Ana Liza Cardoso Neto Oliveira Ortiz

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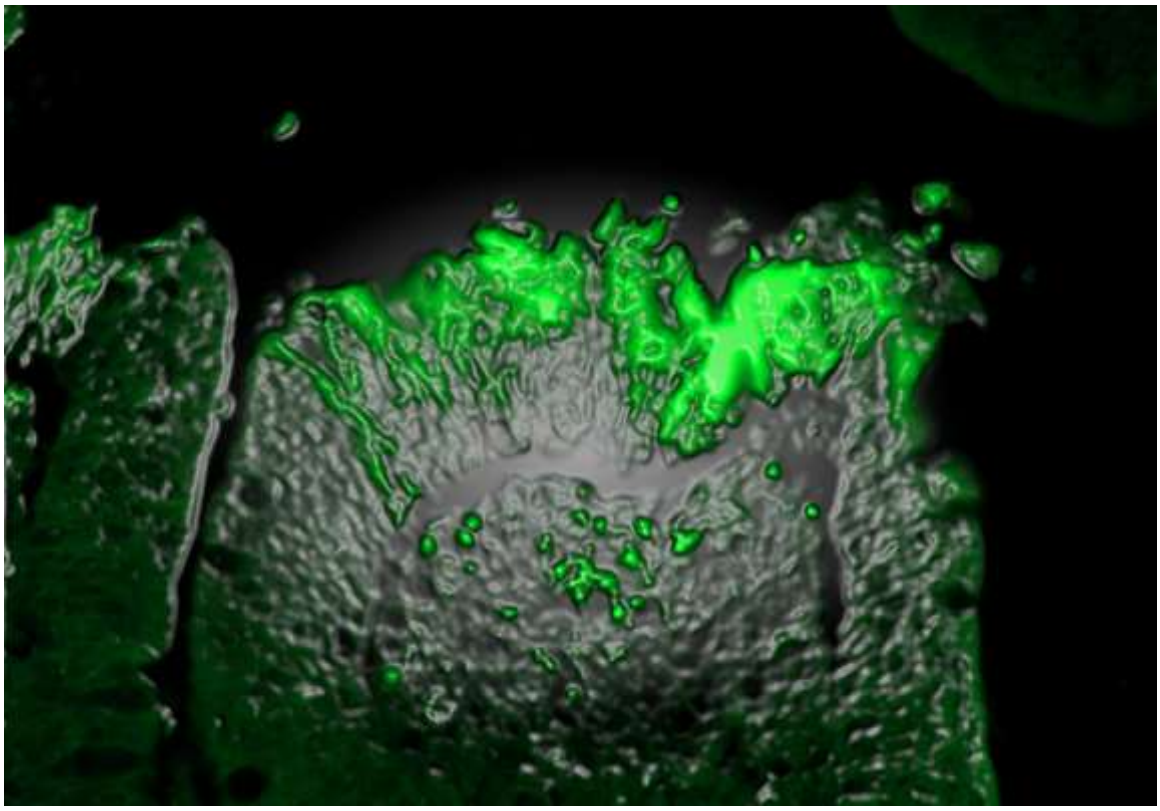

University of León, León, Spain, 2015

University of León
Faculty of Veterinary Medicine
Department of Veterinary Medicine, Surgery and Anatomy

"The influence of administering Ringer's Lactate solution or HES 130/0.4 on the integrity of the small intestinal mucosa in a pig model of controlled haemorrhage"

"La influencia de la administración de solución de lactato de Ringer o HES 130 / 0,4 en la integridad de la mucosa del intestino delgado en un modelo porcino de hemorragia controlada"

Ana Liza Cardoso Neto Oliveira Ortiz

THIS DISSERTION IS SUBMITTED FOR THE DEGREE OF DOCTOR IN VETERINARY SCIENCES

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The doctrines presented in this work

are the sole responsibility of the author.

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Resumen General

La hipovolemia pre-quirúrgica o quirúrgica ocurre con frecuencia tanto en medicina humana como en medicina veterinaria, y puede llevar a hipoperfusión intestinal. La hipoperfusión intestinal está asociada con un incremento de la morbilidad. Se desconocen en profundidad los efectos de las distintas soluciones intravenosas utilizadas para restaurar la volemia y perfusión tisular sobre la integridad de la mucosa intestinal en situaciones clínicas, y si algunas de estas pueden estar asociadas con un menor daño de la mucosa intestinal. Este estudio analiza los efectos de dos soluciones fisiológicas, Ringer lactato e hydroxietil almidón (HES) 130/0.4 en la mucosa del intestino delgado en cerdos bajo anestesia general sometidos a sangrado agudo.

Veintiocho cerdos sanos de la raza Large White, de tres meses de edad, fueron sometidos a sangrado agudo severo (30ml/kg) bajo anestesia total intravenosa con propofol y remifentanilo. Los cerdos fueron asignados de forma aleatoria a tres grupos: Grupo 1 (n=11) recibió solución Ringer Lactato (25ml/kg) tras el sangrado; Grupo 2 (n=11) recibió solución HES 130/0.4 (20ml/kg) tras el sangrado; los animales en el grupo 3 (n=6) no tuvieron sangrado o restauración de volumen. Todos los cerdos fueron eutanasiados con cloruro de potasio tras el estudio y el intestino delgado fue recogido para análisis histopatológico. Edema intestinal, congestión, hiperemia, hemorragia, infiltrado inflamatorio, degeneración celular, necrosis y desprendimiento epitelial fueron evaluados y clasificados. Porcentaje de perdida de mucosa (%ML) y el ratio cripta:intersticio (C:I) también fueron analizados. Adicionalmente, los eventos apoptóticos fueron investigados mediante inmunohistoquímica con anticuerpo para citocromo C, método TUNEL y ensayo de inmunofluorescencia con M30Cytodeath.

Los resultados de este estudio sugieren, que en una situación de hemorragia controlada, HES 130/0.4 puede minimizar el porcentaje de perdida de mucosa en el intestino delgado, en comparación con una solución Ringer Lactato, cuando estas son usadas para reemplazo de volumen. Los datos en este estudio también sugieren que el duodeno puede ser el segmento de intestino delgado más sensible a hipovolemia causada por sangrado severo. La hiperemia en el intestino delgado fue significativamente mayor con HES 130/0.4 comparada con solución Ringer Lactato; esto sugiere que la administración de HES 130/0.4 podría restaurar la perfusión

intestinal con mayor eficiencia y estar asociada con una menor porcentaje de perdida de mucosa. A pesar de las diferencias muy significativas en histopatología para el porcentaje de perdida de mucosa, los métodos inmunohistoquímicos usados para evaluar los eventos apoptóticos en el epitelio del intestino delgado (anticuerpo citocromo C, método TUNEL y ensayos de inmunofluorescencia M30 Cytodeath), no revelaron diferencias significativas en el porcentaje de apoptosis entre grupos.

General Abstract

Perioperative and/or intraoperative hypovolaemia occur frequently in both human and veterinary critical care medicine and may lead to intestinal hypoperfusion. Intestinal hypoperfusion, in its turn, is associated with increased morbidity. Little is known about the effects of different intravenous solutions that may be used in the attempt to restore the volaemia and tissue perfusion, on the integrity of the intestinal mucosa in this clinical situation and whether some with would be associated with decreased damage of the intestinal mucosa. This study analysed the effect of two physiological solutions, Ringer's lactate and hydroxyethyl starch (HES) 130/0.4, on the small intestinal mucosa in pigs under general anaesthesia submitted to severe acute bleeding.

Twenty-eight healthy Large White pigs, with three months of age, were submitted to severe acute bleeding (30 ml/kg) under total intravenous anaesthesia with propofol and remifentanil. Pigs were randomly allocated in three groups: Group 1 (n=11) received Ringer Lactate solution (25ml/kg) after bleeding; Group 2 (n=11) received HES 130/0.4 solution (20ml/kg) after bleeding; animals in Group 3 (n=6) did not undergo bleeding or volume replacement. All pigs were euthanised with intravenous KCl after the study and the small intestine was collected for histopathological analysis. Small intestinal oedema, congestion, hyperaemia, haemorrhage, inflammatory infiltration, cellular degeneration, necrosis, and epithelial detachment were evaluated and classified. Mucosal loss percentage (%ML) and crypt:interstitium ratio (C:I) were also analysed. In addition, apoptotic events were investigated by performing immunohistochemistry using Cytochrome c antibody, TUNEL method and M30Cytodeath immunofluorescence assay.

The results of this study suggest that, in a situation of controlled haemorrhage, HES 130/0.4 can minimize the percentage of mucosal loss on the small intestine, when compared with Ringer Lactate solution, when used for volume replacement. Data in our study also suggest that the duodenum may be the small intestine segment most sensitive to hypovolaemia caused by severe bleeding. Hyperaemia in the small intestine was significantly higher with HES 130/0.4 compared to Ringer Lactate; this suggests that HES130/0.4 administration may more efficiently restore intestinal perfusion and therefore be associated with decreased %ML. Despite the very significant differences in

histopathology for the %ML, the immunohistochemical methods used to evaluate apoptotic events in the small intestine epithelium (cytochrome c antibody, TUNEL method and M30Cytodeath immunofluorescence assays, revealed no significant differences in the percentage of apoptosis between groups.

CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. Introduction

In both human and veterinary clinical practice, keeping an adequate intravascular volume is very important following trauma and/or haemorrhage, and in patients undergoing major surgery. If sustained hypovolemia occurs, it may be responsible for the initiation of complex pathophysiologic processes that may lead to an inadequate tissue perfusion (Lang et al. 2001; Boag & Hughes 2005). Several physiological solutions have been proposed to achieve and/or maintain hemodynamic stability in these situations, namely blood and its components (as human albumin), synthetic colloids (dextrans, gelatins, hydroxyethyl starch - HES), or crystalloids (as lactated Ringer's solution) (Lang et al. 2001; Hiltebrand et al. 2009). Several HES solutions have been developed with different molecular weights (MWs) and degrees of substitution (DSs). Among them, a more recently developed HES preparation with an intermediate MW (130 kd) and a very low DS (0.4) was approved in several countries in recent years for facing hypoperfusion associated to hypovolemia. It was supposed to have convincing advantages in pharmacokinetics and pharmacodynamics and was presented as possibly the safest synthetic colloid. Waitzinger and colleagues found no clinically relevant plasma accumulation and related side effects after single and multiple-dose infusion in healthy human volunteers (Waitzinger et al. 1998; Waitzinger et al. 2003). In a study in a rat model, Bepperling and colleagues demonstrated significantly lower tissue storage after multiple infusions with HES 130/0.4 when compared with conventional HES preparations (Leuschner et al. 2003). Furthermore, HES 130/0.4 was assumed to have less effect on coagulation impairment and minimal or none HES plasma accumulation, and HES tissue storage, usually reported for other HES with higher MWs and DSs (O Langeron et al. 2001; Jungheinrich et al. 2002; Neff et al. 2003; Kozek-Langenecker 2008; Staikou et al. 2012).

In three recent large randomized controlled trials (Brunkhorst et al. 2008; Myburgh et al. 2012; Perner et al. 2012) in human critical patients, the use of HES was associated with higher rates of acute kidney failure or dialysis, and to an increased 90 day mortality rate in one study (Perner et al. 2012). Based on these results, on the 27th of

June 2013, marketing authorizations for all HES products were suspended by the European Medicines Agency, in the United Kingdom. Nevertheless, this decision was not consensually well accepted by all clinicians, and some pointed out limitations of the studies (Chappell & Jacob 2013). It is mentioned that there is lack of evidence of the same adverse effects in surgical patients, which was what presumably accounted for the Food and Drug Administration's (FDA) decision not to withdraw HES solutions completely in the USA (FDA recommended that HES products should not be used in critically ill patients or in those with pre-existing renal dysfunction but did not withdraw them completely). As in surgical patients relatively small volumes of colloid are commonly used to face severe bleeding, it may not be correct to generalize the results obtained in critically ill patients (Nolan & Mythen 2013). Although the research on HES effects on organ integrity has been widely directed toward the kidney, intestinal hypoperfusion was associated with increased postoperative morbidity after major surgical procedures, and the effects of the type of fluid (crystalloid or colloid solution) on the small intestine are largely unknown (Hiltebrand et al. 2009). Severe hypoperfusion can cause small intestine mucosa injury with increased intestinal permeability, which may result in the entrance of bacteria and lipopolysaccharides to the systemic circulation, and consequent sepsis and multiorgan dysfunction syndrome (Mythen & Webb 1994a; Mythen 2005; Mythen 2009). In a recent study, the effects of hydroxyethyl starch 130/0.4 in oxidative stress and in the inflammatory response in a rodent haemorrhagic shock model, significantly reduced MDA (malondialdehyde) levels and MPO (myeloperoxidase) activity in the liver, intestine, lungs and brain, and also inhibited the production of TNF (tumour necrosis factor)-alpha in the intestine two hours after resuscitation, suggesting that hydroxyethyl starch 130/0.4 treatment after haemorrhagic shock ameliorated oxidative stress and the inflammatory response in this rat model (Chen et al. 2013). The effects of the different volume replacement solutions on the intestinal mucosa after severe haemorrhage are largely unknown. The present study investigated if a specific volume replacement solution, a crystalloid or a colloid, would minimize the small intestinal mucosal lesions after severe bleeding in a pig model under general anaesthesia.

1.2. Hypovolaemia, Hypoperfusion and Hypovolaemic shock

The perfusion of cells and tissues consists in the delivery of oxygenated erythrocytes to these sites (Beebe & Funk 2001). Hypoperfusion occurs when there is an imbalance between the oxygen needs of cells and tissues, and the levels of oxygen delivered (Meregalli et al. 2004). Hypoperfusion can occur locally – regional hypoperfusion – or affect the entire body. The most common causes of hypoperfusion are hypovolaemia, which is defined as a decrease in the circulatory volume, with consequent decrease of the cardiac output, and impairment in cardiac function, with decreased delivery of oxygenated blood to the organs, tissues and cells (Boag & Hughes 2005; Burchard KW 2013). A more broad list of causes of hypoperfusion can be found in Table 1. A state of shock occurs when the blood pressure decreases significantly and the perfusion of cells and tissues is seriously compromised, achieving a state in which tissue perfusion is not able to maintain the normal aerobic metabolism of the cells (Gutierrez et al. 2004; Boag & Hughes 2005).

Table 1. Causes of hypoperfusion (adapted from Burchard 2013).

Causes of hypoperfusion	
Decreased venous return	Hypovolaemia
	Pericardial tamponade
	Tension pneumothorax
	Increased abdominal pressure
	Bowel obstruction
	Tension pneumoperitoneum
	Severe bleeding
	Diagnostic laparoscopy
	Ascites
Positive end-expiratory pressure	Increase of intrathoracic pressure with decrease of cardiac output
Decreased myocardial function	Congestive heart failure
	Cardiogenic shock

Four pathophysiological types of shock have been described: hypovolaemic, usually caused by haemorrhage or dehydration; cardiogenic, which can be due to myocardial infarction, cardiomyopathy, valvular disease and/or severe arrhythmias; obstructive shock, commonly caused by pulmonary embolism, pericardial tamponade,

restrictive pericarditis or aorta dissection (in humans); and the distributive shock, related with systemic inflammatory response, which may occur in severe pancreatitis, neoplasia, sepsis, burns and severe tissue trauma (Boag & Hughes 2005; Vincent et al. 2012). In emergency medicine, severe haemorrhage frequently leads to severe hypovolaemia, and haemorrhagic shock easily occurs. Even when a state of shock is not achieved, some degree of decreased systemic perfusion is frequent in critical ill patients, as it can be related with several different conditions, which makes hypoperfusion a clinical situation that needs to be recognised and adequately treated before a serious impairment of the cellular function occurs (Boag & Hughes 2005).

1.2.1. Hypoperfusion/Hypovolaemic Shock

By definition, shock is a state of systemic tissue hypoperfusion caused by a reduced cardiac output and/or reduced effective circulating blood volume. In shock, there is impaired tissue perfusion, leading to cellular hypoxia (Kumar et al. 2015). According to its causes, there are three general categories of shock: cardiogenic shock, hypovolaemic shock and shock associated with systemic inflammation, which development may be related with a several insults, especially infectious agents, burns, trauma and/or pancreatitis. The hypovolemic shock, which is the category of shock that will be addressed in this thesis, is caused by the presence of a low cardiac output related to low blood volume, as occurs in severe haemorrhage or fluid loss, such as in severe burns. Decreased circulating blood will cause decreased vascular pressure and consequent tissular and cellular hypoperfusion (Kumar et al. 2015).

