pressure, pulmonary capillary wedge pressure, cardiac index), and colloid osmotic pressure. These values were not significantly different, suggesting 6% HES 130/0.4 and 10% HES 200/0.5 have similar volume replacement/expansion properties to each other. It is likely that the reason for these similarities in volume expansion is the similar number of HES molecules in vivo, which determine the colloid osmotic pressure, not the size of the molecules. A recent study by Barros et al compared the effect of a 1:1 volume replacement dose of 6% HES 130/0.4 to 3:1 volume replacement dose of lactated Ringers solution (LRS) administered to splenectomised dogs with haemorrhagic shock. Six percent HES 130/0.4 provided greater blood volume expansion effect compared to LRS, 45 minutes and 90 minutes after administration. This study also measured oxygen extraction ratio (O_2ER), which is an objective measure of shock. Haemorrhagic shock increased O_2ER significantly from base line, and both solutions decreased O_2ER back to base line 5 minutes after fluid administration. However, 45 minutes after administration of LRS, the O_2ER increased significantly from base line values, while O_2ER remained the same 45 minutes after 6% HES 130/0.4 administration. This study suggests that one third of a dose of 6% HES 130/0.4 compared to LRS is superior for the treatment of haemorrhagic shock in splenectomised dogs.

2.4.2. Other indications for the use of hydroxyethyl starch solutions

Hydroxyethyl starch solutions are also used as a continuous infusion to maintain colloid osmotic pressure in dogs with hypoalbuminaemia.[15,16] Hypoalbuminaemia results in a decrease in colloid osmotic pressure,[82] as albumin accounts for the majority of the plasma colloid osmotic pressure in a non-linear relationship.[94,95] It has been shown by Smiley and Garvey,[83] that the administration of 6% HES 670/0.75 (dose ranging from 9 to 27mL/kg), was associated with a subjective improvement in clinical signs of peripheral oedema in 18 of 26 dogs, and resulted in a significant increase in colloid osmotic pressure in all 13 dogs in which colloid osmotic pressure was measured. However this study

did not have a control population, therefore improvement in peripheral oedema and colloid osmotic pressure may have occurred with administration of a crystalloid solution or no solution at all. Another study by Moore and Garvey[82] found 6% HES 670/0.75 improved colloid osmotic pressure immediately after administration of a single dose, but decreased back to base line 12 hours after administration in dogs with hypoalbuminaemia.

In people, there was no difference in colloid osmotic pressure after 6% HES 200/0.5 or 6% HES 130/0.4 administration.[67,93] Currently, there are no studies which determine the colloid osmotic pressures in dogs after administration of 6% HES 130/0.4 or 10% HES 200/0.5 as a bolus or as a continuous rate infusion.

Another interesting finding specific to 6% HES 130/0.4 is its potential anti-inflammatory effects and capability of decreasing capillary permeability. Recent experimental studies on septic animal models have shown that HES solutions, compared with crystalloid and gelatine-based colloid solutions, can decrease capillary permeability,[85,86] down regulate the expression of adhesion molecules, inhibit neutrophil recruitment, and decrease cytokine production.[87-89,96,97] In particular, 6% HES 130/0.4 has been shown to be superior to 10% HES 200/0.5 in regards to these effects.[85] Research in this area is still in its early phases, and recommendations for the use of 6% HES 130/0.4 specifically to decrease capillary permeability and inflammation cannot be recommended.

2.5. Adverse Effects of hydroxyethyl starch solutions

2.5.1. Introduction

Despite the many advantages of HES solutions, the use of HES solutions is controversial due to an association with adverse effects.[98-100] One of the major adverse effects is the risk of blood loss and increased transfusion requirements, mainly due to development of platelet dysfunction. In addition, there is evidence for risk of developing acute kidney injury (AKI) in people with sepsis.[101] Other rare

complications include allergic reactions, development of pruritus, as well as metabolic acidosis, depending on the carrier solution.[100] Due to these adverse effects that HES solutions can cause, and with the additional costs of these solutions compared with use of crystalloid solutions, a complex and ongoing debate is continuing in the human medical literature.

2.5.2. Platelet dysfunction

It is well documented that HES solutions can cause both human[50,102-105] and canine[25,26,37] platelet dysfunction. Proposed mechanisms include binding of vWF,[28,29] HES molecules coating platelet surfaces, blocking access to platelet surface protein integrin $\alpha_{IIb}\beta_3$, preventing platelet adhesion and aggregation.[108]

2.5.2.1. Platelet dysfunction in people

Stogermuller et al found that 10% HES 200/0.5 decreased expression of integrin $\alpha_{IIb}\beta_3$, in vitro and in vivo in healthy volunteers, but not P-selectin or glycoprotein Ib receptor expression.[109] Franz et al used platelet flow cytometry to examine the effect of various HES solutions (HES 130/0.4, 200/0.6, 70/0.5 and 400/0.8) on agonist induced activation of integrin $\alpha_{IIb}\beta_3$ complex and P-selectin, in healthy volunteers.[49] There was decreased expression of integrin $\alpha_{IIb}\beta_3$ receptors, but not P-selectin, with all but 6% HES 130/0.4 administration. These findings suggest that HES molecules may cause platelet dysfunction by decreasing the availability of functional receptors required for normal adhesion and aggregation. Interestingly, although a variety of HES solutions affected integrin $\alpha_{IIb}\beta_3$ receptor expression in this study, 6% HES 130/0.4 did not, suggesting it may be a superior solution to other HES solutions if platelet dysfunction was of concern.

Another proposed mechanism is a non-specific binding of HES molecules to platelet surfaces. Deusch et al found using flow cytometry that HES molecules with fluorescent labelling were found to coat platelet surfaces independent of integrin $\alpha_{IIb}\beta_3$ binding.[108] Although the exact mechanism is unknown, this mechanism of platelet dysfunction is often described as platelet coating. Although

impaired intracellular calcium signalling due to HES administration was previously thought to contribute to platelet dysfunction, this has been more recently disproven.[110]

2.5.2.2. Platelet dysfunction in dogs

There are four studies concerning the association of HES solutions with platelet dysfunction in dogs. Two published studies assessed the effect of 6% HES 670/0.75 on canine platelet function using the PFA-100, in vitro and in vivo.[25,26] Canine blood diluted at a ratio of 1:3 with 6% HES 670/0.75 in vitro increased CT,[26] and a 20 mL/kg dose of 6% HES 670/0.75 administered to healthy dogs in vivo also increased CT.[25,26] Both of these studies support that 6% HES 670/0.75 causes canine platelet dysfunction, although the effect of dilution alone on CT in the second study was not specifically addressed. One other study assessed the effect of 10mL/kg of 6% HES 670/0.75 on platelet function in anaesthetised dogs, using platelet aggregometry, and found no difference in platelet aggregation compared to administration of 10ml/kg of LRS.[37]

More recently, 6% HES 130/0.42 was compared with saline solution in vitro.[111] This particular solution is a low MW, low MS HES solution with a slightly higher MS than 6% HES 130/0.4, and derived from potato starch instead of maize. This study found that platelet dysfunction occurred with 6% HES 130/0.42 at a 1:3 dilution with blood as measured by the PFA-100. A direct comparison of all fluid solutions would be ideal, however this is difficult due to different availability of products in different countries. In addition, in vivo studies are warranted for further investigation of 6% HES 130/0.42 on platelet function in dogs.

No studies have investigated the effect of 6% HES 130/0.4 and 10% HES 200/0.5 specifically on canine platelet function.

2.5.3. Effect on von Willebrand Factor and Factor VIII

In people HES solutions cause a decrease in circulating vWF and FVIII in healthy volunteers and clinical patients.[67,106,107,112] Although dilution alone can cause a decrease in any factor

concentration,[113] the decrease in vWF and FVIII was found to be beyond a dilutional effect.[106,107] The cause of this dilution-independent decrease in vWF is unclear; some authors have suggested that it may be due to binding of vWF to HES molecules, resulting in increased clearance of vWF.[71,114] The decrease in FVIII is likely due to the decrease in vWF, which is a carrier protein for FVIII. Decrease in vWF and FVIII results in impaired ristocetin cofactor activity and prolongation of activated partial thromboplastin time.[112] Increase in CT, as measured by the PFA-100, has been attributed to a decrease in vWF in people[115] and in dogs.[23]

Despite these earlier findings with the use of slowly degradable HES solutions in people, 6% HES 130/0.4 was found not to decrease vWF or FVIII in clinical patients.[93,116] One limited study in anesthetised dogs found that 10ml/kg administration of 6% HES 130/0.4 resulted in no significant change in FVIII or vWF, although this finding may be due to the low dose administered.[37]

2.5.4. Fibrin formation and fibrinolysis

Hydroxyethyl starch solutions were found to impair thrombin-fibrinogen interactions and fibrin clot formation.[117,118] The effect of HES solutions on fibrinolysis is controversial. Some studies showed increased fibrinolysis in vitro[119] and in vivo,[120] while others found no effect.[112] A recent study showed that 6% HES 130/0.4, when added to canine blood in vitro, resulted in hypocoagulable parameters on TEG consistent with dose-dependent alteration in fibrinogen concentration and inhibition of platelet function.[121] Further studies are warranted to investigate the effect of 6% HES 130/0.4 on fibrinolysis.

2.5.5. Acute kidney Injury

Hydroxyethyl starch solutions, including HES 130/0.4, are known to cause acute kidney injury (AKI) in people. There are very few studies which have investigated the exact mechanism of AKI secondary to HES 130/0.4 administration.[122-124] One of the most commonly suggested mechanisms is the development of osmotic nephrosis-like lesions, which includes proximal tubular cell swelling and

vacuolisation, due to accumulation of proximal tubular vacuoles via pinocytosis. This was demonstrated in a septic rat model where renal histopathology was compared after HES 130/0.4, gelatine or 0.9% NaCl administration.[122] This study found that septic rats which received HES 130/0.4 had greater dilation of peritubular capillaries, increased renal tubular cell death (verified by cell swelling and fragmentation of cell nuclei), and large numbers of vesicles within the proximal tubular epithelial cells, compared to rats which received 0.9% NaCl. This study also demonstrated increased neutrophil gelatinase-associated lipocalin, which is an early marker of kidney injury in rats which received HES 130/0.4 compared with 0.9% NaCl. This same group of investigators also performed an in vitro study where different concentrations of HES 130/0.4 were applied to harvested proximal tubular cells and incubated over 21 hours.[123] Hydroxyethyl starch 130/0.4 caused tubular cell injury in a dose dependent manner, when compared to 0.9% NaCl, and fluorescent images revealed small vesicular-like structures and large agglomerates which was similar to the earlier study's histological findings. Contrary to these findings, however, a similar in vitro model demonstrated that HES blunted the damage induced by incubation with tumour necrosis factor α.[124]

A porcine study with histologic examination of kidneys after HES 130/0.42 administration also showed tubular epithelial cell vacuolisation suggestive of osmotic nephrosis-like lesions.[125] This study also demonstrated significantly increased macrophage infiltration and tubular damage with HES 200/0.5, but not HES 130/0.4, when compared to the control solution of lactated Ringer's. These results may indicate some advantages of using a lower MW and MS solution. In an ovine endotoxaemic shock model, intraluminal protein precipitates and cellular injury was present after HES 200/0.5, HES 130/0.4 and 0.9% NaCl administration, however was significantly greater with HES 200/0.5 administration, also supporting the advantages of lower MW and MS solutions.[126]

The only in vivo study in people which investigated the mechanism of AKI with HES administration found that after administration of HES to brain-dead kidney donors, histopathology demonstrated osmotic-nephrosis-like lesions in the tubules.[127] However only three kidneys were examined, and the

type of HES solution was not specified. Therefore, although it is known that HES solutions can cause kidney injury in people, there is limited understanding of the mechanism of this process. There are currently no studies which demonstrate kidney injury in dogs, therefore, although precautions should be taken given the current literature, it is unknown if kidney injury occurs in dogs with HES administration.

2.6. Clinical use of hydroxyethyl starch 130/0.4

The use of HES 130/0.4 has become controversial in people due to adverse effects described in the earlier chapters. In 2013, HES 130/0.4 was taken off the market in the United Kingdom by the Medicines and Healthcare Products Regulatory Agency, and the United States Food and Drug Administration has recommended against its use in critically ill patients or those with pre-existing renal dysfunction. In addition, the most recent 2012 Surviving Sepsis Campaign Guidelines recommends 'against the use of HES for fluid resuscitation of severe sepsis and septic shock'.[128]

These changes in guidelines have evolved from four studies.[101,129-131] The first of these, the Efficacy of Volume Substitution and Insulin Therapy in Severe Sepsis (VISEP) trial, was a multicentre randomised study comparing 10% HES 200/0.5 with a balanced crystalloid solution, Ringers lactate. This study was also investigating insulin therapy in severe sepsis, and was terminated early due to hypoglycaemia in patients with strict insulin control. The results therefore included 537 patients with severe sepsis in a multidisciplinary intensive care unit. They found that administration of HES 200/0.5 resulted in a less positive fluid balance, greater increase in central venous pressure (CVP) and central venous oxygen saturation; however the multivariate analysis found HES 200/0.5 was an independent predictor for renal replacement therapy requirement and 90 day mortality in a dose dependent manner.[130] However, these results are not directly applicable to HES 130/0.4 due to difference in concentration, MW and MS.

Following this, The Scandinavian Starch for Severe Sepsis/Septic Shock (6S) trial was published, which was a multicentre blinded trial, including 798 patients with septic shock randomised to receive HES 130/0.42 in a balanced solution or Ringer's acetate for blood volume expansion within the first 3 days of the trial.[129] Thereafter, both groups received open-labelled HES 130/0.42 and or Ringer's acetate at the attending clinician's discretion. The HES 130/0.42 group was associated with greater 90 mortality rates, a greater number of patients dependent on renal replacement therapy, and significantly more transfusions.

The largest study, The Crystalloid versus Hydroxyethyl Starch Trial (CHEST),[132] included 7000 medical and surgical ICU human patients randomised to receive fluid resuscitation with either HES 130/0.4 or 0.9% NaCl. The median dose of HES 130/0.4 administered was 5ml/kg/day for 4 days. This study, as with the VISEP study, found a greater increase in CVP and lower positive fluid balance in the HES 130/0.4 group. However, this group received greater volumes of blood products, had a greater need of renal replacement therapy, and had greater adverse effects, mostly pruritus. This study however did not show a difference in mortality, possibly because people who were unlikely to survive were excluded, and the mortality was less than the calculated power required to find significant difference between the two groups. It should be noted as well that this study included a general intensive care unit population, not a septic population like the other studies.

The CRYSTMAS study randomised 174 people with severe sepsis to receive either HES 130/0.4 or 0.9% NaCl for fluid resuscitation.[131] The mean volume of HES 130/0.4 administered was 1379mls over a four day period. There was no difference in blood volume expansion, need for renal replacement therapy or mortality in this study. The lack of significant difference may be due to the low numbers of people enrolled, and hence lack of power to detect significant difference. Despite these results, there was no benefit in the use of HES 130/0.4 and with the additional costs of this product compared with a crystalloid solution its use was not supported by the results of this study.

Although several meta-analyses have also supported these findings of greater transfusion requirements, increased risk of acute kidney injury and increased risk of mortality with low MW and MS HES solutions,[133-134] there are limited studies investigating the safety of these products in other patient populations such as hypovolaemic shock, trauma or perioperative patients.[134] The only study specifically investigating people sustaining trauma is by James et al, in which 115 people with severe blunt or penetrating trauma were randomised to receive HES 130/0.4 or 0.9% NaCl for fluid resuscitation.[135] In people with penetrating trauma, the volume administered was less, lactate clearance was faster, and there was a lower incidence of kidney injury with HES 130/0.4 administration. In the blunt trauma group, there were greater transfusion requirements in the HES 130/0.4 group, however, the HES 130/0.4 group also had higher trauma scores. This study was discontinued early due to slow recruitment of cases and did not measure mortality as a primary end point.

The major concern for the use of HES 130/0.4 in the perioperative period is the risk of inducing coagulopathy and higher transfusion requirement. There are minimal studies investigating the effect of HES solutions in the perioperative and postoperative period. A pooled analysis of randomised clinical trials including people undergoing orthopaedic, cardiac and urologic surgery compared the effect of HES 130/0.4 and HES 200/0.5 on coagulation parameters, blood loss and transfusion requirements.[136] Two hundred and twenty eight people were included in the HES 130/0.4 group and 221 people were included in the HES 200/0.5 group. This study found a greater prolongation in activated partial thromboplastin time, lower vWF concentration, lower platelet count, and greater blood loss and transfusion requirements in the HES 200/0.5 group, despite the HES 200/0.5 receiving a lower volume of the study fluid. This study suggests that there is less risk of haemorrhage with the use of HES 130/0.4 compared with HES 200/0.5, however, this study did not compare the effect to crystalloid solutions. A meta-analysis of randomised trials compared the risk of postoperative bleeding between use of HES 130/0.4 with albumin administration in people undergoing cardiopulmonary bypass. This study included 18 trials with 970 people. They found that HES 130/0.4

increased the risk of postoperative blood loss by 33.3%, and also resulted in a significantly greater number of re-operations and increased transfusion requirements; however there was no difference in mortality between the two groups.

Although there are some advantages in using HES 130/0.4 for blood volume expansion, the adverse effects such as AKI, haemorrhage and increased transfusion requirements should be considered. Despite the significant amount of literature in people describing these adverse effects, the exact mechanism causing these adverse effects are still under investigation. Currently there is minimal evidence for or against the use of HES 130/0.4 in dogs, although it should be used with caution. Further knowledge is required to determine if these adverse effects occur in dogs and if they are clinically relevant, in addition to a better understanding of the mechanisms contributing to these adverse effects.

2.7 Reference list for literature review

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3. Chapter 3: EFFECT OF HYDROXYETHYL STARCH 130/0.4 AND 200/0.5 SOLUTIONS ON CANINE PLATELET FUNCTION IN VITRO

3.1. Abstract

Objective - To determine whether dilution of blood samples from healthy dogs with 2 hydroxyethyl starch (HES) solutions, HES 130/0.4 and HES 200/0.5, would result in platelet dysfunction as Measured by closure time (Ct) beyond a dilutional effect.

Sample - Citrated blood samples from 10 healthy dogs with a Ct within reference limits (52 to 86 seconds).