Oxygen is used to drive the existing metabolic pathways in the cells through which they extract energy from nutrients. A constant blood flow is critical to distribute oxygen and nutrients to the cells and also to remove all residual products from metabolism, such as carbon dioxide (CO2); it is also important for keeping homeostasis (Aehlert 2011). The decrease of the blood flow will lead to a relative or absolute decrease in cardiac preload, resulting in low cardiac output, inadequate tissue perfusion, and decreased oxygen delivery to tissues, which will cause impairment of the cell function. The delivery of oxygen varies with the cardiac output and the blood oxygen content level. The cardiac output depends on the heart rate and stroke volume, which is the volume of blood delivered to the organism by the left ventricle in a heartbeat. Stroke volume, in its turn, is affected by cardiac pre- and afterload and by myocardial

contractility. The term shock is used when the perfusion of tissue in general is weak. The most problematic consequences of shock are cell failure that results in systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Pachtinger & Drobatz 2008; Mazzaferro 2013).

In hypovolaemic shock, the loss of circulatory blood volume varies between 15 and 80%. When it exceeds 40% hypotension is observed. The severity of shock is determined primarily by the duration and severity of the blood volume loss. In the pathophysiology of hypovolaemic shock, the primary events are directly related with the loss of circulating blood volume, followed by secondary mechanisms, which are activated by the organism in the attempt to compensate this loss (Groeneveld, 2013).

1.2.1.1. Overall Circulatory Changes

When hypovolaemia results in decreased cardiac preload and low filling pressures, cardiac output decreases, with consequent decrease of the vascular pressure. Baroreceptors will respond to decreased pressure by increasing medullary sympathetic nervous output and epinephrine/norepinephrine release, which will increase the cardiac output and cause arteriolar vasoconstriction in the vast majority of the tissues, as an attempt to increase vascular pressure (exceptions are organs such as the heart, brain and kidneys, which initially will have the blood flow preserved). Further help to regulate the pressure will be given by left atrial volume receptors and hypothalamic osmoreceptors, which will interfere in the water and sodium balance. The presence of a reduced plasma volume will prompt the release of antidiuretic hormone/vasopressin (ADH) and water retention and will stimulate the production of angiotensin II by the renin-angiotensin system, which will lead to aldosterone release and sodium retention. In addition, ADH and angiotensin II also promote vasoconstriction, contributing to the increase of the peripheral resistance. There will also be a shift if fluid movement from the interstitium into the vascular space to help to increase the circulating blood volume. The activation of these mechanisms will lead to increased heart rate (tachycardia), increased cardiac output and increased vascular pressure (Mosier 2012). In the presence of short term, mild hypovolaemia, these mechanisms of compensation are usually successful, with return to homeostasis; however, in severe or prolonged hypovolaemia which inhibits the capability of the heart to increase the output, shock enters in a progressive state, which may be irreversible and lead to death (Mosier 2012).

Hypovolaemia is characterized by an oxygen deficit in the tissues - hypoperfusion. Hypoperfusion commonly leads to cellular hypoxia and, in some situations, as in haemorrhagic shock, it can cause severe impairment of the cellular metabolism, due to the severe decrease in the oxygen levels available. In hypoxia, the cells will first start producing energy just using glycolysis, as the other energy generating processes (citric acid cycle and oxidative phosphorylation or the electron transport chain) do not continue under anaerobic conditions. In glycolysis, much less energy is produced when compared to the aerobic processes. During that process, the cell produces pyruvate and spends nicotinamide adenine di-nucleotide (NAD+), and then disposes off the pyruvate and regenerate NAD+, by converting pyruvate to lactate, with simultaneous production of hydrogen ions, leading to metabolic acidosis (Boag & Hughes 2005). Hypoxia, energy depletion and acidosis will result in serious cellular imbalances, with deregulated ionic homeostasis, abnormal intracellular signalling and reduced cellular function, finally leading to death, which may bring additional local inflammation and impairment of the organic function. The severity and duration of hypoperfusion and the degree of oxygen metabolism impairment and cellular damage are directly related with the patient's outcome. When haemorrhage occurs, in an early stage of systemic hypoperfusion, the blood supply to vital organs as the brain and heart are maintained in detriment of non-vital ones, as the muscle, however other organs, as the skin and the gastrointestinal tract, may already be suffering an important degree of cellular hypoxia (Boag & Hughes 2005).

1.2.1.2. Oxygen levels

The vital problem that arises during hypoperfusion is the insufficient supply of oxygen (O_2) to the tissues and cells. The O_2 supply is determined by the cardiac output and the amount of O_2 in the arterial blood, i.e., the concentration of haemoglobin in arterial blood and the oxygen saturation of the haemoglobin. The oxyhaemoglobin dissociation curve determines the saturation of haemoglobin with O_2 for a given oxygen partial pressure (PO_2) in the blood. During hypovolaemia, a decrease in haemoglobin concentration, in oxygen saturation or both, exacerbates the effect caused by decreased cardiac output in impaired oxygen delivery to tissues (Parrillo & Dellinger 2013). .

When there is decreased oxygen supply to the tissues, PO_2 decreases and there is increased oxygen extraction in attempt to compensate for a decreased intake. However this increased oxygen extraction reaches its maximum if the input of oxygen falls to values below 8 to 15 ml/kg/min, which is considered the critical value in humans. However, the critical value of the oxygen supply can vary between species and their specific basal needs of O_2. During a decrease in the oxygen supply below this critical value, the extraction of O_2 falls below the tissue needs, resulting in cellular ischemia. Consequently the organism will use the anaerobic metabolism to meet energy needs, with the development of lactic acidosis (Parrillo & Dellinger 2013).

1.2.1.3. Macrocirculation

In hypovolaemia, a decrease in intravascular volume results in a lower venous return to the heart and a decreased ventricular filling (cardiac preload). Consequently, there is a decreased stroke volume, cardiac output and blood pressure. A decrease in cardiac output with low haemoglobin levels due to haemorrhage, results in decreased delivery of oxygen to tissues and may lead to shock. Initially, a decrease in cardiac output in hypovolemic shock, results in a redistribution of peripheral blood flow. This redistribution results partly from the self-regulation in the attempt to maintain regional blood flow, in which endothelial cells and the production of endogenous vasodilators, including endothelial nitric oxide synthase-derived nitric oxide (NO), heme-oxygenase derived carbon monoxide, hydrogen sulphide and metabolic byproducts in the tissues, such as CO_2, potassium and adenosine. The nitric oxide relaxes the smooth muscle of the vessel walls, by stimulating guanylate cyclase and cyclic guanosine monophosphate (cGMP). The carbon monoxide also acts by stimulating cGMP. The release of nitric oxide may decrease the reactivity to endogenous and exogenous vasoconstrictors, even in the early stages of hypovolemic shock (Parrillo & Dellinger 2013). Factors that will oppose vasoconstriction include catecholamines (released by the activation of the sympathetic nervous system and the adrenal medulla), direct sympathetic stimulation of the vessel wall, angiotensin II (released through the renin-angiotensin-aldosterone system activation) and vasopressin (released by the pituitary gland). Endothelin is a potent endothelium-derived vasoconstrictor, released following catecholamine stimulation and/or hypoxia, and its release can contribute to vasoconstriction (specially

in renal and hepatic vessels). A decreased cardiac filling may also reduce cardiac atrial natriuretic peptide secretion, reducing the vasodilation and diuretic effects of this factor. The overall result of the volume redistribution and increased peripheral vascular resistance is that the blood flow to intestines, skeletal muscle and skin is diverted to vital organs such as heart and brain, resulting in and increased oxygen supply to organs with highest metabolic requirements.

1.2.1.4. Microcirculation

Vasoconstriction secondary to sympathetic nervous system activation during hypovolaemia occurs in arteries and medium-sized arterioles but not in the terminal arterioles. The blood flow is relatively maintained in terminal arterioles possibly by vasodilating metabolic responses to the decrease in the flow of nutrients. Yet, there is usually a decrease in capillary flow. In hypovolaemic shock there is induced expression of adhesion molecules on primed neutrophils and in the vascular endothelium, which, in association with a reduced blood flow, can promote the adhesion of neutrophils to the endothelium. The adhesion of these cells to the endothelium may impair the flow of red blood cells, particularly in capillaries and post-capillary venules. It has also been suggested that capillary leukostasis is pressure dependent and not receptor dependent, and therefore reversible when the pressure is restored. Endothelial cells may also become oedematous and impair/impede the capillary flow of erythrocytes and leukocytes. Vasoconstriction also occurs in the venous vasculature, more frequently in large venules and particularly in the splanchnic area, being also mediated by increased activity of the sympathetic nervous system and vasopressin and angiotensin II release.

During hypovolemic shock, pre and post -capillary resistance increases, resulting in a decrease in capillary hydrostatic pressure and fluid resorption from the interstitial space. The intracellular water is also believed to be mobilised, unless, at a late stage , the sodium -potassium pump fails, with consequent cell swelling (Parrillo & Dellinger 2013).

The mobilization of interstitial fluid and cellular spaces can be promoted by plasma hyperosmolarity, by an increase in glucose concentration. In addition, the lymphatic vessels may show increased pumping capacity, increasing the return of fluid to the systemic circulation. The lymphatic return of interstitial protein and fluid may

contribute to the restoration of circulating protein and fluid volume. Haemorrhage and hypovolaemic shock are associated with a decrease in the haematocrit and also in plasma proteins, due to the transfer of fluid from the interstitial space into the intravascular space. The refilling of the intravascular space gradually decreases after a sudden decrease in circulating volume, with a decrease in oncotic pressure, hypoproteinemia and with an increase in hydrostatic pressure, forming a new state of equilibrium in the capillary exchange, via an adjustment of the hydrostatic and oncotic pressure, which control the transport of fluids and proteins. Increased sympathetic discharge following stimulation of the sympathetic nervous system leads to contraction of the spleen and release of blood cells into the circulation, as an attempt to correct the decreased haematocrit (Parrillo & Dellinger 2013). In combination, the mechanisms mentioned partially compensate a decrease in the circulating blood volume and reduce cardiac output, promoting venous return to the heart.

1.2.1.5. Cell injury in hypovolaemic shock

During hypovolemic shock, the inadequate supply of oxygen to the tissues leads to a drop in the mitochondrial production of high-energy phosphates (ATP), with a higher consumption than production of these compounds. The decrease in the redox status and high-energy phosphates during hypovolemic shock appears to be more pronounced in some tissues (diaphragm, liver, kidney and intestine) than in others (heart and skeletal muscle). A drastic decrease in high-energy phosphates (ATP) during ischaemia leads to irreversible cell damage, while a less severe decrease may only result in prolonged programmed cell death - apoptosis. In animals with hypovolemic shock and critically ill patients, circulating levels of ATP may be reduced and the degradation products of ATP (adenosine, inosine, hypoxanthine, xanthine) may be elevated, suggesting that ATP degradation occurs as a result of lack of oxygen in tissues. Similarly, reperfusion is associated with restoration of energy charges, depending on the effect of ischemia, the need for oxygen and the level of reperfusion. However, part of the mitochondrial dysfunction after trauma or hypovolemic shock, it is considered independent of the lack of oxygen. About 60% of the energy produced by the mitochondrial respiratory chain is required to feed the sodium-potassium pump, through which the gradient in electrolyte concentrations and the electric potential across the cell

membrane are controlled. In the absence of enough ATP due to the reduction in production associated with a lack of oxygen, the sodium-potassium pump is inhibited and there is activation of the Na+/H+ exchanger, resulting in an influx of Na+ and efflux od H+ and K+, leading to cellular uptake of fluid. An increase in cell membrane permeability may also contribute to the fluid influx into the cell. Finally, the influx of calcium (Ca2 +) to the interior of cells and there is mitochondrial inhibition of cellular respiration, which contribute to cellular oedema and cellular damage. Due to the influx of calcium to the cells, the free calcium levels may decrease the hypovolemic shock. The intracellular lysosomes lose their integrity and release their proteolytic enzymes contributing to cell death. These enzymes may eventually reach the systemic circulation and cause damage in remote organs (Myers et al. 2012; Parrillo & Dellinger 2013). Organ reperfusion following a transient episode of ischemia, results in reperfusion injury. Reperfusion injury limits the possibility of recovering of microvascular flow by the tissues, limiting also the recovering of the organ function, even if the cardiac output and blood pressure return to normal values (Myers et al. 2012; Parrillo & Dellinger 2013).

The cell injury in hypovolaemic shock may be irreversible and culminate with cell apoptosis or necrosis. Cell apoptosis frequently occurs as a component of injury in hypoxia. In general, the apoptotic process is usually self programmed and energy dependent, and is initiated by cell signalling, controlled and integrated by regulatory molecules and has a common execution phase by caspase enzymes, followed by the removal of the dead cell. During the initiation, caspases become active and, in the execution phase, these enzymes act to cause cell death (Myers et al., 2012). Initiation occurs principally by signalling from two distinct pathways that will converge at a point during the process: the extrinsic pathway, also called receptor-initiated, and the intrinsic or mitochondrial pathway. Both pathways converge to activate caspases, however they involve distinct molecular interactions (Myers et al., 2012). These are the two main apoptotic pathways described however an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell has also been reported more recently (Martinvalet et al. 2005; Elmore 2007; Lieberman 2010).

The initiation of the extrinsic pathway (receptor-initiated pathway) is done by engagement of cell death receptors to the cells. Death receptors contain a “death domain” (domain involved in protein-protein interactions), which delivers apoptotic

signals. These death receptors are members of the TNF receptor family. 1 TNR receptor (TNFR1) and Fas are well known death receptors hypoxia (Myers et al., 2012). Giving as an example the pathway of apoptosis after Fas engagement, when its ligand FasL cross-links Fas, the minimum of three Fas molecules come together and their death domains, located in the cytoplasm, form a binding site for FADD (Fas associated death domain), which is an adapter protein that also contains a death domain. Multiple caspase-8 come to the area and cleave one another, generating active caspase-8. Active caspase-8 will start a cascade of caspase activation. The active caspases will then mediate the execution phase of apoptosis. The extrinsic pathway can be inhibited by the FLIP protein, by binding to caspase-8 (Myers et al., 2012).

The intrinsic pathway is activated when there is increased mitochondrial permeability, with release of proapoptotic bodies to the cytosol. The production of members of the Bcl-2 family proteins, which are antiapoptotic, is stimulated by growth factors and other survival signals. The two main antiapoptotic proteins of this family are Bcl-2 and Bcl-x. These antiapoptotic proteins normally are located in the mitochondrial membranes and in the cytoplasm. When cells suffer any kind of disturb/stress, are deprived of survival signals, or the DNA is damaged, Bcl-2 and Bcl-x are lost from the mitochondrial membrane and are replaced by membrane proapoptotic proteins from the same family, as Bak, Bax and Bim. With the decrease of Bcl-2 and Bcl-x levels, there is an increasement of the permeability of the mitochondrial membrane, and the release of different proteins into the cell cytoplasm, that can activate the caspase cascade. Cytochrome-c is one of these proteins, and when free in the cytoplasm, it will bind to apoptosis activating factor-1 (Apaf-1), and, as a complex will activate the caspase-9 (Myers et al., 2012). The loss of Bcl-2 and Bcl-x from the cells also allow the activation of Apaf-1 (these proteins, when present in normal conditions, directly inhibit Apaf-1 activation) (Myers et al., 2012).

Other mitochondrial proteins, as apoptosis-inducing factor (AIF), also go into the cytoplasm and neutralize inhibitors of apoptosis. After these processes within the cell, the final result is the initiation of a caspase cascade (Myers et al., 2012).

In summary, the base of the intrinsic pathway of apoptosis is a balance between proapoptotic and protective molecules and the release of apoptosis inducers, which are normally located in the mitochondria. However, there is also evidence that this pathway of apoptosis can be triggered independently of the mitochondria, although this process

is not well defined (Myers et al., 2012). Hypoxia-induced apoptosis is believed to mainly relies on intrinsic, mitochondrial pathways (Weinmann et al. 2004).

Extrinsic and intrinsic pathways may often overlap and not be completely distinct, as it is already known to happen in hepatocytes, where Fas activates Bid, which is a proapoptotic protein belonging to the Bcl family, and that subsequently activates the mitochondrial intrinsic pathway (Myers et al., 2012).

The final phase of apoptosis is the execution phase, and is mediated by a proteolytic cascade, which is initiated by all the described initiating mechanisms. The proteases involved in this phase are, as previously mentioned, from the caspase family. The "c" of caspase comes from "cysteine" protease, which is an enzyme with cysteine in its active site, and "aspase" is related with the ability of these enzymes to cleave aspartic acid residues. The caspase family is divided in the initiator and the executioner group, based on the order of activation of each enzyme during apoptosis. Examples of initiator caspases are caspase-8 and caspase-9; caspase-3 and caspase-6 are examples of executioners (Myers et al., 2012). Caspases are normally in an inactive status, and activation is necessary by cleave, so apoptosis can be initiated. The process of apoptosis is initiated after cleavage of one initiator caspase, which leads to the sequential activation of the other caspases. Execution caspases will cleave the cytoskeletal and nuclear matrix. Leading to breakdown of the nucleus. In the nucleus, the caspases that are usually activation targets are the ones involved in transcription and DNA replication and repair, as is the case of caspase-3, which when active activates a cytoplasmic DNAase that induces the internucleosomal cleavage of DNA (Myers et al., 2012).

1.2.2. General clinical assessment of tissue perfusion

Initially, a physical examination with focus on the respiratory, cardiovascular and neurologic systems is the best way of assessing any critical care patient in human or veterinary medicine, in order to detect the presence of any degree of hypoperfusion. In veterinary medicine, to assess the heart rate, mucous membranes colour, capillary refill time (CRT), amplitude and duration of the pulse in the proximal and distal limbs, and to perform a cardiac auscultation is essential in a first approach to the patient with potential hypoperfusion. This assessment can be more complete if peripheral venous filling, presence or absence of jugular pulse, apex beat and body temperature and temperature of the extremities are also evaluated (Boag & Hughes 2005).

Routinely, volaemia and peripheral tissue perfusion are monitored using vital signs such as blood pressure and pulse rate, as well as by monitoring end organ function, measuring the urine output and peripheral perfusion (SPO2). A simple and quick measurement, the non-invasive systemic blood pressure (SBP), is commonly used for assessing the severity of hypovolaemia. However it may be of limited value when the arterial blood pressure is very low or in the presence of a compensatory phase, with consequent masking of hypoperfusion through all of the body´s compensatory mechanisms previously described. Therefore, this method is very useful for initial triage of possible hypovolaemia but its limitations need to be considered and the measurements interpreted together with other monitoring parameters when possible (Boag & Hughes 2005).

In veterinary medicine, the measurement of the PCO_2 in the oral mucosa or at sublingual level is considered a good indicator of tissues oxygenation and predictor of outcome (Boag & Hughes 2005; Ristagno et al. 2006). In addition, for the monitoring of peripheral tissue perfusion, SPO_2 is routinely used in both veterinary and human medicine.

The monitoring of the cerebral perfusion in critical and surgical patients may also provide a higher change of improved clinical evolution and outcome. Near-infrared spectroscopy (NIRS), such as the INVOS monitor, allows the patients to be monitored for changes in the regional oxygen saturation, which will reflect the balance between cerebral oxygen supply and demand (Cavus et al. 2010; Grocott et al. 2010). In addition, to monitor the cortical electrical activity may provide information about the cerebral perfusion, as hypoperfusion may induce changes in cortical neuronal activity. Cortical neuronal activity may be quantified by electroencephalography (EEG). When there is a marked decrease (more than a half) of the cerebral blood flow, there is acute slowing of the raw-EEG frequency. Bispectral index (BIS) is a statistically based, processed EEG complex parameter that integrates multiple descriptors of the EEG into a single variable. Its measurements are derived from the frequency, amplitude and coherence of the EEG, which have been shown to relate to consciousness and unconsciousness. BIS values range from 100 for completely awake patients, to zero, when there is absence of brain electrical activity. It is therefore used to indicate the level of cerebral activity noninvasively. Furthermore, it has been suggested that a decrease in the values of BIS may indicate cerebral hypoperfusion (Cavus et al. 2010). When used

for monitoring depth of the anaesthesia, a BIS value between 40 and 60 is considered to be indicative of an appropriate anaesthetic level (Kissin 2000).

1.2.3. Hypoperfusion, hypovolaemic/haemorrhagic shock and its consequences

Hypovolaemia is one of the most common and potentially reversible crises in emergency and critical care medicine. As mentioned previously, hypovolaemia is present when there is a decrease in the normal circulating volume (Burchard 2013) and even minor degrees of hypovolaemia may cause serious hypoperfusion and lead to organ dysfunction (Hillman et al. 1997). Cells with high metabolic rates, as the cells of the brain, heart and kidneys, are especially important. Initially, these organs have their blood supply protected by autoregulation, with a redistribution of the blood supply from non-vital organs such as the skin and muscle, as hypoperfusion can be better tolerated in these organs due to its low metabolic rate. However, there comes a stage when even these cells are affected. The gastrointestinal tract is the first system to be affected by hypoperfusion, while vital organs still have its blood supply protected. Even very small degrees of hypovolaemia may lead to visceral hypoperfusion (Hillman et al. 1997), and may result in translocation of bacteria and bacterial breakdown products (Koziol et al. 1988), which in turn may lead to multiorgan dysfunction syndrome (MODS) and, potentially, to death (Koziol et al. 1988; Mythen & Webb 1994a). In ischaemia, cytokines are also released contributing to the MODS (Hillman 2004).

1.2.4. Organism Response to Hypovolaemia and Hypoperfusion

Different compensatory mechanisms occur in hypovolaemia as an attempt to keep the arterial blood pressure (ABP) within normal range. The ABP results from the cardiac output (CO – heart rate x stroke volume) and peripheral vascular resistance; haemorrhage leads to a decreased venous return to the heart, with consequent decrease of the cardiac output. In the presence of decreased blood volume, the baroreceptors will initiate different compensatory mechanisms, as catecholamine release and vagal parasympathetic centre inhibition, which result in the increasement of the heart rate, cardiac contractility and also in increased systemic vascular resistance, in order to normalise the values of the ABP. At this stage, which is called compensated hypovolaemia, the heart rate and contractility are slightly increased and the capillary

refill time is faster than normal. The pulse profile in this phase is referred to as "hyperdynamic", among other terminologies (Boag & Hughes 2005).

1.2.4.1. The General Organ's Response to Acute Haemorrhage

The body responds to acute haemorrhage by activating hematologic, cardiovascular, renal, and neuroendocrine systems.

In this clinical situation, hematologic system activates the coagulation cascade and the contraction of the bleeding vessels, by the release of local thromboxane A2. Platelets are also activated by local thromboxane A2 release and work to form an immature clot on the bleeding site. The damaged vessel has exposed collagen, which leads to fibrin deposition and stabilization of the clot. Approximately 24 hours are needed for complete clot fibrination and mature formation (Stephenson 2007).

The cardiovascular system initially responds to hypovolaemic shock by increasing the heart rate, the myocardial contractility, and constricting peripheral blood vessels. This is secondary to an increased release of norepinephrine and decreased baseline vagal tone, regulated by the baroreceptors in the carotid arch, aortic arch, left atrium, and pulmonary vessels. The cardiovascular system also responds by redistributing blood to the brain, heart, and kidneys, with decreased blood flow to the skin, muscle, and gastrointestinal tract (Stephenson 2007).

The renal system responds to haemorrhagic shock by stimulating an increase in renin secretion from the juxtaglomerular apparatus. Renin converts angiotensinogen to angiotensin I, which is converted to angiotensin II by the lungs and liver, and angiotensin II is responsible for the vasoconstriction of arteriolar smooth muscle and stimulation of aldosterone secretion by the adrenal cortex. Aldosterone is responsible for active sodium reabsorption and subsequent water conservation (Stephenson 2007), as intent to reverse haemorrhagic shock. The neuroendocrine system responds to haemorrhagic shock by causing an increase in circulating antidiuretic hormone (ADH). ADH is released from the posterior pituitary gland in response to a decrease in blood pressure, detected by baroreceptors, and a decrease in the sodium concentration, detected by osmoreceptors. ADH indirectly leads to an increased reabsorption of water and salt by the distal tubule, the collecting ducts, and the loop of Henle (Stephenson 2007).

These mechanisms are effective in maintaining vital organ perfusion in severe blood loss, however, without fluid and blood resuscitation and/or correction of the underlying pathology causing the haemorrhage, cardiac perfusion eventually decreases, and multiple organ failure occurs (Stephenson 2007).

1.2.4.2. Decompensated hypovolaemia

If hypovolaemia persists and progresses, a stage of decompensated hypovolaemia occurs, as the initial compensatory mechanisms start to fail. In decompensated hypovolaemia, the tissue perfusion is seriously compromised. The patients at this stage have marked tachycardia, may have quiet heart sounds on auscultation due to the decreased blood volume, have usually pale to white mucous membranes, and a prolonged or absent CRT. The femoral pulse is weak (short and narrow), metatarsal pulses are not present anymore, and the CO plateau falls when the HR is approximately 180 bpm higher compared with the normal range for the patient (Boag & Hughes 2005). In decompensated hypovolaemia, there is a significant decrease in cardiac output and blood flow to the liver, small intestine, and kidneys. However, blood flow to the brain and heart is initially preserved.

Systemic oxygen delivery and consumption are markedly reduced, as is regional delivery and consumption in the liver, small intestine, and kidney. The liver shows the most severe reductions in oxygen consumption. The breakdown of the intestinal barrier function may result in the entrance of intestinal microorganisms and endotoxins into the systemic circulation, leading to the activation of inflammatory pathways with elevation of pro-inflammatory mediators including interleukin-6, the levels of which are consistently elevated in septic, injured or postoperative patients, and TNFα, implicated in the destruction of endothelial cells and the development of disseminated intravascular coagulation, fluid shift, and multiple organ dysfunction. In human medicine, hypothermia, acidosis, and coagulopathy have been identified as rapidly fatal in critically injured patients if non-treated. At a later stage, the blood supply to the heart and lungs is compromised, causing further reduction of the cardiac output and decreasing even more the oxygenation. As compensatory mechanisms fail, tachycardia changes to bradycardia and progresses to circulatory arrest. In the failure to start

aggressive volume resuscitation, the death of the patient will occur (Vercueil, M Grocott, et al. 2005).

1.2.4.3. Hypovolaemic shock and its effects in the intestine

When hypovolaemic shock occurs, an increased sympathetic activity and increased levels of vasopressin and angiotensin II mediate the redistribution of the blood flow from the gastrointestinal system to other organs. Vasoconstriction may overwhelm nitric oxide and other mechanisms that lead to vasodilation, and there may be an impairment of the endothelium dependent vasodilation after oxidant endothelial injury. The ischaemia affecting the intestine is further worsened by the countercurrent mechanism that occurs in the villus (Parrillo & Dellinger 2013). In the villus, venules run parallel and closely adjacent to the central arteriole, which allows a countercurrent exchange of oxygen to occur; this countercurrent exchange of oxygen is increased when there is a decreased flow velocity, as occurs in hypovolaemic states, with more oxygen being transferred from the arteriole to the adjacent venules in the basal portion of the villus, likely aggravating the sensitivity of the villus tip to hypoperfusion (Vollmar & Menger 2011; Williams et al. 2015). After intestinal ischaemia, the portal blood flow decreases and there is an increase in portal blood lactate levels. Ischaemia is known to lead to apoptosis and necrosis of enterocytes of the intestinal epithelium, with apoptosis having been reported as the major form of cell death occurring after short term ischaemia or ischaemia/reperfusion (Ikeda et al. 1998). The intestinal mucosal damage may eventually lead to leakage of fluid from the vascular space to the intestinal lumen, and diarrhoea may also aggravate fluid loss in prolonged hypovolaemic shock (Parrillo & Dellinger 2013).

Mucosal damage in hypoperfusion may lead to impairment of the mucosal barrier function, increasing the risk of translocation of bacterial agents and toxins from the intestinal lumen to the systemic circulation (Deitch 1990; Deitch et al. 1996; Samel et al. 2002; Qiao et al. 2009). The intestinal permeability for small molecules is also higher. The impairment of the intestinal barrier function with translocation of bacteria and toxins appears to contribute to death in hypovolaemic shock (Deitch 1990). During hypovolaemic shock there is also decreased absorption of nutrients, such as carbohydrates, amino acids and lipids (Parrillo & Dellinger 2013).

1.2.5. Clinical approach to hypovolaemia and hypoperfusion due to acute haemorrhage

The main goal in the treatment of hypoperfusion is to restore the volaemia, which implies to restore also the oxygen levels in circulation and the oxygen available for the tissues and cells. The early restoring of the oxygen levels to organs, tissues and cells will prevent further damage and decrease the risk of multiorganic failure. The restoring of the circulating volume is performed through fluid therapy, with administration of volume replacement solutions. Several solutions are presently available in the market for this goal, and the type of solution and rate of administration must be decided based on the specific conditions of the patient. However there is still no consensus on the optimal fluid solution and optimal resuscitation strategy in cases of severe haemorrhage. Different types of fluids available are isotonic crystalloid solutions, hypertonic saline solution, colloid solutions and blood derived products. The choice of the type of fluid should be performed by knowing the characteristics of each solution, possible adverse effects and conditions of each individual patient (Boag & Hughes 2005). The patient should be monitored continuously in order to assure that the therapy is adequate.

1.2.5.1. Ischaemia/Reperfusion injury

Hypovolaemia may lead to an insufficient blood flow to provide adequate oxygenation of the organs and tissues – ischaemia – which leads to decreased oxygen to be available for the tissues and cells – hypoxia. Correction of hypovolaemia is urgent but reperfusion after hypoperfusion/ischemia is also associated with oxidative stress and systemic inflammatory response, with consequences to the organs and tissues (D L Carden & Granger 2000; Yang et al. 2002; Chen et al. 2013).

1.2.5.1.1. Organ injury in ischaemia/reperfusion

After an episode of ischaemia followed by reperfusion, damage in multiple organs may occur, even when these were not directly involved in the primary ischaemic insult. This clinical condition is referred to as multiple organ dysfunction syndrome (MODS), being a common cause of death in critical patients. The development of MODS is reported to be the result of the reperfusion of the intestines, liver and skeletal muscle, aortic occlusion-reperfusion, and circulatory shock (D L Carden & Granger 2000). Splanchnic vasoconstriction and variable degrees of mesenteric ischaemia, which occur as physiological response to hypovolaemia and hypovolaemic shock in order to preserve

the perfusion of vital organs, may contribute to the pathogenesis of MODS, as in ischaemia, there is loss of the barrier integrity of the intestinal mucosa, with translocation of bacteria and lipopolysaccharide (LPS) into the circulation. Bacterial products and other mediators stimulate enteric macrophages that may cause macrophage activation, and also the release of inflammatory cytokines, as the TNFα. The inflammatory mediators may activate vascular endothelial cells and circulating leukocytes, and increase the expression of leukocyte adhesion molecules, promoting the interaction between neutrophils and endothelial cells, multifocally (D L Carden & Granger 2000) (Figure 1). Reactive oxygen species and inflammatory leukocytes seem to be directly involved in the development of MODS, as well as oxidants and activated leukocytes.