Procedures - Blood samples were diluted 1:9 and 1:3 with 6% HES 130/0.4 and 10% HES 200/0.5 solutions and saline (0.9% NaCl) solution. Dilutions at 1:9 and 1:3 mimicked 10 mL/kg and 30 mL/kg doses, respectively, ignoring in vivo redistribution. Closure time was measured with a platelet function analyzer and compared among dilutions.

Results - A dilutional effect on Ct was evident for the 1:3 dilution, compared with the 1:9 dilution, but only HES 200/0.5 increased the Ct beyond the dilutional effect at the 1:3 dilution, to a median Ct of 125 seconds (interquartile range, 117.5 to 139.5 seconds). No effect of HES or dilution on Ct was identified at the 1:9 dilution.

Conclusions and Clinical Relevance - 1:3 dilution of blood samples from healthy dogs with HES 200/0.5 but not HES 130/0.4 significantly increased Ct beyond the dilutional effect, suggesting that IV administration of HES 200/0.5 in dogs might cause platelet dysfunction.

3.2. Introduction

Hydroxyethyl starch solutions are artificial colloid solutions used in dogs to treat shock by expanding blood volume. The use of HES is controversial because of its association with platelet dysfunction in people[1–5] and dogs,[6–8] and in human medicine, its use has been associated with an increase in intraoperative blood loss and transfusion requirements.[1,9–12] This effect is particularly relevant to solutions with a high molecular weight and molar substitution,[1,9,10] such as the commonly used solution HES 670/0.75, which has a high molecular weight of 670 kDa and a high molar substitution of 0.75. A platelet function analyzer can be used to assess platelet function in high shear stress conditions.[13] The device measures closure time (Ct), which is the time taken in seconds for a small aperture, coated with a platelet agonist, to be closed by platelet plug formation.[13] An increase in Ct beyond the reference interval of 52 to 86 seconds,[14] through use of cartridges coated with collagen and ADP as a platelet agonist, indicates platelet dysfunction in dogs. Dilution of blood that results in a decrease in Hct or PCV (< 25%[15] to 30%[14,16]) and platelet count (< 100 x 10^9 cells/L[14]) can also increase Ct. Two studies[6,7] assessed the effect of HES 670/0.75 on canine platelet function by use of the PFA-100. Canine blood samples diluted at a ratio of 1:3 with HES 670/0.75 solution led to an increase in Ct,[6] and HES 670/0.75 administered IV to healthy dogs at a dose of 20 mL/kg also resulted in an increase in Ct.[6,7] The findings of both studies support the hypothesis that use of HES 670/0.75 can cause platelet dysfunction in dogs, although the effect of dilution alone on Ct in the second study was not specifically addressed. In another study[8] in which HES 670/0.75 was administered to anesthetized dogs at a dose of 10 mL/kg IV, platelet aggregometry revealed no difference in platelet function, compared with platelet function after administration of lactated Ringer's solution. Because of the complications associated with the use of HES 670/0.75 in people, HES solutions with lower molecular weights and molar substitutions, such as HES 130/0.4 and HES 200/0.5, were developed to minimize adverse effects, such as platelet dysfunction. Evidence for a difference between these 2 HES solutions, however, is mixed in regard to their effect on platelet function in people. Whereas some studies[2,17,18] have indicated that administration of HES 130/0.4 does not cause platelet dysfunction, others have shown that use of HES 130/0.4 causes platelet

dysfunction to the same[4] or lesser[5] degree than that caused by HES 200/0.5. No consensus has been reached on the clinical implication of using an HES solution of lower molecular weight and molar substitution. Although a study[19] has shown some differences in blood loss and transfusion requirements among people treated with HES solutions of different molecular weights and molar substitutions, several meta-analyses have failed to show a significant difference.[1,20,21]

Little research has been conducted to investigate the effect of HES solutions of low molecular weight and molar substitution on platelet function in dogs. One study[22] revealed that dilution of canine blood samples with HES 130/0.42 at a ratio of 1:3 resulted in an increase in Ct beyond a dilutional effect.[22] The product HES 130/0.4, which has a low molecular weight and molar substitution and is commercially available in North America and Australia, has different properties from HES 130/0.42. The product HES 200/0.5 is also available in Australia. Whether use of HES 130/0.4 causes platelet dysfunction in dogs is unknown, as is whether use of HES 200/0.5 would cause worse platelet dysfunction because of its higher molecular weight and molar substitution than HES 130/0.4. The purpose of the study reported here was to measure Ct in samples of canine whole blood diluted with HES 130/0.4 and HES 200/0.5 in vitro and to compare the results with those of whole blood diluted with saline (0.9% NaCl) solution. We hypothesized that dilution alone would result in a dilution-dependent increase in the Ct and that whole blood samples diluted with HES 200/0.5 would have a longer Ct than those diluted with HES 130/0.4, beyond any dilutional effect.

3.3 Material and methods

Animals—Ten healthy adult dogs (6 males and 4 females) owned by hospital staff members were included in the study after owner consent was obtained. The median age of the dogs was 4 years (IQR, 2 to 8 years), and the median body weight was 28.2 kg (IQR, 16.2 to 31.2 kg). All dogs were considered healthy on the basis of the absence of the following factors: disease or illness within 1

month prior to study inclusion; history of coagulopathy or disease that may affect hemostasis; treatment with NSAIDs, propofol, synthetic colloids, or antimicrobials within 1 month prior to study inclusion; and abnormal findings during physical examination. Each dog had a PCV > 40%; platelet count > 200 X 10^9 cells/L as measured with a hematologic analyzer[a] and verified by manual count; mean Ct within the reference interval of 52 to 86 seconds,[14] as measured in duplicate with the platelet function analyzer[a] by use of cartridges coated with collagen and ADP[b]; and results of automated CBC analysis[c] within reference intervals. The study protocol was approved by the Animal Ethics Committee of Murdoch University.

Blood sample collection and processing—The skin over a jugular vein was aseptically prepared for venipuncture in all dogs. A 21-gauge butterfly catheter was placed in the prepared vein through a stab incision and connected to allow blood collection (4.5 mL) into each of 4 evacuated tubes containing 3.2% trisodium citrate. The first tube in each set was discarded, and the remaining 13.5 mL of citrated whole blood was used for the experiment.

Each citrated whole blood sample was diluted with 6% HES 130/0.4,[d] 10% HES 200/0.5,[e] and saline solution at a ratio of 1:9 (0.2 mL of solution to 1.8 mL of whole blood) and 1:3 (0.5 mL of solution to 1.5 mL of whole blood) and was maintained at 38.0°C in a water bath. Within 2 hours after sample collection, Ct for each diluted blood sample was measured with the platelet function analyzer and collagen and ADP–coated cartridges in duplicate. When duplicate measurements had a covariance > 15%, or when the analyzer indicated an error such as flow obstruction, a new sample at the same dilution was created, and a repeated measurement was made. Results that had a covariance > 15% were then excluded from analysis.

Statistical analysis—Closure time was used as the response variable of interest, and values were assessed for a normal distribution with the Shapiro-Wilk test. Because the test revealed that the values were not normally distributed, Cts were reported as median (IQR). Reciprocal transformation resulted in a normal distribution, so 1/Ct was used for the response variable in statistical tests. A

generalized linear mixed model was developed by use of statistical software,[f] with fixed effects of solution (3 levels) and dilution (3 levels) and a random effect for dog. When a significant ($P < 0.05$) interaction of solution and dilution was identified, post hoc comparisons were performed across solutions and dilutions with the least squares means method and Bonferroni correction, with values of $P < 0.005$ considered significant.

3.4. Results

Blood samples were successfully collected from all 10 healthy dogs and were processed in accordance with the experimental protocol. The median predilution PCV was 48% (IQR, 42% to 50%), and platelet count was 276 X 10^9 cells/L (IQR, 192 X 10^9 cells/L to 440 X 10^9 cells/L). The PCV and the platelet count of all blood samples diluted with saline solution remained > 32% and > 86 X 10^9 cells/L, respectively (Table 3-1). One blood sample diluted 1:9 with saline solution was excluded from statistical analysis because of a dilution error.

A dilutional effect on Ct was identified for the 1:3 dilution, compared with the 1:9 dilution, for each type of solution tested, but only samples diluted with HES 200/0.5 had an increase in Ct beyond a dilutional effect at the 1:3 dilution ($P = 0.002$). No effect of HES or dilution on Ct was evident for the 1:9 dilutions (Table 3-2). Fourteen of the 140 (10%) measurements needed repeated measurement because of a high covariance or machine error; however, no sole measurement was repeated more than twice.

Table 3-1 - Median (IQR) PCV and platelet count for 1:3 and 1:9 dilutions with 0.9% NaCl solution of whole blood samples from 10 healthy dogs.

Dilution	**PCV (%)**	**Platelet count (X10^9 cells/L)**
None	48 (42–54)	276 (192–440)
1:9	41 (30–50)	243 (100–288)
1:3	36 (32–42)	213 (86–326)

Table 3-2 - Median (IQR) platelet Ct for 1:3 and 1:9 dilutions with 0.9% NaCl solution, HES 130/0.4, and HES 200/0.5 of whole blood samples from 10 healthy dogs.