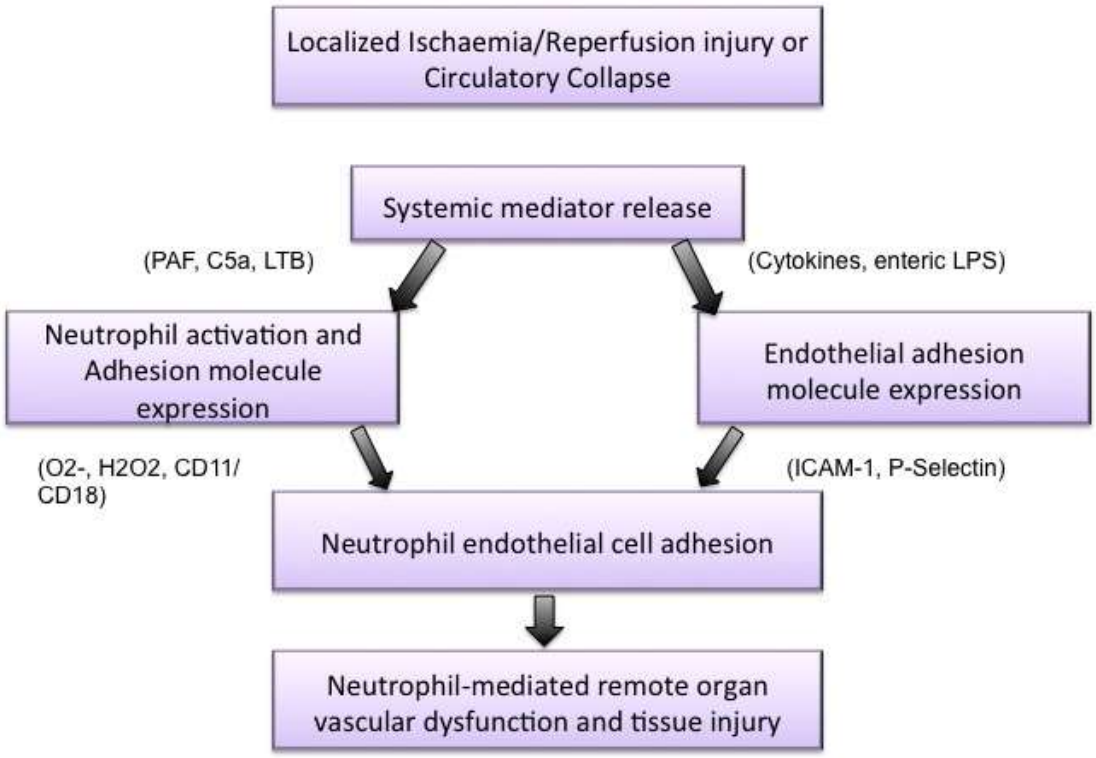

Figure 1. Mechanisms involved in the development of organ injury following ischaemia and reperfusion. The ischaemia followed by reperfusion stimulate the release/availability of inflammatory mediators, which leads to leukocyte activation, with neutrophil and endothelial cell adhesion molecule expression and vascular dysfunction (adapted from Carden & Granger 2000).

1.2.5.1.2. Cell damage in ischaemia/reperfusion injury

Hypoxia is one of the main causes of cell injury and death. When cells are damaged due to hypoxia, all the processes that happen when injury is not lethal, and

cells are allowed normal blood flow after an episode of hypoxia (frequently due to ischaemia), are complex and known to result in further cellular damage – ischaemia-reperfusion injury. This damage is thought to be related to the calcium influx after reperfusion. Restored blood flow to areas with cells which are still viable results in membrane damage. After reperfusion, calcium enters in the damaged cells and activates at least one endogenous, membrane-bound phospholipase – phospholipase A. These phospholipases break down the phospholipids of the inner mitochondrial membrane and other cell membranes and their activation also generates arachidonic acid, which is the substrate for many lipid mediators of inflammation. Proteases that cause cytoskeleton and membrane damage, adenosine triphosphatases (ATPases), which accelerate the decrease of ATP, and endonucleases that cause chromatin degradation are also activated by calcium (Myers et al. 2012).

In ischaemia-reperfusion injury, apart from the effects of the calcium influx, the combination of free radicals that are generated in hypoxia with normal oxygen levels, originates reactive oxygen species, as peroxynitrite from NO. Peroxynitrite is normally present in endothelial and parenchymal cells and is involved in vasodilatation, inhibition of platelet aggregation and prevention of leukocyte adhesion. There is also an increase is free radicals, derived from the increase of inflammatory cells in reperfused areas, in their majority neutrophils, after release of cytokines, as TNF-α (Myers et al. 2012).

Reactive oxygen species (ROS) may cause serious membrane injury to the cell and organelles – free radical injury. Free radicals are molecules with an impaired electron, being for this reason highly reactive; most, but not all, are reactive oxygen radicals. Proteins, membrane lipids and nucleic acid may suffer free radical injury. In addition, free radicals may be responsible for the lipid peroxidation of plasma and organelle membranes (Myers et al. 2012).

1.3. Perfusion Monitoring in Organs and Tissues

In the routine of intensive care, volaemia and peripheral tissue perfusion are monitored using vital signs such as blood pressure (BP) and pulse rate (PR), as well as by monitoring end organ function, measuring the urine output and peripheral perfusion (SPO_2) (Hillman 2004). In both veterinary and human emergency and critical care medicine, it is extremely important to assess organs and tissues' perfusion. Local

hypoperfusion may develop before a state of systemic hypoperfusion, as in the case of the GI tract (Chiara et al. 2001; J.-X. Chang et al. 2005), and its early detection will allow the institution of the correct treatment. The hypoperfusion of individual organs and tissues may increase the morbidity, and in some cases, even the mortality among patients (Boag & Hughes 2005; Hillman et al. 1997). One of the first organ systems to suffer from hypoperfusion in hypovolaemia is the gastrointestinal system, which may lead, as stated before, to increased morbidity and may also lead to progressive organ dysfunction (Chiara et al. 2001; Boag & Hughes 2005; Lu et al. 2014).

1.3.1. Lactate

In hypoperfusion, when there is tissue hypoxia and cells start using anaerobic metabolism for energy generation, lactate is generated from pyruvate, in order to allow energy production to continue. In the presence of sufficient oxygen, aerobic combustion of 1 mole of glucose results in formation of 38 mole of ATP, which can be hydrolysed to supply power to vital and metabolic cell functions. In the absence of oxygen, glucose taken up by cells cannot be processed due to insufficient absorption of pyruvate in the Krebs cycle, with a low transformation rate. The pyruvate dehydrogenase is partially inactive but can intervene at late reductions. The pyruvate is converted to lactate and the ratio of lactate/pyruvate increases together with a decrease in the mitochondrial oxidation-reduction potential. With anaerobic glycolysis within the cytosol 2 moles of ATP are formed for one per mole of glucose. The hydrolysis of ATP originates hydrogen ions (H +), leading to intracellular and latter extracellular metabolic acidosis (Parrillo & Dellinger 2013). The lactate quickly diffuses across cellular membranes and, for this reason, an elevation on intracellular lactate results in increased interstitial and blood lactate. Whenever the rate of lactate production exceeds that of lactate extraction, hyperlactatemia develops. These are the basic mechanisms for the onset of lactic acidosis during the hypovolemic shock, with lactate levels in arterial blood above 2 mmol/l being associated with acidosis (normal value is <1.2 mmol/L, in humans (Loftus 2010) and <2.2 mmol/l in pigs (Swindle 2007)); lactate is therefore considered a useful parameter for assessing the severity and duration of the reduction of oxygen in the tissues (Boag & Hughes 2005).

However, as there are many causes of hyperlactatemia, it is difficult to identify a critical level of oxygen delivery associated with lactic acidosis. There are several

limitations in the use of lactate to assess hypoperfusion: lactate is a late marker of hypoperfusion, as tissue hypoxia is already present when hyperlactatemia occurs; due to the capacity of the liver and other tissues to oxidize lactate, normal lactate levels may occur in the presence of regional hypoperfusion; and hyperlactatemia may occur secondary to other disease conditions in the absence of hypoperfusion (Boag & Hughes 2005).

1.3.2. Arterial blood pressure (ABP) and central venous pressure (CVP)

Arterial blood pressure (ABP) and central venous pressure (CVP) are commonly assessed for haemodynamic monitoring. ABP can be measured using different methods, from non invasive to invasive techniques. Doppler and oscillometric are non-invasive techniques, while placement of an arterial catheter is considered an invasive technique that, when the catheter is connected to an electronic pressure transducer, allows direct continuous measurement of ABP. The peak of ABP is immediately after cardiac systole, falling from this point to a nadir at the end of diastole. The mean arterial pressure (MAP) is the sum of the diastolic pressure and a third of the difference between systolic and diastolic pressures. The difference between systolic and diastolic blood pressures is the pulse pressure, which gives us the amplitude of the pulse. (Boag & Hughes 2005). In humans, normal ranges for systolic, diastolic and mean arterial blood pressures are 90-140 mmHg, 60-90 mmHg and 70-105 mmHg. Although there may be some degree of individual patient variation and specific clinical condition, it is considered that with a mean arterial blood pressure of 65 mmHg or below, tissue hypoperfusion is likely to occur (Parrillo & Dellinger 2013). In pigs, the normal arterial blood pressure values are not consensual, however a mean arterial blood pressure value of less than 30 mmHg is considered to be associated with significant tissue hypoperfusion (Fish 2008).

As definition, CVP consists of the blood pressure at the level of the cranial vena cava, in relation to atmospheric pressure (Reems & Aumann 2012). This value is considered a reflection of the right atrial pressure. The right atrial pressure is, in its turn, used to estimate the end-diastolic volume at the left ventricle, which is called preload. And the CVP values are influenced for both the cardiac function and the venous return (Reems & Aumann 2012). The CVP determination has been used to assess the central venous blood volume, and measures the blood pressure in the thoracic vena cava, close

to the right atrium of the heart. The CVP is measured using a pressure transducer that is connected to a central venous catheter placed in one of the thoracic central veins – internal jugular or subclavian veins (Zanotti Cavazzoni & Dellinger 2006), and gives information on the volume of blood returning to the heart and the ability of the heart to pump the blood into the arterial system (Reems & Aumann 2012). Until recently, CVP low values was assumed as an indicator of low intravascular volume, and therefore, an indicator for fluid administration in order to expand the circulating volume and ameliorate tissues' perfusion. However, it has been interpreted recently more as an indicator of right ventricular function and as not so reliable as an indicator of central blood volume (Marik et al. 2008).

Both cardiac function and venous return are dependent on the total blood volume, vascular tone, cardiac output (volume of blood pumped by the heart per minute), right ventricular compliance and intrathoracic pressure. Several of these variables (as for example ventricular compliance) may be changed in disease states, therefore interpretation of CVP values needs to be carefully performed in the presence of a influencing condition. The CVP should only be assessed together with a measurement of CO, which can be measured directly by thermodilution using a Swan-Ganz catheter, as performed in the present study.

1.3.3. Cardiac Output

The cardiac output (CO) is the amount of blood the heart pumps in one minute, being expressed in litres/minute, and is equal to the product of the stroke volume and the heart rate. It is dependant of the heart rate, contractility, preload, and afterload, being the preload the degree of myocardial distension prior to shortening, and the afterload the force against which the ventricles must act in order to eject blood, being dependent on the ABP and vascular tone (Vincent 2008). The decrease of the cardiac output in the presence of hypovolaemia, will trigger a series of compensatory physiological mechanisms towards preventing hypovolaemic shock, namely increased stimulation of the sympathetic nervous system and epinephrine/norepinephrine release, as mentioned in detail in the topic, describing the overall circulatory changes in hypovolaemia (1.2.1.1).

The cardiac output may be determined using different methods, such as applying the Fick Principle, with measure of the arteriovenous oxygen content differences across the lungs and the rate of oxygen uptake in order to estimate the pulmonary blood flow (which in the absence of intrapulmonary or intracardiac shunts should be equal to the systemic blood flow, and therefore to cardiac output); using the thermodilution method, using a pulmonary artery catheter, which is a method available since 1970's, when Swan, Ganz and colleagues reported that pulmonary artery catheter (PAC) insertion could be performed by the use of specially designed balloon tipped catheter (Mathews & Singh 2008). In the thermodilution method, a bolus of 5 to 10 ml of cold 0.9%NaCl is injected through the proximal port of a pulmonary artery catheter into the right atrium and temperature changes are measured by a thermistor in the pulmonary artery; a plot of temperature change through time will give a thermodilution curve from which the cardiac output can be determined (using the Stewart-Hamilton equation). The degree of change in the temperature is inversely proportional to the cardiac output: in increased blood flow and cardiac output there will be minimal temperature change; with decreased blood flow and cardiac output marked temperature change will be observed. Nowadays there are modern pulmonary artery catheters that allow a continuous measurement of cardiac output (Parrillo & Dellinger 2013).

The cardiac output may also be measured using dye (indocyanine green) or indicator dilution (lithium), with a similar concept that the one behind the thermodilution method. Among may other methods for which the description is not in the aim of this thesis, it can also be determined by arterial pulse pressure analysis, being a less invasive technique when compared with the ones previously described, as it can be performed through an arterial line, which is available in most critical care patients (Parrillo & Dellinger 2013).

Although in critically ill patients and commonly in hypovolaemic shock and resuscitation, the main treatment goal is to make sure that the oxygen delivery to the tissues and cells is sufficient to allow an efficient cell metabolism, by re-establishing an adequate cardiac output, as the cardiac output requirements are different for each patient an clinical condition, a normal value od cardiac output cannot be specified, and it needs to be assessed in combination with several other parameters, such peripheral saturation of oxygen, mean arterial blood pressure, etc. (Parrillo & Dellinger 2013).

The cardiac index is a parameter that relates the cardiac output in one minute to the body surface area, and its normal range in a human adult is considered to be 2.5-4.0 L/min/m^2. The acute loss of 10% of the circulating blood volume is usually well tolerated by the organism, with the cardiac index being minimally decreased; compensatory mechanisms start to fail with volume losses of 20-25% and, with the loss of 40% or more of the circulating volume, there will be consequent marked hypotension and cardiac index and tissue perfusion may fal to less than half of the normal values (Parrillo & Dellinger 2013).

1.3.4. Monitoring of the peripheral tissue oxygenation and perfusion

Besides the initial clinical assessment, optical methods are the most commonly used non-invasive methods to assess tissues' perfusion. These methods apply light with different wavelengths directly to the tissue to assess various tissue states. Different research techniques apply optical methods to assess microcirculation, oxygen levels (SPO_2), pCO_2, and microvascular function in tissues. Routinely used optical methods to monitor peripheral perfusion are finger/skin photoplethysmography (pulse oximetry) and near-infrared spectroscopy (NIRS). The results from these monitoring methods are usually interpreted together with the clinical assessment and additional peripheral perfusion measurements (Lima & Bakker 2005; van Genderen et al. 2012).

With pulse oximetry, arterial haemoglobin oxygen saturation and pulse rate are measured and monitored, by using two wavelengths of light, red and infrared, that are transmitted through the skin/mucous membranes, and are traduced in a pulsatile photoplethysmographic waveform (van Genderen et al. 2012). These two different wavelengths - 660 nm (red light) and 940 nm (infrared light) - are emitted through the vascular bed of the tissues. As haemoglobin (Hb) absorbs more light at 660 nm and the oxyhaemoglobin (HbO_2) absorbs more light at 940 nm, and a detector measures the intensity of the transmitted light at each wavelength, the peripheral oxygen saturation (SpO_2) is given by the ratio between the red light and the infrared light absorbed. As other tissues also absorb light, the pulse oximetry distinguishes the pulsatile component of arterial blood from the nonpulsatile component of other tissues, and this nonpulsatile component is discarded. The pulsatile component is used to calculate the arterial oxygen saturation. The overall haemoglobin concentration can be measured by a third wavelength (800 nm), with a spectrum that resembles that of both Hb and HbO_2, and the

variation in intensity of this light can be used to determine the variation in arterial blood volume - pulsatile component (Lima & Bakker 2005). The normal range for the peripheral oxygen saturation is between 95 and 100% and values lower than 95% should be interpreted in association with other hemodynamic parameters, such as the systolic and mean blood pressure to investigate the possibility of organ ad tissue hypoperfusion.

Near-infrared spectroscopy (NIRS) is a noninvasive technique that enables the determination of tissue oxygenation based on the spectrophotometric quantitation of oxyhaemoglobin and deoxyhaemoglobin within a tissue. Although this technique can be applied to any tissue, it is primarily used to monitor peripheral oxygenation of muscle tissue in critically ill patients (van Genderen et al. 2012). NIRS allows continuous and noninvasive monitoring of tissue oxygenation. The pulsation of arterial blood causes a pulsating volume variation. Peripheral perfusion index is calculated as the ratio between the arterial pulsatile component and the nonpulsatile component. NIRS also uses the principles of light transmission and absorption to measure the concentrations of hemoglobin oxygen saturation, having a better tissue penetration than pulse oximetry. In addition, it allows a general assessment of oxygen levels in all vascular compartments (arterial, venous, and capillary). Depletion of local available O_2 is monitored by NIRS as a decrease in HbO_2 and a simultaneous increase in Hb, whereas total Hb remains constant (Lima & Bakker 2005).

1.3.5. Monitoring of the cerebral oxygenation and perfusion - Jugular bulb venous oxygen saturation ($SVjO_2$) and Near-infrared spectroscopy (NIRS)

The brain is one of main organs of humans and animals, however is often less monitored in emergency and critical care medicine when compared with others, especially in veterinary medicine. Appropriate monitoring of the cerebral perfusion in critical care and surgical patients may allow the optimization of the clinical outcomes (Grocott et al. 2010). In human medicine, monitors of depth of anesthesia, multichannel electroencephalography (EEG) monitoring, motor and sensory evoked potentials, jugular venous bulb saturation, and direct oxygen tissue monitors are nowadays more often available. Different studies have been also performed on the application of some of these monitoring methods in animals (March & Muir 2005; Martín-Cancho et al.

2006; Engbers et al. 2014).

1.3.5.1. Near-infrared spectroscopy (NIRS)

Near-infrared spectroscopy (NIRS), such as the INVOS monitor, may be used to noninvasively and continuously monitor changes in the regional oxygen saturation, reflecting the balance between cerebral oxygen supply and demand (Grocott et al. 2010). In normal physiological conditions, changes in cerebral blood flow result in changes in oxygen delivery to the peripheral cortex, which could be detected by NIRS. NIRS can determine the saturation of the cerebral tissue using similar principles to pulse oximetry.

The oxygen saturation of the blood present within the brain is determined by the differential absorption of multiple wavelengths of near-infrared light by oxygenated and deoxygenated haemoglobin (Grocott et al. 2010). In humans, cerebral oxygen saturation recorded with the INVOS monitor has been reported to have a very good correlation with the invasive methods of assessing cerebral oxygenation (Grocott et al. 2010).

1.3.5.2. Jugular bulb venous oxygen saturation ($SvjO_2$)

The measurement of the $SvjO_2$ is performed for assessment of the cerebral global oxygenation state and it has been used in human medicine in several different procedures, as for example during neurosurgery and in neurointensive care, in order to detect and monitor the global cerebral oxygenation, being an indirect indicator of cerebral oxygen utilization. Normal values in humans are between 55 and 75%. When the demand of oxygen exceeds the supply, oxygen extraction will increase and SvjO2 will consequently decrease; when the oxygen available exceeds the demand, SvjO2 will increase. It can also be used to direct therapies, as for example to monitor the degree and duration of hyperventilation therapy, and in human medicine it has also been used to investigate cerebral injury in cardiac surgery. $SvjO_2$ measurement is known to be sensitive in detecting cerebral ischemia, however this method is not often performed possibly due to the fact that it is an invasive procedure, and it is frequently replaced by noninvasive monitoring of oxygen saturation using cerebral oximetry, and by direct tissue pO_2 monitoring (Grocott et al. 2010). The brain venous blood oxygen saturation monitoring provides information about the overall brain oxygenation but it does not provide information about regional cortex blood supply, as small regional areas of ischaemia may be present without a noticeable variation in the global oxygen demand

and consumption within the brain that would be enough to cause SvjO2 values to be out of the normal range (Gopinath et al. 1994; Grocott et al. 2010).

1.4. Clinical approach of hypoperfusion

The main goal when approaching hypoperfusion is maintaining the intravascular volume in order to avoid/minimise the harmful effects on body tissues (Hillman et al. 1997; Boag & Hughes 2005). Many physiologic solutions are available for volume replacement in during clinical hypoperfusion, and the most commonly used are crystalloid and colloid solutions. These solutions have different properties that will determine the way in which volume replacement will restore the tissue oxygenation.

1.4.1. Crystalloids – Isotonic crystalloids

Crystalloid solutions are commonly the primary fluid used for IV therapy, in both human and veterinary medicine. Crystalloids contain electrolytes (e.g., sodium, potassium, calcium, chloride) but lack the large proteins and molecules found in colloids. These solutions come in many preparations and are classified according to their "tonicity." A crystalloid's tonicity describes the concentration of electrolytes dissolved in the water, as compared with that of body plasma. When the crystalloid contains the same amount of electrolytes as the plasma, it has the same concentration and is referred to as "isotonic", such as 0.9% Isotonic Saline and Ringer Lactate solutions. If a crystalloid contains more electrolytes than the body plasma, it is more concentrated and is considered "hypertonic" (e.g. 7% NaCl). A crystalloid solution that has a lower concentration of electrolytes than the body plasma is a hypotonic solution (e.g. 5% dextrose in water and 0.45% NaCl). Crystalloid solutions also contain a buffering anion of lactate or acetate.

It has been shown that crystalloids may affect the coagulation system through hemodilution. Different studies reported that 0.9% Isotonic Saline at lower dilutions (10%) has a procoagulant effect, but also acts as an anticoagulant at higher dilutions (Ruttmann et al. 1999; Coats et al. 2006). Other authors replicated these findings by demonstrating an increased reaction time, which corresponds to an early clot formation, and reduced α -angle, which is indicator of the rate of solid clot formation, in thromboelastography (TEG) at higher dilutions (40-60%), which are consistent with the

hypocoagulable state (Roche et al. 2006). Coats and colleagues (Coats et al. 2006) proposed a dilutional effect, as a primary explanation for the coagulation impairment However, other studies report that the coagulation impairment is more likely related with changes on thrombin formation and fibrin polymerisation by the large volume administered (Brazil & Coats 2000; Soresen & Fries 2012). This hypercoagulability could be explained by the presence of an imbalance between decreased antithrombin III activity and thrombin generation. The induced haemodilution, which also results in platelets aggregation, leads to thrombin generation, which subsequently binds with antithrombin III, with the consumption of this clotting factor resulting in hypercoagulability (Ruttmann et al. 1998).

Ringer Lactate solution is another isotonic crystalloid that contains sodium chloride, potassium chloride, calcium chloride, and sodium lactate in sterile water, and there are slight variations in the composition for Ringer Lactate as supplied by different manufacturers. With Ringer Lactate solution, when used alone to replace a large blood loss, normal O_2 consumption is maintained by increased cardiac output and increased oxygen extraction with a low venous oxygen content, what can make the patient vulnerable to any further insult (Baue et al. 1967). Massive blood loss frequently causes metabolic acidosis, due to hypoperfusion and consequently increased anaerobic metabolism. Different resuscitation fluids can lower the acid load in an indirectly or directly way: in the first one, the production of acid can be minimized by increasing the intravascular volume and maintaining adequate tissue perfusion; in the second one, the acid load can be reduced if the fluid solution contains an acid buffer, or a source of buffer. Whole blood, plasma, or albumin solutions contain protein, which can act as a buffer; Ringer Lactate contains a potential source of bicarbonate to act the same way (Khala et al. 2009). As for Isotonic saline, Ringer Lactate solution has also been reported as having effects on coagulation. This solution has a hypercoagulable effect at 20% dilution, but an hypocoagulable effect with prolonged reaction time and reduced α - angle at dilutions >40% (Roche et al. 2006; Petroianu et al. 2000).

Isotonic saline and Ringer Lactate have several differences. The pH of 0.9% Isotonic Saline is only 5 and, along with the high sodium and chloride load, is responsible for the development of the hyperchloraemic metabolic acidosis (Duchesne et al. 2010). Ringer Lactate with the pH of 6.5 is a more alkalising solution (Santry & Alam 2010; Moore 2011). The Ringer Lactate solution, due to its calcium citrate

content cannot be used for infusion with red blood cells, as it leads to the clotting of the blood filters (Duchesne et al. 2010); in addition, the presence of potassium may worsen hyperkalaemia (Schreiber 2011). Both products seem to have advantages and disadvantages, and a relatively recent review states that there is no obvious advantage of one of these crystalloids over the other (Harris et al. 2012).

Due to the lack of intrinsic colloid osmotic pressure, crystalloids are known to enter the interstitial space (Hillman et al. 1997), and according to clinical and experimental studies, less than 30% of the substituted volume remains inside the intravascular space. This effect is one of its disadvantages and may potentially result in pulmonary oedema and peripheral oedema (Moore, 2011), interfering with tissue oxygen exchange.

1.4.2. Colloids

A colloid is a fluid with an osmotic pressure similar to plasma and that, for this reason, is mainly kept in the intravascular space. When compared with crystalloids, colloids result in minimal expansion of the interstitial fluid space (Hjelmqvist 2000) and have been, until recently, routinely used as plasma volume expanders for treating severe hypovolaemia (Langeron et al. 2001).

1.4.2.1. Pharmacology of Colloids

Colloids are homogenous noncrystalline preparations consisting of large molecules or ultramicroscopic particles of one substance dispersed through a second substance, and which particles cannot be separated out by normal filtration or centrifugation and that do not precipitate. There are two major groups of colloids: natural colloids and semisynthetic/artificial colloids. Natural colloids include human albumin solution, plasma protein fraction, fresh frozen plasma and immunoglobulin solutions, all derived from plasma. Semisynthetic colloids consist of derivatives of three major groups of molecules: gelatines, dextrans and starches. These colloid molecules are then suspended in a solvent, being isotonic saline the most commonly used solvent; however, hypertonic saline, hypertonic glucose and isotonic balanced electrolyte solutions have also been studied and are available for clinical use. The nature of the carrier solution is an important element when considering the physiological effect of individual solutions (Vercueil et al. 2005).

Colloids that contain molecules of uniform size are known as monodisperse colloids. Human albumin solution has historically been considered the perfect monodisperse colloid, having more than 95% of particles with a uniform molecular weight (MW of 69 kDa). Semisynthetic colloids are polydisperse, with a wide distribution of particle MWs. Colloids may be described by their MWs, which are usually proportional to the size of the molecule, except in gelatines (Vercueil et al. 2005).

The colloid MW is important especially because of its relationship to pharmacokinetics; small particles with a low MW exert a greater oncotic effect and, for a given number of molecules, will have a lower viscosity than larger molecules. In the other hand, they have a shorter intravascular persistence before being filtered at the glomerulus or, less frequently, lost into the interstitium. Larger molecules are retained in the intravascular space longer but, as there are fewer of them, they exert less osmotic force across the semipermeable membrane of the endothelium, having less volume expansion effect (Vercueil et al. 2005).

1.4.2.2. Hydroxyethyl starch – HES

Hydroxyethyl starch (HES) is a polydispersed solution of ethoxylated amylopectin. There are several solutions of HES available, with different MWs. These solutions have been commonly used as plasma volume expanders in human (Gattas et al. 2012) and veterinary medicine (Glover et al. 2014) and are composed by an amylopectin, a waxy starch derived from either maize or sorghum, which is not broken down by amylase (Hjelmqvist 2000; Glover et al. 2014).

There are different concentrations of HES solutions – 3%, 6% and 10% - and the concentration of the solution used will have an important influence on the initial volume effect (Glover et al. 2014). Balanced HES solutions became available in Europe where the use of high-MW starches started to be very rare. A large variety of HES solutions were available, especially medium MW starches, presented in normal saline, such as Haesteril® (200 kDa; DS, 0.5) and Elohes® (6% HES 200 kDa; DS, 0.62) and relatively recently, lower MW tetrastarches such as Voluven® (130 kDa; DS, 0.4), became available, and was adopted by many. It seemed to have a better safety profile (Olivier Langeron et al. 2001), however, in the last few years, studies reported that the

use of HES 130/0.4 in sepsis and critically ill patients may be associated with renal function impairment (Myburgh et al. 2012; Perner et al. 2012).

Voluven® (Fresenius Kabi, Bad Homburg, Germany) is a relatively recently developed HES type, with an average molecular weight of 130.000 Dalton and a degree of substitution of 0.4 (HES 130/0.4), being a tetrastarch. Its molecular weight distribution is the narrowest of all available HES types, which means that the proportion of very large and very small molecules was significantly reduced, compared with other preparations. Voluven®, after multiple dosing, was not seen to accumulate in plasma, which is contrast to all other HES types (Leuschner et al. 2003; Waitzinger J et al. 2003), and renal excretion was seen to be increased (Waitzinger J et al. 1998). These pharmacological properties were assumed to allow decreased tissue storage when compared with other HES formulations (Lang et al. 2001; Lehmann et al. 2007). The lower DS was assumed to be responsible for a fast elimination rate and lower tissue oedema than with higher MW HES solutions with a high DS (Lang et al. 2001). In vitro and in vivo coagulation seemed to be less compromised by Voluven® when compared with other HES specifications (Tigchelaar et al. 1998; Waitzinger J et al. 1998; Langeron et al. 2001; Jungheinrich et al. 2002; Neff et al. 2003; Kozek-Langenecker 2008; Staikou et al. 2012). In addition, Voluven® was reported to have a volume effect of approximately 100% of the infused volume and with 4 to 6h duration (Waitzinger J et al. 1998).

1.4.2.2.1 Distribution and clearance of HES solutions

The clearance of HES solutions is in great part through renal excretion (approximately 70%). Other routes of elimination include extravasation and uptake with transient storage in the reticuloendothelial cells of the liver, spleen and lymph nodes and by excretion in the bile. In dogs, deposition of HES in intravascular and interstitial spaces, in hepatocytes, proximal renal tubular cells has also been demonstrated in histopathology, however with a short-term duration and without impairment of organ function. The HES that is accumulated in these locations, including in the reticuloendothelial cells of the liver, spleen and lymph nodes, seems to be gradually redististributed into the circulation and/or to be catabolized by proteolytic enzymes, in the case of HES present in the reticuloendothelial cells. A higher MW seems to be associated with higher tissue storage (Glover et al. 2014).

1.4.2.2.2. Adverse Effects Reported

Different adverse effects of the administration of HES solutions have been reported, that appear to be related with cumulative dose of these products. These adverse effects include volume overload, coagulopathies, acute kidney injury, proinflammatory effects and allergic reactions. Allergic reactions are fairly rare, but are reported in humans, with emphasis for the foamy macrophage syndrome (hydrops lysosomalis generalisatus) and delayed onset refractory pruritus, both reported in humans. The pruritus seems to occur due to the deposition of HES in the Langerhans cells (Bork 2005). The foamy macrophage syndrome is an acquired lysosomal storage disease that has been reported particularly in people that require chronic plasmapheresis that uses HES solution as diluent (Auwerda et al. 2002).

Coagulopathies have been reported in humans and animals following administration of HES solutions. There are several confirmed and proposed mechanisms by which HES solution may affect coagulation, explaining possible effects in platelet function, decline in factor VIII coagulant and in vWF concentration and activity, and possible relation with acquired fibrinogen deficiency (Glover et al. 2014).

HES was thought to have no influence on renal function as assessed by urine output, sequential organ failure assessment (SOFA) score, and creatinine (Sakr et al. 2007), however, as previously mentioned, two recent large randomized controlled trials in humans (Myburgh et al. 2012; Perner et al. 2012), reported that HES 130/0.4 was associated with higher rates of kidney failure or dialysis and to an increased 90 day mortality rate in one of the studies (Perner et al. 2012), in critical care and septic patients, respectively.

Since recent studies suggested that HES could be nephrotoxic in patients with sepsis and critically ill patients, different other studies on HES effects in different clinical situations have been published. One randomized clinical trial evaluated the possible nephrotoxicity of 6% HES 130/0.4 in patients with normal renal function undergoing hip arthroplasthy, using the measurements of urinary excretion of neutrophil gelatinase-associated lipocalin (n-NGAL), and no evidence of a deleterious effect of the intraoperative use of 6% HES 130/0.4 on renal function was found (Kancir et al. 2014). Other study, of retrospective nature, studied the effect on the kidney of the use of 6% HES 130/0.4 during liver transplantation, and found that patients that received this

colloid had an increased possibility of developing acute kidney injury when compared with patients receiving 5% albumin during liver transplantation (Hand et al. 2015).

A recent study on the effect of the molecules of HES on the human proximal tubule cells in vitro, reports that HES molecules have an harmful effect on renal proximal tubular cells, which seems to be independent of the molecular weight or origin of the solution (potato or corn derived). All HES solutions used in the study (70, 130 and 200 kDa of molecular weight) decreased cell viability in a dose dependent way, with minimal differences in the harmful effects between different preparations (Bruno et al. 2014).

Many recent studies on the use of HES in healthy patients in different clinical and surgical conditions still report clinical benefits from its use, such as efficient improved prevention of hypotension and symptomatic hypotension, more efficient intravascular volume expander to maintain tissue perfusion than conventional crystalloids, among others (Mercier et al. 2014; Hung et al. 2014; El-Fandy et al. 2014; Cui et al. 2014; Miao et al. 2014).

The intestine has an extremely import role in the metabolism, inflammation and sepsis, however information about the effects of HES solutions on the intestinal mucosa and overall function and barrier integrity is very limited. A small number of studies on the effects of HES in the intestine have been performed in different species (Feng et al. 2007; Lobo et al. 2008; Kimberger et al. 2009; Lu et al. 2015; Wong et al. 2015).
In rats with polymicrobial sepsis, intestinal permeability was significantly decreased after HES 130/0.4 administration when compared with saline administration in septic rats, being also associated with reduced levels of inflammatory mediators (Feng et al. 2007).