Dilution	**0.9% NaCl**	**HES 130/0.4**	**HES 200/0.5**
None	70 (66–73)^{A}	70 (66–73)^{A}	70 (66–73)^{A}
1:9	71 (62–83)^{a,A}	83.5 (71–91)^{a,B}	80.5 (73–84)^{a,A}
1:3	92 (85–100)^{a,B}	101 (94–112)^{a,b,C}	125 (118–140)^{b,B}

$^{a–c}$Within a row, values with the same superscript letter are not significantly (ie, $P \geq 0.005$; Bonferroni corrected) different. $^{A–C}$Within a column, CTs with the same superscript letter are not significantly (ie, $P \geq 0.005$; Bonferroni corrected) different. The reference interval used for CT was 52 to 86 seconds.[14]

3.5. Discussion

The present study revealed that a 1:3 dilution of canine blood with HES 200/0.5 but not HES 130/0.4 led to an increase in platelet Ct beyond a dilutional effect. This in vitro dilution was chosen to mimic the dilution achieved in vivo through IV administration of a fluid bolus of 30 mL/kg, ignoring redistribution and excretion in dogs. Findings suggested that HES 130/0.4 had less of an adverse effect on platelet function than did HES 200/0.5. We also found that a 1:3 dilution resulted in an increase in Ct beyond the upper reference limit, no matter which solution was used. The increase in Ct associated with dilution with any solution can be explained by a decrease in PCV and platelet count. A negative relationship between Ct and PCV in dogs has been described.[14,16] Changes in Hct can affect platelet function because of changes in blood flow dynamics altering platelet interaction with the endothelium,[23] which may also hold true for contact with the aperture of the platelet function analyzer used, as well as a decrease in ADP release from erythrocytes.[24] A decrease in

platelet count may have also contributed to the increase in Ct in the diluted samples in the present study, which has been identified in previous studies[14,16] involving dogs. This effect may have been enhanced by dilution of von Willebrand factor.[25] Several theories may explain the mechanism by which HES 200/0.5 dilution caused platelet dysfunction in the present study, including binding of von Willebrand factor,[26,27] HES molecules coating platelet surfaces, and a decrease in expression of platelet surface protein integrin αIIbβ3, which all may reduce platelet adhesion and aggregation.[28] An in vitro study[18] of human platelet function revealed a decrease in expression of integrin αIIbβ3 receptor with dilutions involving HES 200/0.5 but not HES 130/0.4.[18] The differences identified between HES 200/0.5 and HES 130/0.4 might be related to solution-specific pharmacological properties, in particular molecular weight and molar substitution.[27,29] The molecular weight of an HES solution is the mean manufactured molecular weight of the polydispersed solution, and the molar substitution is the number of hydroxyl groups substituted with hydroxyethyl groups on the glucose subunit of HES molecules.[29] Hydroxyethyl starch solutions of higher molecular weight and molar substitution have a slower rate of degradation and excretion in vivo than do other HES solutions, and this phenomenon has commonly been cited as the main reason that treatment with these solutions causes greater platelet dysfunction.[29,30] However, these pharmacokinetic properties may not be relevant to in vitro studies. It is possible that cleaving of HES molecules by amylase may still occur in vitro, as it does in vivo, decreasing the HES molecular weight within the sample; therefore the rate of degradation may still be relevant. Some evidence exists that high molar substitution can considerably affect the degree of whole blood coagulation in vitro, independent of molecular weight.[31] But there is little evidence regarding the effect of differences in molecular weight and molar substitution, independent of each other, on platelet function specifically in vitro. Another property related to a slower rate of HES degradation and greater platelet dysfunction is a high C2:C6 ratio; [31] however, this was unlikely to be applicable in the present study given that HES 200/0.5 has a lower C2:C6 ratio (5:1) than does HES 130/0.4 (9:1). The present study did not

elucidate the exact mechanisms of HES-induced platelet dysfunction, given that it was designed only to determine whether platelet dysfunction occurred, as detected by the platelet function analyzer.

One additional difference between the 2 HES solutions evaluated was the concentration of HES. The HES 200/0.5 solution had a greater concentration of HES molecules (10%), compared with HES 130/0.4 (6%). The 10% solution was chosen for comparison because it is the solution available for clinical use in Australia. Although it is unknown which solution had the greater number of HES molecules because of the polydiversity among brands and individual bags of solution, HES 200/0.5 may have provided more individual HES molecules, enhancing platelet dysfunction. Such differences between solutions also create variations in the degree of redistribution in vivo, such as differences in plasma volume expansion, degrees of dilution of blood constituents, and rates of clearance.[32,33]

These variations contribute to the challenge of designing in vivo studies, not only in interpreting differences between solutions but also in the ability to select a valid control solution for comparing the effect of dilution, given that crystalloid solutions have a different pattern of distribution. Therefore, in vitro studies such as the present study, although limited by many factors including the absence of any platelet interaction with the endothelium, also have an advantage in that the effect of various solutions can be compared without the complicating factors of redistribution and metabolism. Other complicating factors common to in vivo studies designed to assess the effect of HES solutions on platelet function include variability in doses used, differences in the disease status of patient populations, and choice of method of platelet function analysis, making comparisons of findings across studies difficult. We chose 1:9 and 1:3 dilutions with the aim of indirectly comparing results of the present study with those of another study,[6] which demonstrated that whole blood diluted with HES 670/0.75 led to an increase in CT beyond a dilutional effect when a 1:3 but not 1:9 dilution was used. The 1:9 dilution mimicked a 10 mL/kg bolus dose and the 1:3 dilution mimicked a 30 mL/kg bolus dose administered IV to a dog with an estimated blood volume of 90 mL/kg (ie, euvolemia). The effect of the same in vitro dilutions on Ct in dogs was investigated in another

study[22] involving an HES solution of low molecular weight and molar substitution, HES 130/0.42, which has some differences in properties compared with HES 130/0.4. A significant increase in Ct was identified with HES 130/0.42 diluted 1:3 with canine whole blood beyond a dilutional effect, which differs from our findings, despite the 2 solutions being very similar. Several factors could account for this difference. Hydroxyethyl starch 130/0.4 is a waxy, maize based colloid, whereas HES 130/0.42 is a potato-based colloid. The 2 solutions also differ in molar substitution, which may account for the difference in Ct. A conservative value of $P < 0.005$ was used for pairwise comparisons made in our study to reduce type I error, which may have precluded detection of more differences than were identified. Differences in study subjects and subject numbers as well as a lower predilution Ct in our study may have also contributed to different results. For comparison, an in vitro study[3] in humans compared the effect on coagulation between these 2 solutions through use of thromboelastography and platelet aggregometry and found no difference between the solutions, but HES 130/0.4 and HES 130/0.42 have not been compared directly in dogs.

The clinical relevance of the effect of HES solutions with low molecular weight and molar substitution on platelet function in humans remains controversial despite the abundance of literature on the topic. Early in vitro studies indicated that blood samples diluted with HES 130/0.4 caused less platelet dysfunction as measured through various techniques such as platelet aggregometry,[4,34] Ct analysis,[4,5,18] and flow cytometry,[18] compared with that caused by HES solutions of higher molecular weight and molar substitution. However, several clinical trials[1,20,21] comparing the effects of HES 130/0.4 with HES 200/0.5 on blood loss and transfusion requirements in orthopedic surgery, cardiopulmonary bypass patients, and intensive care patients have yielded conflicting evidence, compared with the in vitro studies. Meta-analyses and literature reviews[1,20,21] have shown that no conclusion can be made regarding the benefit of administration of HES 130/0.4 versus HES 200/0.5 or crystalloid solutions in humans because of the small sample sizes and the quality of these human trials and because several reports were retracted for scientific misconduct. In addition, although the manufacturers of HES 130/0.4 have recommended a dose limit of 50 mL/kg/d,

the European Society of Intensive Care Medicine released a consensus statement, in which no dose recommendations for HES 130/0.4 could be made given the minimal evidence for its safety regarding blood loss, acute kidney injury, and risk of death.[35] Most recently, the Surviving Sepsis Campaign consensus statement has recommended against the use of HES solutions in septic human patients because of the lack of clear benefit and evidence of harm caused to patients,[36] but large clinical trials have yet to be performed in veterinary medicine.

The present study demonstrated that dilution of canine blood samples 1:3 with saline solution, HES 130/0.4, and HES 200/0.5 led to an increase in Ct attributable to a dilutional effect alone. Hydroxyethyl starch 200/0.5, however, led to an increase in Ct beyond a dilutional effect when diluted 1:3 with blood, consistent with platelet dysfunction. Interestingly, we found that HES 130/0.4 did not cause platelet dysfunction at this same dilution, which suggests that this solution may be a superior choice when platelet dysfunction is of concern. However, in vivo studies are warranted to investigate the risk of clinical use of this solution in dogs.

3.6. Footnotes

a. Platelet Function Analyzer-100, Dade Boehring Inc, Miami, Fla.

b. Dade PFA Collagen/ADP Test Cartridge, Siemens Healthcare, Marburg, Germany.

c. ADVIA 120 hematology system, Bayer Diagnostics Mfg Ltd, Dublin, Ireland.

d. Voluven 6% (hydroxyethyl starch 130/0.4), Fresenius Kabi Deutschland GmbH, Friedberg, Germany.

e. Starquin 200 10%, Biomed Ltd, Point Chevalier, Auckland, New Zealand.

f. SAS, version 9.3, SAS Institute Inc, Cary, NC.

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4. Chapter 4: PLATELET CLOSURE TIME IN ANESTHETIZED DOGS WITH HEMORRHAGIC SHOCK TREATED WITH HYDROXYETHYL STARCH 130/0.4 OR 0.9% NACL

4.1 Abstract

Objective - Hydroxyethyl starch (HES) 130/0.4 is used to treat shock in dogs. This study measured platelet closure time (PCT) in dogs during hemorrhagic shock and after HES 130/0.4 or 0.9% NaCl administration.

Animals - Eleven healthy greyhounds

Procedure - Dogs were anesthetized and 48mL/kg of blood removed to induce hemorrhagic shock. Dogs received 20 mL/kg of HES 130/0.4 (*n*=6) or 80 mL/kg of 0.9% sodium chloride (NaCl) (*n*=5) intravenously over 20 minutes. Platelet closure time was measured using the Platelet Function Analyser-100 with collagen and adenosine-diphosphate cartridges at: T0 = 60 minutes after induction of anesthesia prior to hemorrhage, T1 = during hemorrhagic shock and T2 = 40 minutes after completion of fluid bolus. Packed cell volume (PCV) and platelet count were concurrently measured.

Results - Hemorrhagic shock did not significantly change PCT, with no difference between T0 and T1. Both the HES 130/0.4 and 0.9% NaCl group had a significantly increased mean PCT at T2 to 91.4 seconds (95% CI 69.3-113.4) and 95.5 seconds (95% CI 78.2-112.8), respectively, compared to T1. The magnitude of change was significantly greater for the 0.9% NaCl group than the HES 130/0.4 group. There was no difference in the magnitude of change in PCV and platelet count between the two groups. The PCV and platelet count were > 25% and > 100,000/µL respectively in all dogs, except for dogs in the HES 130/0.4 group at T90 where platelet counts were <100,000/µL.

Conclusion and Clinical Relevance - Hemorrhagic shock in greyhounds under anesthesia did not cause a significant change in PCT. Both HES 130/0.4 and 0.9% NaCl administration after induction of

shock increased PCT. These results do not support that HES 130/0.4 causes relevant platelet dysfunction beyond hemodilution, however, this study did not assess risk of bleeding.

4.2 Introduction

The use of hydroxyethyl starch (HES) solutions for the treatment of shock is controversial because of adverse effects reported on both human[1-5] and canine[6-8] platelet function, and its association with increased intra-operative blood loss and transfusion requirements in people.[1,9-12] Many studies support that these complications are associated with HES solutions of high molecular weight (MW) and high molar substitution (MS).[5,6,13] Therefore, the use of HES 130/0.4, with a low MW of 130 KDa and a low MS of 0.4, has become more commonplace in people due to its minimal effect on platelet function.[2,13,14] However, there is some contradicting evidence that platelet dysfunction still occurs with HES 130/0.4.[4,5]

Studies investigating the use of HES solutions in dogs have most commonly used the Platelet Function Analyzer-100 (PFA-100) to measure platelet function.[6,7,15] It measures platelet closure time (PCT), which is the time taken in seconds for a small aperture, to be closed by platelet plug formation.[16] An increased PCT indicates platelet dysfunction, however, dilution of blood that decreases hematocrit (< 25% – 30%[17-19]) and platelet count (< 100,000 platelets/µL[19]) can also increase PCT.

There have been two recent in vitro studies using the PFA-100 to assess the effect of low MW and low MS HES solutions on canine platelet function.[15,20] One study using HES 130/0.42 found that it caused a mild prolongation in PCT[20] whereas one using HES 130/0.4 found no effect.[15] As in vitro studies cannot assess the pharmacokinetic and pharmacodynamic effects of these fluids, and exclude any platelet interaction with the endothelium, it is unknown to what degree HES 130/0.4 may have on canine platelet function in vivo. Also, these solutions are usually used in dogs suffering

from shock and it has been found in people that trauma-induced shock can cause platelet dysfunction itself.[21-24] Therefore, shock may further enhance any platelet dysfunction that HES 130/0.4 causes.

The objective of this study was to determine if hemorrhagic shock increases PCT above baseline in anesthetized greyhounds. The secondary objective was to determine if 20 mL/kg of HES 130/0.4 increases PCT beyond the effect of hemorrhagic shock or hemodilution.

4.3 Materials and methods

This study was approved by the university's Animal Ethics Committee. Eleven healthy adult greyhound dogs were included in this study. Dogs were healthy based on physical examination and complete blood count. The dogs were not fasted and had free access to water. Each dog was randomized to receive either HES 130/0.4 (treated group, *n* = 6) or 0.9% sodium chloride (NaCl) (control group, *n* = 5) according to an assigned sealed envelope.

The dogs were premedicated with 0.2mg/kg of methadone[a] given intramuscularly. Thirty minutes after premedication, a 20-gauge 1-inch over-the-needle catheter was aseptically placed in the right cephalic vein, and anesthesia was induced with 2.5mg/kg of alfaxalone[b] given intravenously. Animals were placed in left lateral recumbency, orotracheally intubated and anesthesia was maintained with isoflurane[c] and 100% oxygen via a circle breathing system. Vaporiser settings were adjusted to achieve an end-tidal isoflurane concentration of 1.4% measured using an agent monitor.[d] Mechanical ventilation was provided using a volume controlled time cycled ventilator[e] at a rate of 10 breaths per minute with an initial tidal volume of 20 mL/kg, which was subsequently adjusted if required during the instrumentation period to achieve a $PaCO_2$ of 35-40 mmHg. No further adjustment was made throughout the remainder of anesthesia. Hartmann's solution[f] was administered via the cephalic catheter at a rate of 10 ml/kg/hr until the time of administration of the

treatment or control solutions. A 12-gauge 6-inch over-the-needle catheter was aseptically placed in the right jugular vein, with the distal end measured beyond the thoracic inlet. A 14-gauge 4-inch over-the-needle catheter was surgically placed in the left femoral artery. The catheters were connected via fluid filled transducers[g] to a multivariable monitor[h] and calibrated to atmospheric pressure. Heart rate (HR), electrocardiogram, central venous pressure, and pulse oximetry were monitored with a multivariable monitor throughout the experiment. Body temperature was maintained between 36°C and 38°C by applying warm air to the upper surfaces of the dog.[i]

Sixty minutes after induction of anesthesia, hemorrhagic shock was induced by removing 48 mL/kg of blood over a 30 minute period from the femoral arterial catheter. A pilot study in greyhounds using the same anesthetic protocol was performed prior to this study, which determined that removal of 48 mL/kg of blood induced shock, as assessed by an increase in HR, a direct MAP of less than 60 mmHg, and an increase in oxygen extraction ratio (O_2ER).

Immediately after 48 mL/kg of blood was removed, intravenous Hartmann's solution administration was discontinued and the dogs received either 20 mL/kg of HES 130/0.4[j] or 80 mL/kg of 0.9% NaCl over 20 minutes. Hydroxyethyl starch 130/0.4 was delivered by a pressure bag via the cephalic catheter and 0.9% NaCl was delivered by 2 pressure bags via the cephalic and jugular catheters. The pressure applied to the pressure bags was monitored throughout to ensure the fluid was delivered continuously during the 20 minute period.

Cardiac output (CO) was measured by the lithium dilution cardiac output[k] technique as previously described,[25] with modification of the dose of lithium chloride to 0.005 mmol/kg and withdrawing blood via the femoral artery. Measurements were made in duplicate. When two results varied more than 15%, repeated measurements were made and the results varying more than 15% were excluded from analysis.

Blood was collected simultaneously from the femoral arterial and jugular venous catheters into lithium heparin syringes, and blood gas analysis[l] was performed within 5 minutes of blood collection. Venous blood was collected from the jugular venous catheter into sodium citrate tubes for the measurement of PCT. Platelet closure time was measured in duplicate using the PFA-100,[m] with collagen and adenosine diphosphate cartridges[n] and co-variance of less than 15% between measurements were accepted. Where duplicate measurements had a co-variance of greater than 15%, or if the analyzer indicated an error such as flow obstruction, measurement was repeated using the same sample and the two closest samples with a CV < 15% were included. Platelet closure time was measured at three time points: T0, at base line during general anesthesia immediately prior to hemorrhage; T1, during hemorrhagic shock, which was immediately after removing 48 mL/kg of blood; and T2, 40 minutes after completion of fluid administration (Figure 1).

Data also collected simultaneously at these time points included HR, direct MAP, arterial and venous blood gas, hemoglobin concentration (Hb), PCV, and CO. At the conclusion of data collection, euthanasia was performed using intravenous pentobarbital in accordance with the ethics committee approved protocol. The O_2ER was calculated at each time point using the formulae in Table 1.

Statistical analysis - For assessment of the effect of shock on PCV, platelet count, O_2ER and PCT, paired comparisons were made across all greyhounds for data at T0 vs T1 using a paired t-test.

For assessment of the effect of fluid therapy on PCV, platelet count, O_2ER and PCT, comparisons were made across both treatment groups for data at T1 vs T2 using a paired t-test. Where both treatments had a similar result (i.e. increase or decrease), the magnitude of the change was compared between treatments using an unpaired t-test with a Satterthwaite adjustment for unequal variances when indicated. Significance was determined at $p \leq 0.05$ for all tests. SAS v 9.3 was used for the analysis (SAS Institute, Cary, NC).

Figure 4-1 - Time points for platelet closure time measurements: T0, at base line during general anesthesia immediately prior to hemorrhage; T1, during hemorrhagic shock, which was immediately after removing 48 mL/kg of blood; and T2, 40 minutes after completion of fluid administration of either hydroxyethyl starch (HES) 13/0.4 or 0.9% sodium chloride (NaCl).