In rabbits that underwent splanchnic hypoperfusion, the administration of a combination of Lactated Ringer solution combined with hydroxyethyl starch solution was associated with less intestinal oedema when compared with the infusion of a combination of Lactated Ringer solution and saline. In this study, the intestinal oedema was evaluated in a segment of ileum, which was isolated and perfused (Lobo et al. 2008). In pigs, goal-directed fluid therapy with hydroxyethyl starch significantly increased microcirculatory blood flow and tissue oxygen tension in both healthy and perianastomotic colon compared to goal-directed or restricted crystalloid fluid therapy (Kimberger et al. 2009).

A study in a rabbit model of sepsis investigating the effect of fluid resuscitation with hydroxyethyl starch (HES) 130/0.4 in the intestinal mucosal barrier function, fluid resuscitation with 6% HES 130/0.4 was associated with decreased intestinal mucosal injury compared with a non-treated sepsis model – there was mild atrophy of the intestinal mucosa, regularly arranged villi, grossly intact epithelium, increased number of goblet cells, cystic space was present below the upper villous epithelium, and mild oedema and lymphocyte infiltration of the lamina propria was seen. In the non-treated sepsis model, evident atrophy of the intestinal mucosa, villi shedding, degeneration, necrosis and shedding of epithelial cells was observed. Exposure, oedema, and lymphocyte and neutrophil infiltration of the lamina propria were also presented, and there was also lymphocyte and neutrophil infiltration, submucosal capillary congestion, and purulent exudate on the serosal surface (Lu et al. 2015).

The results of a more recent study using an isolated perfused model of the mouse small intestine suggested that the vascular perfusion with clinically relevant concentrations of HES significantly impairs the endothelial and epithelial barrier integrity and the metabolic function of the intestine, when compared with albumin (Wong et al. 2015). In this study, mice that had their small intestine perfused with 3% HES 130/0.4 solution showed a significant loss of vascular fluid, an increased fluid accumulation in the intestinal lumen, an enhanced translocation of fluorescein isothiocyanate–dextran from the vascular to the luminal compartment and a significantly impaired intestinal galactose uptake. On electron microscopy analysis, changes where detected in intestinal morphology, with aggregation of intracellular vacuoles within the intestinal epithelial cells and enlarged intercellular spaces being observed (Wong et al. 2015).

Overall, the effects of HES in the intestinal mucosa and barrier function are still unclear.

At the light of the studies that associate HES administration with renal injury and early mortality, the marketing authorization of HES solutions has been suspended in Europe. In United States of America, these solutions are still legally available however additional recommendations for both patients and health professionals were added to the product information by the Federal Drug Administration (FDA), in order to promote the adequate use of these solutions when the benefits are more than the risks, minimizing the adverse effects.

1.5. Ischaemia/reperfusion injury

IR injury is an important mechanism of intestinal mucosal injury in people and animals suffering from acute and chronic intestinal ischemic disorders (Blikslager et al. 2007). Mesenteric ischaemia can be occlusive (caused by occlusion of major arteries) or nonocclusive (consequence of shock or vasoconstriction and resultant low-flow). Mucosal injury following occlusive acute mesenteric ischaemia is very severe; nonocclusive acute mesenteric ischaemia results in less severe mucosal injury, however it may be exacerbated by reperfusion injury (Parks & Granger 1986) and increases morbidity and mortality rates on recovery (Levy et al. 1990; Hiltebrand et al. 2009).

Ischemia/reperfusion injury has been investigated in different animal species, as cats, rodents, and pigs, with a number of distinct forms of ischemia (Parks & Granger 1986; Schoenberg et al. 1991; Kubes et al. 1992; Stringa et al. 2012). In a feline model, mesenteric blood flow reduced to 20% of baseline was used to prime tissues for reperfusion injury while causing relatively little mucosal injury itself (Kubes et al. 1992). In this study, reperfusion injury to the intestinal mucosa was attributed to the activation of neutrophils located within the mucosa. Other studies also in feline models, suggested that xanthine oxidase-elaborated superoxide was largely responsible for initiating reperfusion injury (Grisham et al. 1986). In other study, in mice genetically engineered to overexpress superoxide dismutase, reperfusion injury was decreased, providing support for the role of superoxide suggested by prior studies (Deshmukh et al. 1997).

The first studies characterizing intestinal mucosal lesion in low-flow states were performed by Chiu and colleagues, in 1970, and showed that the extent of the damage affecting the intestinal mucosa is directly related with the decrease in the blood flow, and that a decrease in blood flow causes detachment of the intestinal epithelium in different degrees of severity (Chiu et al. 1970). Intestinal IR is associated with intestinal barrier function loss, bacterial translocation into the circulation, and consequent systemic inflammation (Collard & Gelman 2001; Fink & Delude 2005). In addition, reperfusion of damaged intestinal tissue aggravates tissue damage and is considered to be an effector of local as well as distant inflammation and multiple organ failure (Parks & Granger 1986; Homer-Vanniasinkam et al. 1997; Chen et al. 2004; Vollmar &

Menger 2011), which is a common cause of death in critically ill patients (Baue 2006) (Figure 2).

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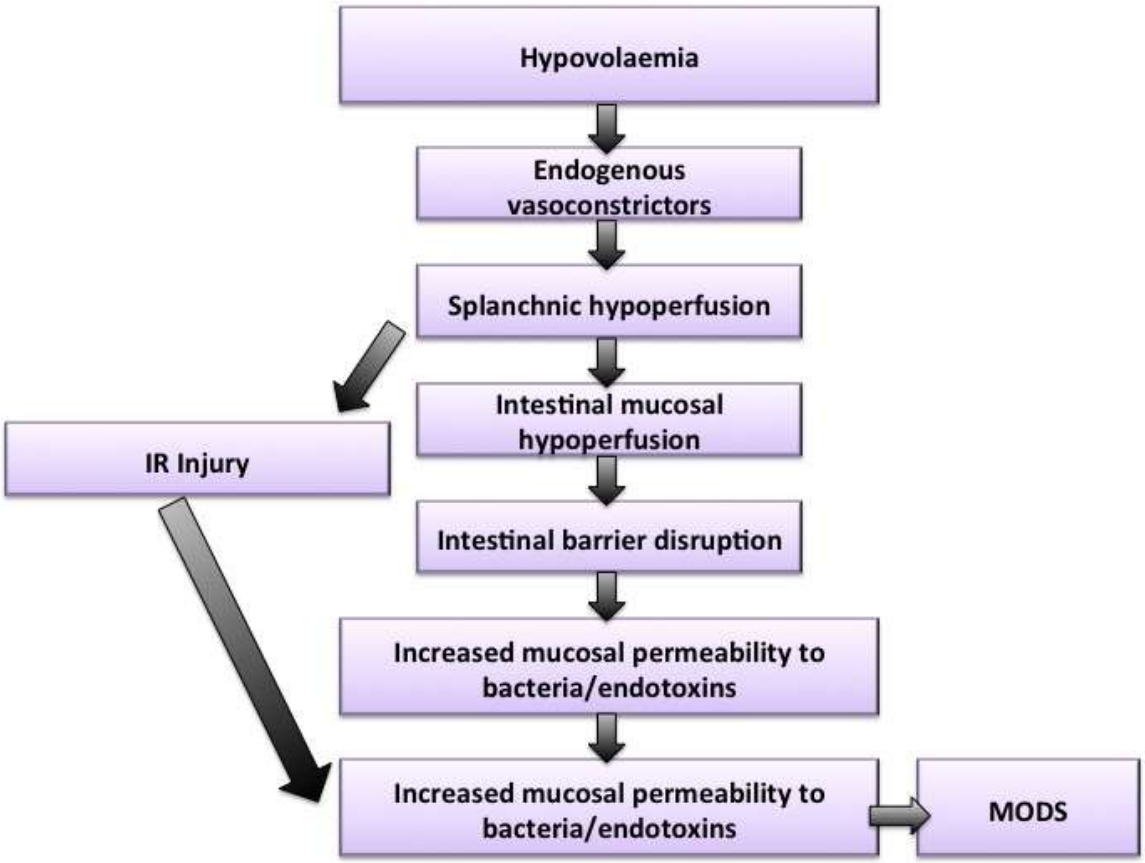

Figure 2. Development of multiorgan dysfunction syndrome (MODS) following intestinal hypoperfusion (adapted from Ackland et al. 2000).

The extent of injury that is developed during ischemia compared with reperfusion seems to be determined in great part by the nature of the ischemic process. Complete ischemia results in rapid mucosal degeneration with relatively little injury during reperfusion; in other hand, marked reperfusion injury seems to occur following low-flow ischaemia (Park et al. 1990). In both cases, mucosal injury has similar features, consisting of villus contraction and epithelial sloughing. Sloughing of epithelium begins at the tip of the villus and continues toward the base of the villus and, in some cases, the crypt depending on the degree and duration of ischemia. Sloughing starting in the tip of the villi towards the base and crypts is likely due the distance of the epithelium from the arterial blood supply.

Anatomical variations in blood supply between species may also play an important role in epithelial ischemic injury. In humans, rodents, cats, and pigs, villi have a central arteriole that arborizes near the tip of the villus and drains into peripheral venules, generating a countercurrent exchange mechanism in which oxygen diffuses from the arteriole into the tissue and adjacent peripheral venules, before reaching the tip of the villus, resulting in a relatively hypoxic villus tip. When arterial blood flow is reduced, the villus tip becomes progressively hypoxic, resulting in epithelial injury. Previous studies occluding the small intestinal mesenteric vasculature for one hour lead to loss of epithelium from the upper third of the villus, and ischemia for two hours resulted in almost complete loss of the epithelium (Blikslager et al. 1997). In the large intestine, although there are no villi, surface epithelium is lost initially during ischemia, followed by crypt epithelial injury as the duration of ischemia increases, following a similar pattern as the small intestine (Blikslager et al. 2007).

The oxygen consumption in the intestine is independent of the blood flow to a certain level, with a constant level of oxygen uptake being normally present, which is possible via compensatory reciprocal changes in intestinal oxygen extraction and increased capillary recruitment (Boley et al. 1978). This is an extremely important mechanism of protection from ischaemic injury (Bulkley et al. 1985). However, in humans, is known that the intestinal oxygen uptake becomes flow dependent, when the intestinal blood flow falls below 30 ml/min per 100 g tissue (Wilcox et al. 1995) or when the systemic blood pressure falls below 50 mmHg (Bulkley et al. 1985); the lack of an adequate level of oxygen leads to tissue ischaemia and consequent impairment of cellular function and cell death (Mallick et al. 2004). Following mesenteric ischaemia, consequent intestinal ischaemia and reperfusion injury occurs after reestablishment of the local circulation.

Ischaemia and reperfusion (IR) injury is associated with microcirculatory derangements, which are characterized by the capillary “no-reflow” and the “reflow paradox” (Vollmar & Menger 2011). The capillary “no-reflow” corresponds to the lack of blood flow upon onset of reperfusion, and the manifestation of “reflow-paradox” is associated with the reestablishment of the microvascular perfusion and reoxygenation. Reperfusion is believed to cause more severe lesions when compared with ischaemia alone, suggesting that the cause of IR injury is a process initiated by the return of oxygenated blood to the ischaemic intestinal tissues (Kurose & Granger 1994). The re-

oxygenation is related with the generation of cytotoxic oxidants, which activate leukocytes and promote leukocyte adhesion to the endothelial lining of venules (McCord 1985; Schoenberg & Beger 1993; Menger & Vollmar 2000). The impairment of the microcirculation seems to take place before the parenchymal cell damage, and is believed to be a crucial point in intestinal failure. Apart from the cause and duration of the ischaemia (Gonzalez et al. 1994; Beuk et al. 1997; Massberg, Gonzalez, et al. 1998), the ischaemia being partial or total is very important and directly related with the severity of intestinal injury (Megison et al. 1990; Beuk et al. 1997; Beuk et al. 2008). Injured intestine will have increased intestinal permeability, related with endothelial and epithelial dysfunction. The impairment of the endothelial function leads to the extravasation of plasma molecules of high molecular weight, therefore contributing to interstitial oedema. The lack of epithelial integrity leads to the loss of barrier function with consequent translocation of intestinal-derived endotoxin and intraluminal bacteria (Gayle et al. 2002; Kalia et al. 2002; Szabó et al. 2006; Szabó et al. 2008).

1.5.1. Mechanisms of acute intestinal ischaemia and reperfusion injury

The non reestablishment of blood flow upon onset of reperfusion (capillary "no-reflow") has been believed to be due to different mechanisms, as intravascular haemoconcentration and thrombosis, leukocyte plugging, swelling of endothelial cells, vasomotor dysfunction, and capillary narrowing caused by an increased interstitial pressure due to oedema (Menger et al. 1997). However, reduced red blood cell velocity, and red blood cell sludging have been reported to be involved in post-ischemic perfusion failure (Gonzalez et al. 1994; Beuk et al. 2000). Increased flow resistance due to leukocyte-vessel wall interactions (House & Lipowsky 1988) was suggested as the mechanism responsible for the decreased red blood cell velocity and consequent reduced small bowel perfusion. Beuk and colleagues reported that the submucosal capillary perfusion and leukocyte adherence in submucosal venules are negatively correlated; furthermore, the same authors described an eightfold improvement in flow through the submucosal capillaries with complete attenuation of leukocyte interaction (Beuk et al. 2008).

Intestinal reperfusion injury, besides failure of perfusion, also includes an important inflammatory reaction, with recruitment of leukocytes in a gradual process, which is initiated by a rolling adhesive interaction with consequent inflammatory

activation and firm adhesion of leukocytes to the endothelium of post-capillary venules. Adhesion molecules of the selectin family are believed to support the leukocyte rolling, with the role of the individual selectins possibly differing in specific organs (Carlos & Harlan 1994). P-selectin is suggested as being involved in leukocyte rolling in the colon (Riaz et al. 2002; Santen et al. 2007).

The leukocyte adhesion in IR is mainly dependent on beta2- integrins and immunoglobulins on the leukocytic and endothelial site, respectively (Riaz et al. 2002). In addition, the circulation of leukocytes is regulated by CXC chemokines (Ajuebor et al. 2002), with macrophage inflammatory protein-2 and cytokine-induced neutrophils chemoattractant having an important role in advocating leukocyte–endothelial interactions (Riaz et al. 2003; Santen et al. 2008). Leukocyte–endothelial cell interaction has been considered a crucial step in the microvascular dysfunction caused by IR (D L Carden & Granger 2000), however more recent studies report that other blood cells, namely T-lymphocytes, have a more important role in the pathogenesis of IR injury (Shigematsu et al. 2002; Watanabe et al. 2002). In post-ischaemia intestine, CD4+ and CD8+ T cells are described to be recruited and seem to boost the post-ischemic infiltration of the affected tissues by neutrophils and to increase the extravasation of albumin (Shigematsu et al. 2002). A role of platelets in the pathogenesis of intestinal I/R has also been reported; these cells adhere to the capillary endothelium and seem to be involved in the impairment of the intestinal microvascular perfusion (Massberg, Enders, et al. 1998). Platelets are also believed to induce tyrosine phosphorylation in endothelial cells; therefore these cells may also contribute to inflammation in intestinal IR (Massberg et al. 1999). In addition, platelets cooperate and synergize with leukocytes during intestinal I/R. The adhesion of platelets to leukocytes through the platelet P-selectin induces nuclear translocation to NF-κB and increases the expression of CD11b/CD18; it also increases MCP-1 and reactive oxygen species (Yeo et al. 1994; Weyrich et al. 1996; Neumann et al. 1997), expanding the inflammatory reaction. It is reported that 26% of platelets that adhere to post-ischemic intestinal venules are attached directly to the endothelium, and that the remaining 74% are bound to leukocytes that are already adherent to the endothelium (Cooper et al. 2004). Angiotensin II type 1 receptors (AT1-R) seem to be also involved (Riaz et al. 2004; Petnehazy et al. 2006). In most recent studies, toll-like receptors (TLRs) have been implicated as a possible link between the innate and adaptive immune system, thereby

mediating intestinal inflammation and injury (Aprahamian et al. 2008; Watson et al. 2008; Moses et al. 2009; Victoni et al. 2010), and these receptors are also considered novel targets for therapeutic intervention in intestinal IR injury (Vasileiou et al. 2010).
Ischemia leads to the production of detrimental metabolites, to the influx of inflammatory cells and epithelial cell death (apoptosis and necrosis). The aggravation of injury in reperfusion is believed to be related to the production of reactive oxygen species and generation of chemoattractants for neutrophils (Gonzalez et al. 2015). In ischaemia, tissues continue to use adenosine triphosphate to produce energy, and there is accumulation of hypoxanthine (the metabolic by-product). Furthermore, in the situation of decreased/lack of oxygen, xanthine dehydrogenase, which would metabolize hypoxanthine in normal conditions, is converted to xanthine oxidase by tissue proteases that are locally produced (Nilsson et al. 1994). After the reestablishment of the tissue perfusion, xanthine oxidase the now available oxygen as an electron acceptor as it metabolizes hypoxanthine, with production of superoxide (Granger et al. 1981; Granger, Höllwarth, et al. 1986; Granger, McCord, et al. 1986; Grisham et al. 1986). Oxygen free radicals induced direct injury and their ability to activate and chemoattract neutrophils leads to the reperfusion tissue injury.