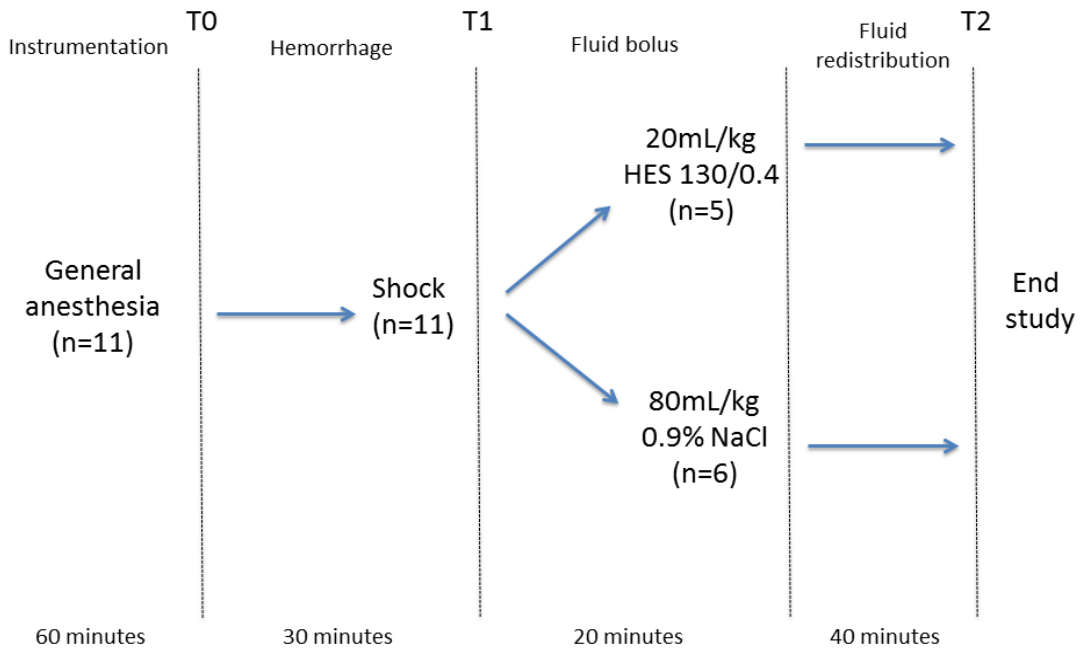

Table 4-1 - Formulae for calculated variables[49]

Calculated variable (units)	**Symbol**	**Formula**
Arterial oxygen content (mL/dL)	CaO_2	(1.34 x [Hb] x SaO_2) + (0.003 x PaO_2)
Venous oxygen content (mL/dL)	CvO_2	(1.34 x [Hb] x SvO_2) + (0.003 x PvO_2)
Cardiac index (L/minute/m^2)	CI	CO/BSA
Oxygen delivery index (mL/minute/m^2)	DO_2	CaO_2 x CI x 10
Oxygen consumption index (mL/minute/m^2)	VO_2	(CaO_2 – CvO_2) x CI x 10
Oxygen extraction ratio (%)	O_2ER	VO_2/DO_2 x 100
Body surface area (m^2)	BSA	10.1 × body weight $(kg)^{0.67}$/100

BSA, body surface area; CO, cardiac output; Hb, hemoglobin concentration; P_aO_2, partial pressure of oxygen in arterial blood; P_vO_2, partial pressure of oxygen in venous blood; SaO_2, hemoglobin saturation in arterial blood; S_vO_2, hemoglobin saturation in venous blood.

4.4 Results

Five entire male and 6 entire female greyhounds were used in this study. The mean body weight was 28.3kg (SD 3.3kg), and the mean age was 3.7 years (SD 1.8 years). Two dogs in the HES 130/0.4

group had PCTs and platelet count at T2 excluded from analysis due to platelet clumping in the sample.

The first objective was evaluated across all dogs. There was no significant change in PCT at T1, compared to T0 (Table 2). There was a significant decrease in PCV and platelet count, and a significant increase in O_2ER, at T1 compared to T0 (Table 2), consistent with hemorrhagic shock.

The second objective was evaluated across treatment groups. Both groups had a significant increase in PCT at T2, compared to T1 (Table 3). The magnitude of change was significantly greater in the 0.9% NaCl group compared to the HES 130/0.4 group. Both groups had a significant decrease in PCV, platelet count and O_2ER at T2, compared to T1 (Table 2), however, the magnitude of change was not significantly different between the two groups.

The PCV of all dogs at each time point was > 25%. All platelet counts where > 100,000/µL, except for dogs in the HES 130/0.4 group at T2 where platelet counts were < 100,000/µL.

Table 4-2 - Mean (95% confidence interval) packed cell volume, platelet count, oxygen extraction ratio (O_2ER) and platelet closure time in greyhounds under general anesthesia (T0) and thirty minutes after 43mL/kg of blood loss (T1).

Variable	**T0**	**T1**	**P value***
Packed cell volume (%)	49.3 (47.5 - 51.0)	46.9 (45.4 - 48.5)	0.0125
Platelet count ($X10^9$ cells/L)	172 (158 - 186)	149.5 (131.3 - 167.8)	0.0001
O_2ER (%)	8.80 (6.90 - 10.5)	60.0 (48.1 - 70.9)	<0.0001
Platelet closure time+ (seconds)	87.7 (69.6 - 105.8)	70.4 (61.7 - 79.0)	0.0805

* Values less than 0.05 were considered significant

+The greyhound reference interval for platelet closure time is 63 to 92 seconds[48]

Table 4-3 - Mean (95% confidence interval) of packed cell volume, platelet count, oxygen extraction ratio (O_2ER) and platelet closure time in greyhounds in hemorrhagic shock (T1), under general anesthesia, that then received either 20 ml/kg of hydroxyethyl starch (HES) 130/0.4 or 80 ml/kg of 0.9% sodium chloride (NaCl) (T2).

Variable	**Group**	**T1**	**T2**	**P value***	**Magnitude of change (P value*)**
Packed cell volume (%)	HES	46.3 (44.3 - 48.4)	29.8 (28.4 - 31.2)	<0.0001	0.766
	NaCl	47.6 (44.2 - 51.0)	30.6 (26.7 - 34.5)	0.0002	
Platelet count ($X10^9$ cells/L)	HES	133 (111 - 154)	69.5 (28.9 - 110)	0.0023	0.065
	NaCl	170 (144 - 195)	128 (110 - 146)	0.0072	
O_2ER (%)	HES	68 (61 - 75)	9 (4 - 14)	<0.0001	0.056
	NaCl	49 (24 - 75)	10 (5 - 16)	0.0071	
Platelet closure time (seconds)	HES	75.1 (59.0 - 91.4)	91.4 (69.3 - 113.4)	0.0443	0.0421
	NaCl	64.6 (56.9 - 72.3)	95.5 (78.2 - 112.8)	0.0077	

* Values <0.05 were considered significant

4.5 Discussion

This study demonstrated no change in PCT during shock but a significant increase in PCT after administration of 20 mL/kg of 6% HES 130/0.4 and 80 mL/kg of 0.9% NaCl. Interestingly, we found that the magnitude of change in PCT was greater after 0.9% NaCl administration, compared to HES 130/0.4 administration. This result does not support that HES 130/0.4 causes platelet dysfunction beyond the effect of hemodilution.

The lack of significant change in PCT during shock in this study is contrary to the majority of recent studies in people that found platelet dysfunction to occur during hemorrhagic shock secondary to trauma.[21-24,26,27] However, one study in people showed a decrease in PCT immediately following traumatic hemorrhage, which corresponded with increased platelet reactivity measured by flow cytometry.[28] Blood samples were collected immediately following trauma in this one study while blood samples in most previous studies were collected later in the emergency room. Our study also chose an early time point in shock to measure PCT, however the fluctuation in PCT that we observed was not statistically significant.

We used O_2ER as an objective measure of shock, as several studies have demonstrated a significant increase in O_2ER during severe shock.[26,29,30] An increase in O_2ER reflects decreasing oxygen delivery, despite oxygen consumption staying relatively constant. An O_2ER greater than 60% is generally considered a marker of severe shock, as this is the time at which oxygen consumption starts to depend on oxygen delivery, depending on the underlying cause of shock. In our study, there was a moderate to marked increase in O_2ER, and all dogs were tachycardic and hypotensive, supporting the presence of shock. The increase in O_2ER was not as marked as expected in all individuals, given the volume of blood loss. This may be due to individual variability, or due to measuring the parameter in early shock, where good cardiovascular compensation is still intact. Another reason for this difference may be due to the greyhound breed utilized in our study. Although O_2ER has not been

compared between greyhounds and non-greyhound breed dogs, cardiovascular function[31] and oxygen carrying capacity[32] are known to be different.

We found a minor, but significant, increase in PCT with both types of fluid administration, which can be explained by hemodilution. A negative relationship between PCT and PCV has been previously described in dogs.[18,19] Changes in hematocrit can affect platelet function due to changes in blood flow dynamics altering platelet interaction with the endothelium,[33] as well as decreased ADP release from red blood cells.[34] The effect of dilution on PCT may also be due to dilution of von Willebrand Factor.[35] In our study, a decrease in platelet count may have also contributed to the increased PCT, which has been described previously in dogs.[18,19] It is interesting that the magnitude of change in PCT was greater in the 0.9% NaCl group, which seems unlikely to be due to greater hemodilution, as there was no significant difference in PCV or platelet count between the groups. However, dilution of other factors that may affect PCT, such as von Willebrand Factor, were not measured.

In contrast to previous studies using higher MW and MS HES solutions,[6,7] our study found that HES 130/0.4 did not significantly increase PCT, compared to 0.9% NaCl. Hydroxyethyl starch solutions with a high MW and MS are known to have a direct adverse effect on platelet function in people, the mechanisms of which have been described elsewhere.[14,36,37] Previous studies in dogs showed HES 670/0.75 to cause platelet dysfunction as demonstrated by an increased PCT in vitro[6] and in vivo.[7] In another study, HES 200/0.5 (medium MW and MS) diluted 1:3 with canine whole blood increased PCT in vitro, however HES 130/0.4 at this same dilution did not increase PCT beyond dilutional effect. [15] This finding supports our result. In contrast, HES 130/0.42 diluted 1:3 with canine whole blood increased PCT beyond dilutional effect in vitro.[20] However, the different properties of HES 130/0.42 make direct comparisons with HES 130/0.4 difficult.

The failure to cause a significant change in PCT with HES 130/0.4, beyond hemodilution, may be due to use of a 20 mL/kg dose in our study. A 1:4 dilution of HES 130/0.4 with canine whole blood caused greater abnormalities in coagulation, as measured by thromboelastometry, compared with a 1:10

dilution, which suggests a dose dependent effect on coagulation.[38] This study did not assess platelet function specifically. We chose a dose of 20 mL/kg of HES 130/0.4, attempting to match the 80 mL/kg dose of isotonic crystalloid solution,[39,40] as it is a common large bolus dose for the treatment of shock in dogs.[41] As we chose this single dose we cannot comment if HES 130/0.4 has a dose dependent adverse effect on PCT.

There are some limitations to this study. The shock model used in this study induced hemorrhagic shock with minimal trauma by removing a fixed volume of blood in a controlled manner. This model is applicable to situations of large volumes of blood loss under general anesthesia. The model is not directly applicable to animals with systemic inflammatory response syndrome, sepsis or trauma where there is likely cytokine release, which may affect the result.

The results of this study may only be referent to greyhounds. Greyhounds are known to have a higher PCV, resulting in higher blood viscosity, and a lower platelet count.[42-44] Some greyhounds are also known to have significant post-operative bleeding, related to increased fibrinolysis[45] or reduced fibrin cross link formation and clot strength, as measured by thromboelastography.[46,47] Despite postoperative bleeding, PCTs were found to be normal in one study.[46] Platelet closure time has also been validated in greyhounds with their own established reference interval.[48] These findings suggest that the greyhound breed-specific variability in coagulation should not heavily influence the PCT.

We did not have a control group that did not receive fluid resuscitation, therefore we do not know if PCT would have changed over time under general anesthesia or due to prolonged shock. We cannot determine if the changes we saw in PCT after treatment with fluid resuscitation were due to hemodilution or the late effects of shock. Inclusion of a group that were resuscitated with auto-transfused fresh whole blood may have answered this question.

In conclusion, we demonstrated that hemorrhagic shock did not cause platelet dysfunction, as measured by PCT, in greyhounds under general anesthesia. We found that 20 mL/kg of HES 130/0.4

and 80 mL/kg of 0.9% NaCl administered over a 20 minute period significantly increased PCT. The magnitude in increase of PCT was greater with 0.9% NaCl administration, indicating HES 130/0.4 does not cause platelet dysfunction beyond a dilutional effect. Although the changes in PCT were significant, they were minor, therefore may not be clinically relevant. However, this study did not assess the risk of bleeding due to platelet dysfunction.

4.6. Footnotes

a Methone, Parnell Australia Pty Ltd, NSW, Australia

b Alfaxan®, Jurox Pty Ltd, NSW, Australia

c ISO Veterinary Companies of Australia, NSW, Australia

d Capnomac Ultima, Datex-Ohmeda Medical supplies Australia, NSW, Australia

e Model TH-1 Beijing Read Eagle Technology Co Ltd, Beijing, China

f Baxter Healthcare Pty Ltd Australia, NSW, Australia

g DTX Plus™ Argon Critical Care Systems, Singapore

h Surgivet V9203 Sound Medical, VIC, Australia

I Bair Hugger warming unit Model 505, Critical Assist, VIC, Australia

j Voluven 6% (hydroxyethyl starch 130/0.4), Fresenius Kabi Deutschland GmbH, Friedberg, Germany

k LiDCO Cardiac Sensor Systems, LiDCO Ltd, London, UK

l ABL 725 Radiometer Pacific Pty Ltd, VIC, Australia

m Platelet Function Analyzer-100, Dade BoehringInc, Miami, Florida, USA

n Dade PFA Collagen /ADP Test Cartridge, Siemens Healthcare, Marburg, Germany

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5. Chapter 5: SUMMARY AND CONCLUSION

We found that HES 200/0.5 causes canine platelet dysfunction, as measured by the PFA-100 in vitro at a 1:3 dilution with saline, however HES 130/0.4 did not. We also found that in a greyhound haemorrhagic shock model, 20 ml/kg of HES 130/0.4 administered rapidly to treat shock did not cause platelet dysfunction beyond the effects of dilution. This is consistent with the reports from people, where higher MW and MS solutions has a greater risk of causing platelet dysfunction compared with lower MW and MS solutions.[48]

Our findings are an important addition to the literature as they are specific to canine platelet function. Previous studies have shown that HES 670/0.75 causes platelet dysfunction in dogs in vitro and in vivo[25,26] and, although direct comparisons cannot be made with our study, our findings suggest that HES 130/0.4 would be a more ideal solution to use if platelet dysfunction and haemorrhage were of concern.

Some of the limitations of our studies are that only one platelet function test, CT measured by the PFA-100, was utilised, and multiple methods of platelet function analysis would be ideal in order to investigate the mechanisms of platelet dysfunction. In addition, there were some difficulties in measuring platelet function with the PFA-100, due to mechanical errors and result variability. We minimised these errors by ensuring duplicate measurements were made with minimal covariance, however, it became obvious during the study that use of this equipment requires excellent sample handling. This was especially a problem with the in vitro study, where 14% of samples had to be repeated. One of the reasons for this may have been that samples for the in vitro study required more delay before analysis, to allow mixing with solutions, which may have led to platelet clumping. Another possibility is that, as the in vivo study was performed after the in vitro study, the technique for sample handling may have improved. In future studies, the research group will be analysing samples in triplicate in order to avoid having to repeat samples when there is a mechanical error and

improve accuracy of results. This will also avoid the bias of discarding the result that has the larger covariance.

It is unclear how our results apply to dogs requiring colloid support for disease. The first study was in vitro and may not replicate the clinical situation. The second study was an in vivo study, but is specific to a haemorrhagic shock model under general anaesthesia. Therefore, the results from this study may be applicable to haemorrhage associated with surgery but may not apply to animals sustaining trauma in which tissue injury and inflammation may interfere with platelet function. It is also unknown what the effect of anaesthetic agents such as isoflurane have on platelet function in dogs. An additional limitation of the in vivo study is the use of greyhounds. Although the PFA-100 has been validated in greyhounds,[19] as discussed in chapter 3, results may differ from those in non-greyhound breed dogs.

Further studies are warranted to investigate the clinical applicability of our results. Unfortunately, there is no single platelet function or coagulation test that predicts bleeding in dogs or people and the volume of bleeding can be difficult to determine. Therefore, it would be difficult to determine if bleeding occurs with the use of HES 200/0.5, or even HES 130/0.4, despite the findings of our results. Large scale clinical trials would be required to determine the risk of haemorrhage in dogs with trauma, surgery, and those at risk of coagulopathies with the use of HES 130/0.4.

It is also important to address other potential risks of HES solutions in dogs; most importantly acute kidney injury, which is a serious concern in people with sepsis.[100,127-129] Future studies are justified to determine if HES solutions cause acute kidney injury in dogs, and if this risk is clinically relevant. If it does, the canine model may be of use in determining the mechanism of kidney injury, as this information is currently sparse in the human literature also.

Despite our limited understanding of the effects of HES solutions in dogs, based on the results of our study and those to date in the literature, there should be some caution using HES solutions in dogs

at risk of coagulopathy and kidney injury, and in dogs in septic shock. If choosing a HES solution, our findings suggest there would be some benefit in using a lower MW and MS solution such as HES 130/0.4, as it appears to cause less platelet dysfunction, which may reduce the risk of ongoing haemorrhage.

APPENDICES

Appendix A: Chapter 3 raw data – closure times

Closure times (CT) of all dogs (no dilution), and in vitro CTs of respective blood with one in nine dilution (1:9) and one in three (1:3) dilution, with each of the following solutions: 0.9% sodium chloride (NaCl), hydroxyethyl starch (HES) 130/0.4, and HES 200/0.5.