Xanthine oxidase is a form of xanthine oxidoreductase, a type of enzyme that generates reactive oxygen species. This enzyme has widespread endothelial localization and has the ability to produce superoxide (O_2^-) and H_2O_2, and its plasmatic levels were seen to increase after reperfusion of several organs, following ischaemia. Intravascular generation of oxidants by this enzyme, which may affect the integrity of the vascular endothelium and its ability to bind to the surface of endothelial cells may contribute to multiorgan dysfunction syndrome (MODS). The oxidants generated by the xanthine oxidase may also originate chemotactic factors, which promote the sequestration of leukocytes in the organs. Leukocytes that had been exposed to ischaemia, after reperfusion, may re-enter in the circulation in an activated state. Activated neutrophils are assumed to have an important role in IR injury in multiple organs and believed to cause tissue injury by different proposed mechanisms: occlusion and increased permeability of the microvasculature (Hernandez et al. 1987; del Zoppo et al. 1991), release of reactive oxygen metabolites (Suzuki et al. 1989; Nilsson et al. 1994), cytotoxic enzyme release (Zimmerman & Granger 1990), and mechanical injury induced by migration (Gayle et al. 2002). Neutrophils produce O_2^- and H_2O_2 and when

activated also produce proteases, which are able to degrade the components of the endothelium. Also, is known that neutrophil activation and adhesion molecule expression occurs following prolonged visceral ischaemia, resulting in increased mortality in multiple organ failure (Foulds et al, 1998; Carden and Granger, 2000). The inflammatory mediators produced by tissues following ischaemia, which are released to the circulation, may induce the expression of endothelial cell adhesion molecules (ECAMs) multifocally in the body, and, in this way, promote the adhesion of neutrophils to the endothelial cells and neutrophil related injury in vessels and tissues. TNFα is a known inflammatory mediator released by the tissues that suffered ischaemia, and is able to stimulate the production of inflammatory mediators in multiple local and distant tissues in the body. During reperfusion, there is also activation of the complement cascade. Complement activation products are chemotactic for leukocytes and also promote neutrophil activation, generation of oxidants and adhesion molecules expression (D L Carden & Granger 2000).

The nitric oxide (NO) is also known to be involved in the process of IR injury. The decreased availability of NO that occurs after IR promotes the interaction between the neutrophils and the endothelial cells and also induces oxidative stress in multiple organs. The phospholipid platelet-activating factor (PAF) is present in increased levels in the plasma following IR, and promotes neutrophil activation, adhesion and leukocyte mediated injury in IR, which may be related with initiation or perpetuation of organic ischaemia and failure (D L Carden & Granger 2000).

1.5.2. Histology and biology of the intestine

The intestine is a tubular organ and an internal layer, the mucosa, a middle layer, the muscularis propria, and an external layer, the serosa form the intestinal wall. The intestinal mucosa itself is composed by the epithelium, which consists of a single layer of epithelial cells – simple columnar epithelium – lining the intestinal lumen, a lamina propria, with mesenchymal cells, and the muscularis mucosa, formed by smooth muscle (Bacha & Bacha 2012; Gelberg 2012). Any insult to the mucosa can cause intestinal dysfunction. The mucosa works as a permeable barrier, with a high degree of selectivity, allowing nutrient, electrolyte and water absorption but excluding toxins and pathogens, in normal conditions (Gelberg 2012).

The major cell types lining the intestine are enterocytes, undifferentiated or crypt epithelial cells, goblet cells, Paneth cells, enterochromaffin (neuroendocrine, argentaffin) cells, and microfold (M) cells (Bacha & Bacha 2012; Gelberg 2012).

Enterocytes are tall and columnar cells, with luminal microvilli, containing a surface glycocalyx. The glycocalyx is where the digestive and absortive enzymes are stored. Mature enterocytes do not proliferate, however they give feedback for inhibition of mitosis to the crypt cells by polypeptides inhibitors of mitosis. Cells are attached by tight junctions, which are composed by proteins, being the predominant ones occludin, junctional adhesion molecules and claudins. Many nutrients are absorbed through the lateral intercellular spaces between cells – transcellular absorption. As per physiologic turnover, enterocytes move up the crypt and villus to the villus tip – extrusion zone – where effete enterocytes are discarded to the intestinal lumen by anoikis (apoptosis induced by cell detachment) (Gelberg 2012).

Undifferentiated crypt epithelial cells are progenitor cells that replace all the other epithelial cells and have minimal or no digestive functions. These cells have short and sparse microvilli and are the source of the secretory component that acts as a receptor for IgA and IgM (produced by plasma cells in the lamina propria). These cells are also a source of ion secretion into the intestinal lumen (Gelberg 2012).

Regarding the goblet cells, they mainly secrete mucous and are present in villi and crypts (Gelberg 2012).

Paneth cells are located near the crypt base in some species, as primates, horses and rodents, however it is not certain if they are present in swine. Paneth cells migrate in a different direction, comparing with all the other cells in the intestine – towards the crypts. They have secretory and phagocytic roles (Gelberg 2012).

Enteroendocrine cells, also known as enterochromaffin and argentaffin cells (due to affinity to silver stains), are primarily in the crypts and produce serotonin, glucoinsulotropic peptid, cathecolamines, gastrin, and many others, in response to chemical and mechanical stimuli. These products are secreted directly into the intestinal tissue (Gelberg 2012).

M cells (microfold cells), as per name, have a microfolded surface, and form the dome epithelium that covers the gut-associated lymphoid tissue (GALT). They have an important role in the uptake of protein and peptide antigens from the intestinal lumen and in the transport of these to the GALT, where local dendritic cells and macrophages

take them up. M cells are also a portal of entry for some pathogens, as bacteria and some viruses (Gelberg 2012).

Mesenchymal cells are in the intestinal lamina propria. The number of cells increases with exposure to antigen, but a resident population of these cells exist in normal animals (Gelberg 2012).

The intestinal lymphoid tissue constitutes 25% of the body's lymphoid mass. Is formed by the lymphoid tissue in the lamina propria and the GALT, and is responsible for the non-response to several food antigens ingested. The lymphoid tissue in the lamina propria has a role in the intestinal crypt cell differentiation (Gelberg 2012).

Neutrophils are transient and short-lived within the lamina propria, and are removed from the body by migrating through the wall to the lumen, and digested or excreted from the body in the faeces (Gelberg 2012).

Eosinophils, when present in the lamina propria and submucosa, tend to be indicative of a hypersensitivity reaction (frequently to food antigens or parasites) (Gelberg 2012).

Mast cells comprise approximately 2 to 3% of the cells of the lamina propria and, under normal conditions, help regulate the intestinal epithelial barrier, participating in the control of the blood flow and coagulation, smooth muscle contraction, stimulation of the enteric nervous system, and peristalsis. Mast cells also help regulate acid, electroly and mucous production by enterocytes, and recognize parasites and microorganisms through antibody-dependent mechanisms and receptor recognition. They release pro-inflammatory mediators and attract inflammatory cells (Gelberg 2012).

Globule leukocytes are large granular lymphocytes found in all species that are inter-epithelial or are found within the lamina propria, but their normal function is not certain. Infrequently they can originate neoplasms (Gelberg 2012).

1.5.2.1. Intestinal Barrier

One layer of columnar epithelium and inter-epithelial tight junctions form the intestinal barrier. Inter-epithelial tight junctions, which are located at the apical-most region of the paracellular space (Figure 3), polarize the cell into apical and basolateral regions, what is referred to as fence function, and regulate passive diffusion of solutes and macromolecules, known as gate function (Mandel et al. 1993). This barrier is the

first line of defense against pathogens/deleterious environment from the intestinal lumen (Podolsky 1999). This innate mucosal defense has two components, namely, mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa and mechanisms that ensure rapid repair of defects in the epithelium. Regulation of passive diffusion across the intestinal barrier is centered on selectivity of tight junctions, which allow passage of select solutes that are beneficial to the host, but prevents the passage of antigens, pathogens and bacterial toxins (Podolsky 1999). Furthermore, the epithelium is also a sentinel, serving as a sensor, signaling to resident innate immune cells in the mucosa when infection or injury occur, to recruit regulate the function of innate and adaptive immune system elements essential to fight infection and promote healing (Blikslager et al. 2007).

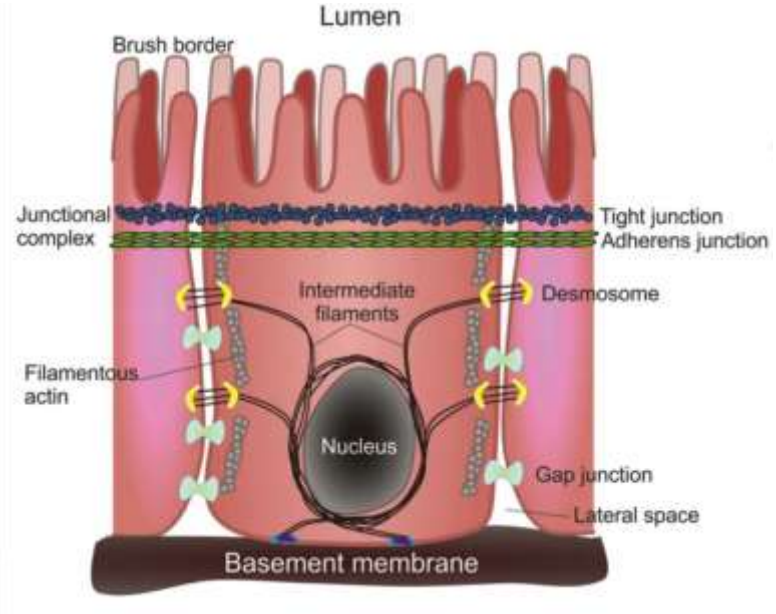

Figure 3. The epithelial barrier - illustration of the intestinal epithelial cell junctional complexes. Tight junctions (located in the upper portion of the cell) connect individual epithelial cells between them, being responsible for the intestinal epithelial barrier function. Parallel transport is performed through the lateral spaces present between cells (adapted from Williams et al. 2015).

1.5.3. Intestinal Apoptosis and Its Role in The Regulation of Barrier Function

Apoptosis, often also referred to as programmed cell death, is the self-induced cell death of individual cells, which can occur in physiologic or pathologic conditions. Besides other pathologic conditions, apoptosis occurs as a component of injury in

hypoxia (Myers et al. 2012; Mitchell 2014). Most studies have linked apoptotic cell death to the pathogenesis of gastrointestinal disease, although other forms of cell death like necrosis might be related with intestinal inflammation (Watson 1995; Barkla & Gibson 1999; Mayhew et al. 1999; Proskuryakov et al. 2003). The physiological and pathophysiological role of necrosis in the gut is largely unknown. Necrosis can be observed under physiological stress, inflammation and infection and is caused by external environmental changes such as the occurrence of toxins, hypoxia, cytolysins and significant changes in temperature, and might play an important pathogenic role in infectious gastrointestinal diseases (Proskuryakov et al. 2002; Vanlangenakker et al. 2008).

The apoptotic process is usually self programmed and energy dependent, and is initiated by cell signalling, controlled and integrated by regulatory molecules and has a common execution phase by caspase enzymes, followed by the removal of the death cell. During the initiation, caspases become active and, in the execution phase, these enzymes act to cause cell death (Myers et al. 2012).

Initiation occurs principally by signalling from two distinct pathways that will converge at a point during the process: the extrinsic pathway, also called receptor-initiated, and the intrinsic or mitochondrial pathway. Both pathways converge to activate caspases, however they involve distinct molecular interactions (Myers et al. 2012).

In the normal intestine, epithelial cells migrate from the base of the crypt to the villi where they will be shed. This phenomenon was first described in 1948 and the term ‘extrusion zone’ used to refer to the specific area where cells are shed, on the villus or colonic surface (Leblond & Stevens 1948; Quastler & Sherman 1959). Several reports quantified and described cell shedding (Clarke 1970; Heath 1996; Montgomery et al. 1999) and several mechanisms of cell shedding have been proposed (Mayhew et al. 1999). It was thought that in mouse and most likely also in humans the whole cell was shed and subsequently would undergo apoptosis, due to loss of attachments to the basement membrane (anoikis) (Potten & Allen 1977; Grossmann et al. 1998; Watson et al. 2005). It has also been suggested that apoptosis or anoikis initiates cell shedding although direct evidence is little (Hall et al. 1994; Merritt et al. 1995; Pritchard et al. 2000).

Two main spots of epithelial cell death were described in the intestine: the villus tip and the crypt region (Edelblum et al. 2006). Aged epithelial cells are thought to die

from anoikis (a form of programmed cell death which is induced in anchorage-dependent cells after detachment from their matrix), after travelling from the crypt base to the villus tip in the small intestine, or to the surface epithelial cuff in the colon (Potten et al. 1997; Grossmann et al. 1998; Watson et al. 2005; Günther et al. 2013). Precise mechanisms controlling this process are still not completely understood, however studies state that this process is actively regulated and involves caspase-3 (Bullen et al. 2006; Marchiando et al. 2011). This theory has been supported by experimental studies, which show that excessive cell shedding induced by TNF administration could be inhibited if caspase activation is blocked (Marchiando et al. 2011). However, mice deficient for central molecules of apoptosis (caspase 3, caspase 8, Fas-associated protein with death domain (FADD), show little if any structural changes in intestinal homeostasis, which implies that apoptosis might not be required for epithelial turnover in the gut in the steady state. According to these findings, epithelial cell shedding might be a rather passive process induced by the spatial constraints of densely packed epithelial cells at the villus tip and shedding associated cell death may be a consequence rather than a cause of shedding. As previously mentioned, spontaneous cell death of IEC has also been described within the crypt region (Watson 1995).

To understand the mechanism of cell shedding is important, as it allows investigations on abnormal or increased intestinal shedding and impact on intestinal barrier function (Figure 4).

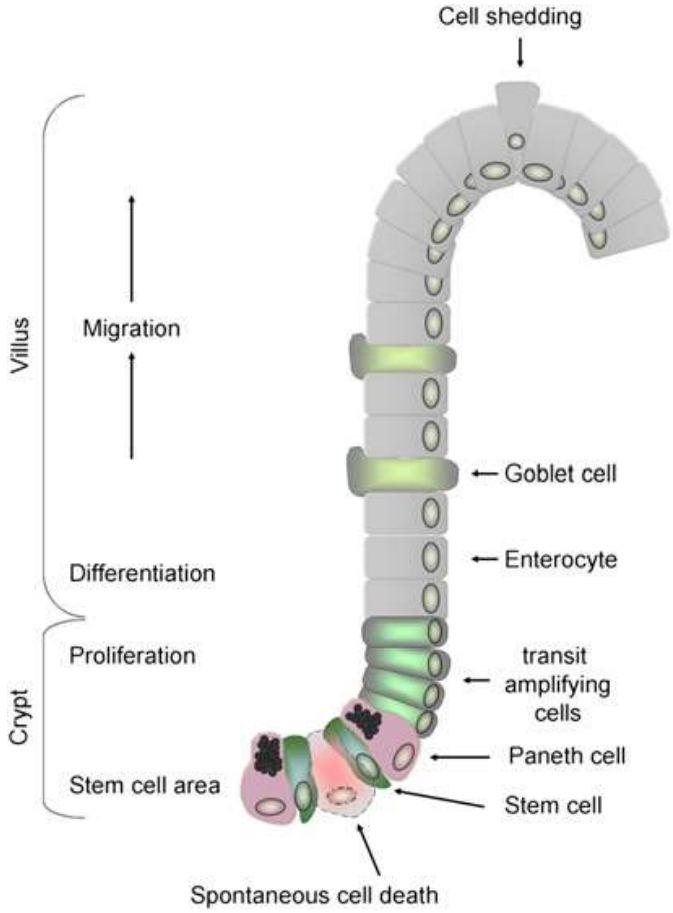

Figure 4. Normal shedding process in the intestinal, epithelial cell layer. The crypt-villous border is where the stem cell progeny is located. In the human intestine, these cells experience four to five stages of cell division and, after that, they stop proliferation and originate the mature epithelial cells. Aged epithelial cells are latter shed into the lumen at the villus tip. In the crypt region, cells that presumably lack the capacity to undergo cell shedding, may suffer cell death at this location (Günther et al. 2013).