Dog number	Solution	Concentration	CT (seconds)
1	No dilution		69
1	No dilution		65
1	0.9% NaCl	1:9	58
1	0.9% NaCl	1:9	56
1	0.9% NaCl	1:3	79*
1	0.9% NaCl	1:3	67
1	0.9% NaCl	1:3	73
1	HES 130/0.4	1:9	87
1	HES 130/0.4	1:9	66
1	HES 130/0.4	1:3	82
1	HES 130/0.4	1:3	77
1	HES 200/0.5	1:9	84
1	HES 200/0.5	1:9	96
1	HES 200/0.5	1:3	95
1	HES 200/0.5	1:3	92
2	No dilution		73
2	No dilution		75
2	0.9% NaCl	1:9	97
2	0.9% NaCl	1:9	74

2	0.9% NaCl	1:3	102
2	0.9% NaCl	1:3	154*
2	0.9% NaCl	1:3	88
2	HES 130/0.4	1:9	86
2	HES 130/0.4	1:9	88
2	HES 130/0.4	1:3	160*
2	HES 130/0.4	1:3	>300*
2	HES 130/0.4	1:3	111
2	HES 130/0.4	1:3	94
2	HES 200/0.5	1:9	99*
2	HES 200/0.5	1:9	71
2	HES 200/0.5	1:9	77
2	HES 200/0.5	1:3	129
2	HES 200/0.5	1:3	131
3	No dilution		72
3	No dilution		71
3	0.9% NaCl	1:9	62
3	0.9% NaCl	1:9	62
3	0.9% NaCl	1:3	104
3	0.9% NaCl	1:3	74*
3	0.9% NaCl	1:3	94
3	HES 130/0.4	1:9	62
3	HES 130/0.4	1:9	72
3	HES 130/0.4	1:3	93
3	HES 130/0.4	1:3	90
3	HES 200/0.5	1:9	81

3	HES 200/0.5	1:9	72
3	HES 200/0.5	1:3	141
3	HES 200/0.5	1:3	99*
3	HES 200/0.5	1:3	121
4	No dilution		69
4	No dilution		65
4	0.9% NaCl	1:9	119*
4	0.9% NaCl	1:9	108*
4	0.9% NaCl	1:3	83
4	0.9% NaCl	1:3	97
4	HES 130/0.4	1:9	126
4	HES 130/0.4	1:9	88*
4	HES 130/0.4	1:9	125
4	HES 130/0.4	1:3	184
4	HES 130/0.4	1:3	205
4	HES 200/0.5	1:9	84
4	HES 200/0.5	1:9	93
4	HES 200/0.5	1:3	>300*
4	HES 200/0.5	1:3	180*
4	HES 200/0.5	1:3	139
4	HES 200/0.5	1:3	94*
4	HES 200/0.5	1:3	140
5	No dilution		71
5	No dilution		66
5	0.9% NaCl	1:9	87
5	0.9% NaCl	1:9	72

5	0.9% NaCl	1:3	96
5	0.9% NaCl	1:3	88
5	HES 130/0.4	1:9	83
5	HES 130/0.4	1:9	84
5	HES 130/0.4	1:3	107
5	HES 130/0.4	1:3	96
5	HES 200/0.5	1:9	114
5	HES 200/0.5	1:9	30
5	HES 200/0.5	1:3	145
5	HES 200/0.5	1:3	123
6	No dilution		71
6	No dilution		58
6	0.9% NaCl	1:9	83
6	0.9% NaCl	1:9	76
6	0.9% NaCl	1:3	86
6	0.9% NaCl	1:3	96
6	HES 130/0.4	1:9	106
6	HES 130/0.4	1:9	71*
6	HES 130/0.4	1:9	93
6	HES 130/0.4	1:3	155
6	HES 130/0.4	1:3	123
6	HES 200/0.5	1:9	96
6	HES 200/0.5	1:9	84
6	HES 200/0.5	1:3	116
6	HES 200/0.5	1:3	100
7	No dilution		77

7	No dilution		66
7	0.9% NaCl	1:9	73
7	0.9% NaCl	1:9	89
7	0.9% NaCl	1:3	107
7	0.9% NaCl	1:3	83
7	HES 130/0.4	1:9	74
7	HES 130/0.4	1:9	70
7	HES 130/0.4	1:3	101
7	HES 130/0.4	1:3	96
7	HES 200/0.5	1:9	99*
7	HES 200/0.5	1:9	71
7	HES 200/0.5	1:9	80
7	HES 200/0.5	1:3	198
7	HES 200/0.5	1:3	100*
7	HES 200/0.5	1:3	154
8	No dilution		73
8	No dilution		67
8	0.9% NaCl	1:9	69
8	0.9% NaCl	1:9	70
8	0.9% NaCl	1:3	90
8	0.9% NaCl	1:3	94
8	HES 130/0.4	1:9	73
8	HES 130/0.4	1:9	87
8	HES 130/0.4	1:3	105
8	HES 130/0.4	1:3	103
8	HES 200/0.5	1:9	71

8	HES 200/0.5	1:9	76
8	HES 200/0.5	1:3	120
8	HES 200/0.5	1:3	116
9	No dilution		87
9	No dilution		66
9	0.9% NaCl	1:9	68
9	0.9% NaCl	1:9	89
9	0.9% NaCl	1:3	115
9	0.9% NaCl	1:3	84
9	HES 130/0.4	1:9	109
9	HES 130/0.4	1:9	79
9	HES 130/0.4	1:3	112
9	HES 130/0.4	1:3	86
9	HES 200/0.5	1:9	84
9	HES 200/0.5	1:9	81
9	HES 200/0.5	1:3	127
9	HES 200/0.5	1:3	121
10	No dilution		86*
10	No dilution		59
10	No dilution		59
10	0.9% NaCl	1:9	95*
10	0.9% NaCl	1:9	65
10	0.9% NaCl	1:9	62
10	0.9% NaCl	1:3	107
10	0.9% NaCl	1:3	86
10	HES 130/0.4	1:9	122*

10	HES 130/0.4	1:9	70
10	HES 130/0.4	1:9	68
10	HES 130/0.4	1:3	147*
10	HES 130/0.4	1:3	100
10	HES 130/0.4	1:3	99
10	HES 200/0.5	1:9	77
10	HES 200/0.5	1:9	74
10	HES 200/0.5	1:3	119
10	HES 200/0.5	1:3	137

Platelet closure time (PCT), sodium chloride (NaCl), hydroxyethyl starch (HES).

*PCT excluded from statistical analysis.

Appendix B: Chapter 3 raw data – packed cell volumes and platelet counts

Packed cell volume (PCV) and platelet count of each dog, and respective measurements with one in nine (1:9) and one in three (1:3) in vitro dilutions with 0.9% sodium chloride (NaCl).

Dog	PCV of dog (%)	PCV of 1:9 dilution with 0.9% NaCl (%)	PCV of 1:3 dilution with 0.9% NaCl (%)	Platelet count of dog (x 10^9 cells/L)	Platelet count of 1:9 dilution with 0.9% NaCl (x 10^9 cells/L)	Platelet count of 1:3 dilution with 0.9% NaCl (x 10^9 cells/L)
1	54	50	42	259	288	326
2	42	37	32	291	100	86
3	48	42	37	292	270	172
4	40	30	30	440	201	173
5	52	41	35	192	240	191
6	48	41	36	260	240	209
7	42	40	33	250	246	216
8	46	40	38	312	283	224
9	50	46	38	216	270	225
10	48	44	36	319	288	241

Appendix C: Chapter 4 raw data – closure times

Closure times (CT) in greyhounds under general anaesthesia (T0), in haemorrhagic shock (T1), and 40 minutes after receiving either 80ml/kg of 0.9% sodium chloride (NaCl) or 20ml/kg of hydroxyethyl starch (HES) 130/0.4 over 20 minutes (T2).

Dog	**Solution**	**PCT T0**	**PCT T1**	**PCT T2**
50811	0.9% NaCl	69	61	71
		87	73	94
90911		72	77	107
		75	69	98
A141011		67	62	72
		70	64	100
B141011		148	65	117
		175	64	116
		95*		73*
A211011		67	50	88
		80	62	92
170611	HES 130/0.4	73	70	Flow obstruction[#]
		60	68	Flow obstruction[#]
80711		85	60	No closure[#]
		78	67	No closure[#]
260811		109	88	83
		108	76	91
				158*
230911		81	72	83

		87	72	84
A071011		90	116	122
		79	90	102
B071011		91	65	91
		79	58	75

* Data excluded from analysis

Data excluded from analysis due to platelet clumping in sample

Appendix D: Chapter 4 raw data - packed cell volume and platelet counts

Packed cell volume and platelet counts of greyhounds under general anaesthesia (T0), during haemorrhagic shock (T1), and 40 minutes after received 0.9% sodium chloride (NaCl) or hydroxyethyl starch (HES) 130/0.4 .

Dog	Solution	PCV (%) T0	PCV (%) T1	PCV (%) T2	Platelet count (x 10^9 cells/L) T0	Platelet count (x 10^9 cells/L) T1	Platelet count (x 10^9 cells/L) T2
50811	0.9% NaCl	54	51	34	179	156	107
90911		47	50	30	169	149	122
A141011		47	45	30	184	164	144
B141011		47	46	33	190	178	133
A211011		47	46	26	201	200	134
170611	HES 130/0.4	50	47	N/A	136	118	N/A
80711		54	48	N/A	174	140	N/A
260811		48	43	29	151	110	38
230911		50	47	29	184	166	86
A071011		48	48	28	141	121	60
B071011		50	45	31	186	143	94

N/A Data not available