1.5.3.1. Intestinal Apoptosis in Ischaemia/Reperfusion

The main cause of mucosal loss during tissue intestinal ischemia-reperfusion lesion in the small intestine is thought to be anoikis (apoptosis of enterocytes, potentially caused by loss of contact between the epithelial cells and extracellular matrix, and between neighbouring epithelial cells) (Ikeda et al. 1998; Derikx et al. 2008; Grootjans et al. 2010). The retraction of the collagen IV positive cells from the basal pole of the epithelial cells at the tip of the intestinal villus causes subepithelial spaces, causing loss of contact with the underlying villous stroma, leading to enterocyte anoikis (Frisch & Francis 1994). A more recent study confirmed the retraction of the collagen IV positive basement membrane that demarcates the lamina propria from the epithelial

cells and also observed that there was shortening of the underlying network of myofibroblasts (Derikx et al. 2008). This seems to lead to the apoptosis that is observed in the villus epithelial cells after ischemia-reperfusion lesion.

The sequence of development of intestinal mucosal injury after ischaemia and reperfusion has been studied intensively in early (J. Chang et al. 2005; Grootjans et al. 2010) and late phases (Chiu et al. 1970; Wagner et al. 1979; Parks & Granger 1986), with a higher number of studies investigating the late phase. One study proposed that interactions between the matrix and cells are critical to regulation of apoptosis (Frisch & Francis 1994). Ikeda and colleagues (1998) reported that both ischemia and ischemia and reperfusion induce intestinal mucosa injury, with detachment of epithelial cells, with 80 % of the detached cells showing morphologic characteristics of apoptosis. In this same study was reported that apoptosis is the major form of cell death occurring after short term ischaemia (30 min) or ischaemia/reperfusion injury in the rat jejunum, and that the distribution of epithelial cell-matrix interactions could be an important part in induction of apoptosis in detached enterocytes (Ikeda et al. 1998).

In the study of Grootjans and colleagues (2010) investigating inflammation induced by IR in the human intestine, immunohistochemical analysis of M30 showed that apoptosis was only seen at the tip of the villi in early stage reperfusion and in the lumen debris later, which indicates that apoptosis was not induced by ischemia alone (Grootjans et al. 2010). So, at the light of more recent findings, apoptosis appears to be importantly involved in the intestinal mucosal reperfusion injury following a period of ischaemia.

1.6. Total Intravenous Anaesthesia and Target Controlled Infusions – Concepts and Application

The general anaesthesia has as main aims to prevent awareness of and response to pain and to provide restraint and immobility of the patient and relaxation of skeletal muscles when necessary for the procedure. These goals need to be achieved without putting at risk the life and safety of the patient. Nowadays is known that a combination of agents is more likely to be more efficient in achieving these aims (Jones 2010).

Using an anaesthetic machine and vaporizer is possible to achieve precise and continuous delivery of volatile anaesthetic agents, such as isofluorane, sevoflurane and desflurane, which are halogenated ether and highly fluorinated methyl isopropyl ethers,

respectively. This is the main concept of volatile anaesthesia. Pain management, pre-medication, sedation and induction are usually achieved by using a combination of other agents and the general anaesthesia in then maintained by continuous and controlled vaporization of the volatile anaesthetic agent (Alibhai 2010).

Continuous infusions of intravenous anaesthetic agents may also be used to provide general anaesthesia. Total intravenous anaesthesia (TIVA), by definition, is the use of intravenous drugs to provide general anaesthesia and analgesia and it appears to have potential advantages over volatile anaesthesia, such as the fact that the hypnotic and analgesic components of anaesthesia can be controlled independently and rapidly adapted to minimize and control awakeness and painful stimuli; the achievement of a greater hemodynamic stability and a more rapid recovery from general anaesthesia; the potential use of less amount of drugs to achieve similar anaesthetic levels, among others (Fragen 1988; Camu & Kay 1991; Morton 1998; De Castro et al. 2003; Eyres 2004)

The development and availability of new intravenous drugs with rapid onset of action, redistribution and clearance, such as propofol and remifentanil, is responsible for the increasing interest of the anaesthesiologists in TIVA. In addition, an improved knowledge on drugs pharmacodynamics and pharmacokinetics allowed the development of target controlled infusion systems, making close titration of the drugs concentrations in the organism possible, increasing the safety of TIVA (Morton 1998; De Castro et al. 2003).

Drug pharmacokinetic models allow the mathematical assessment the time course of drugs within the body, allowing the quantification of its absorption, distribution, metabolism and excretion. Administering a known dose of drug and measuring the resulting plasma concentrations is the principle behind the development of these models. The differences between the amount of drug administered and the concentrations of the drug in the blood will provide information that will allow the estimation of mathematical constants of drug elimination and drug distribution from the blood to peripheral compartments. These constants will then be included in mathematic models, which will allow the approximate calculation of the drug concentrations in the blood accordingly to the amount of drug administered (Dhillon & Kostrzewski 2006).

The vast majority of the drugs have a multicompartment pharmacokinetic behaviour (Sheiner et al. 1979). The pharmacokinetics of propofol and remifentanil, two agents commonly used in general anaesthesia, is usually described by a three-

compartment model (Marsh et al. 1991; Minto, Schnider & Shafer 1997; Schnider et al. 1999). The drug is administered into a central compartment that represents a distribution volume, often expressed in litres or litres per kilograms, including the first pulmonary uptake and the mixture of the drug with the blood. Subsequently, the drug is expeditiously distributed to one peripheral compartment, to which splanchnic and muscle tissue store belong, and slowly distributed to another peripheral compartment, which is the adipose tissue (adipose tissue store). The intercompartmental rate constants k describe the time course of drug exchange until an equilibrium between the central compartment and the two peripheral compartments is achieved: kij is the rate constant for drug transfer from compartment "i" to compartment "j" (k12, k21, …). The pharmacokinetic models must also consider the irreversible elimination of the drug from the central compartment by simple elimination (for example, through the kidneys) and/or by biotransformation (for example, liver and plasma enzymes), which is described by k10 rate constant. After administration, the drug must reach the organ where the drug will act, as for example the brain in the case of anaesthetic drugs, and it will be transported to the effect-site by the plasma. The passage of the drug from the central compartment to the effect compartment is translated by the constant k1e, and the disappearance of the drug from the effect compartment is the determined by the ke0 (Hull et al. 1978; Sheiner et al. 1979; Hill 2004).

Target-controlled infusion (TCI) is a way of delivering intravenous and specifically anaesthetic drugs. The rational for TCI is based on setting a desired "target" concentration, a priori by the anaesthesiologist to achieve a desired clinical effect (Cazalaà et al. 2009). Target-controlled infusion (TCI) incorporates the pharmacokinetic variables of an intravenous drug to facilitate safe and reliable administration (Wietasch et al. 2006). TCI administration of propofol is often used in combination with opioids, some of which alter propofol kinetics. Remifentanil appears to be an ideal analgesic component for total IV anaesthesia (TIVA) in combination with propofol because of its elimination via an independent pathway from that of propofol as well as its rapid elimination and favourable controllability (Mertens et al. 2003; Wietasch et al. 2006).

1.7. Target Controlled Infusion using Propofol and Remifentanil

1.7.1. Hypnotics – Propofol

Propofol is a hypnotic alkylphenol, which is part of the non-barbiturate anaesthetics group. It is oil at room temperature, being insoluble in aqueous solutions but highly lipid soluble. This hypnotic anaesthetic drug is nowadays formulated as a oil-in-water emulsion, containing soybean oil, glycerol and egg lecithin (Ferreira et al. 2010; Kastner 2010). Propofol has a rapid onset and a short duration of action. Its hypnotic action is attributed to the increased inhibition of the synaptic transmission, by interaction with the GABAA receptor subunit, which potentiates the GABA-induced chloride current, by activation of the chloride channels (Hara et al. 1994). It has also been seen to inhibit the NMDA subtype of glutamate receptors (Orser et al. 1995; Yamakura et al. 1995).

1.7.1.1.Metabolism and elimination

Propofol bounds highly to plasma proteins (96-99%) (Schywalsky et al. 2005) with just a very small free fraction acting at the effect-site. Propofol is highly metabolized in the liver (Takizawa et al. 2005), however the clearance rates of this drug exceed hepatic blood flow (Gepts et al. 1987; Simons et al. 1988), and extra-hepatic clearance has also been suggested, as in the lungs, brain, small intestine and kidney (Raoof et al. 1996; King et al. 1999; Dawidowicz et al. 2000). An extrahepatic metabolism of propofol was demonstrated by Veroli and colleagues and by Gray and colleagues (Veroli et al. 1992; Gray et al. 1992). In the liver, the metabolism of propofol is performed through the cytochrome P450 system, originating sulphate and glucuronide conjugates, which are inactive and mainly excreted in the urine. However, the existence of kidney disease does not seem to change the propofol's clearance. The renal clearance of propofol metabolites was reported by Takizawa and colleagues, which also demonstrated that it seems to be dependent on renal blood flow rather than on renal function (Takizawa et al. 2005).

The uptake of propofol by the lungs has been reported by several authors (Matot et al. 1993; Matot et al. 1994; Dutta & Ebling 1998; Kuipers et al. 1999), but the role of the lungs in the metabolism of propofol is still not clear (He et al. 2000; Chen et al. 2006).

As well as the sequestration that occurs in the adipose tissue, propofol is also sequestrated in the lungs, resulting in a slow propofol return into the circulation, and being a justification for its long elimination half-life (Levitt & Schnider 2005). Also, the cardiac output has an important influence on the propofol clearance, possibly because of the influence of the cardiac output in circulatory dynamics (Upton et al. 2000), that conditions the hepatic and renal blood flow. The fact that propofol is metabolized and eliminated very quickly, with minimal accumulation after repeated doses, makes it a hypnotic of choice for using total intravenous anaesthesia (TIVA).

1.7.1.2. Haemodinamic effects

Propofol administration decreases the systemic blood pressure, by reducing the cardiac output and systemic vascular resistance. The hypotension caused by propofol is thought to be related with its inhibitory effects on sympathetic nerve activity and reflex responses to hypotension, with consequent vasodilation and significant decrease of the mean blood pressure (Ebert 2005). However, in addition to this, propofol may also cause direct vasodilation by interfering with calcium mobilization in the blood vessels, by blockade of voltage-gated influx of extracellular Ca^{2+} in the blood vessels (Chang & Davis 1993).

In hypovolaemic patients, the hypotension caused by this drug may be severe. Also, in bradycardic patients, refractory bradycardia or asystole may occur. This seems to be related with the prevalence of the parasympathetic nervous system in face of a sympathetic inhibition (Freysz et al. 1991). Severe bradycardia may be seen when high doses of opioids are combined with propofol. However, previous administration of a parasympatholytic drug may prevent severe bradycardia in these cases (Kastner 2010).
After repeated or continuous propofol administration, respiratory depression with hypercapnia and decreased arterial oxygen saturation may occur, with oxygen supplementation being recommended in repeated or prolonged propofol infusions (Blouin et al. 1993).

Propofol seems to cause depression of the function of the cardiac baroreflex (Sato et al. 2005). This fact, together with the inhibition of the sympathetic nervous system reflex responses, does seem to worsen the haemodynamic depression caused by the opioids administration in patients under propofol anaesthesia, by preventing or decreasing the reflex tachycardia and sympathetic responses to opioid administration,

that would otherwise occur (Ferreira et al. 2010).

Propofol proconvulsant and anticonvulsant properties have also been reported (Borgeat 1997). Its anticonvulsant properties may be related to its depressant action on the central nervous system, to a potentialization of GABA-mediated pre- and postsynaptic inhibition, and to its capacity of causing decreased release of excitatory transmitters, glutamate and aspartate (Borgeat 1997; Ohmori et al. 2004). Regarding its proconvulsant properties, these are thought to be related to intrinsic subcortical glycine antagonism (Dolin et al. 1992). In addition, propofol decreases the cerebral metabolic oxygen requirements, and the cerebral perfusion pressure, with a parallel decrease in the intra-ocular pressure (Kastner 2010)

1.7.1.3. Cerebral effects

The cerebral blood flow velocity and metabolic rate decrease with the administration of propofol, in a dose dependent way (Stephan et al. 1987; Van Hemelrijck et al. 1990; Ramani et al. 1992). Propofol is believed to preserve the cerebral blood flow/cerebral metabolism coupling (Ludbrook et al. 2002), however more significant decreased in cerebral blood flow than in cerebral metabolism have been observed following administration of this hypnotic drug(Ramani et al. 1992; Doyle & Matta 1999), which suggests a direct effect on the brain vasculature (Ludbrook et al. 2002). Findings of a decreased venous blood oxygenation in the jugular vein bulb in anaesthesia with propofol when compared to isofluorane based anaesthesia in patients with brain tumours support this hypothesis (Jansen et al. 1999). Furthermore, it has also been reported that changes in the cerebral blood flow have an influence in the intracarotid requirements of propofol to achieve electrocerebral silence in rabbits (Joshi et al. 2006).

In patients with normal intracranial pressure, propofol causes an approximate decrease of 30% of the intracranial pressure; this effect could be related to a decrease in the perfusion pression of the brain (Ravussin et al. 1988). Also, propofol caused a 30 to 50% decrease in the intracranial pressure in patients with pre-existing increased intracranial pressure (Hartung 1987; Herregods et al. 1988).

A maximum decrease of approximately 60% in the cerebral blood flow velocity related to high doses of propofol has been reported (Ludbrook et al. 2002); the decrease in intracranial pressure may be due to the propofol induced decrease in cerebral blood

flow velocity and in cerebral perfusion pressure. After propofol administration the metabolic rate of the brain suffers decreased approximately 36% (Stephan et al. 1987), apparently with no significant changes in the arteriovenous cerebral oxygen content (Ludbrook et al. 2002), which suggests that the cerebral metabolic autoregulation is preserved during propofol administration [252] probably due to the CBF/cerebral metabolism coupling (Ludbrook et al. 2002).

In propofol-based anaesthesia, there is a dose-dependent decrease in the BIS. The administration of low doses of propofol increases the amplitude and the EEG alpha waves rhythm, followed by a shift to EEG gamma and theta frequency and when higher doses are given, there is burst suppression and a decrease of the amplitude of the EEG (Yate et al. 1986; Hazeaux et al. 1987; Billard et al. 1997; Glass et al. 1997; Liu et al. 1997; Struys et al. 1998; Bousoula et al. 2003; Bruhn et al. 2003; Ellerkmann et al. 2006).

1.7.2. Opioids - Remifentanil

Opioids are potent analgesic drugs that act by directly or indirectly facilitating or inhibiting presynaptic and synaptic transmission of neurons in various regions of the peripheral and central nervous system (CNS). The opioid receptors are proteins coupled to the superfamily of G-proteins in the cellular membranes (Pleuvry 1991). There are three major classes of opioid receptors, which are *mu (μ)*, *delta* and *kappa*. When binding of the opioid to the opioid receptor occurs, on the membrane G-proteins, it will generate a short-term or a longer term of action, (depending on the G-protein effector system associated to the specific opioid receptor). The short-term opioid action tends to be associated to K^+ and Ca^{2+} channels; the long term of action involves other messengers such as the cyclic adenosine monophosphate and the phosphatidylinositol. Opioids are classified as agonists, partial agonists, mixed agonists/antagonists and antagonists. Agonist opioids have high affinity and intrinsic activity for *mu* receptors and examples include morphine, pethidine, hydromorphone, methadone, fentanyl, sulfentanil, alfentanil, remifentanil and codeine. Partial agonists are named this way due to their only partial agonist effect, and include buprenorphine. Mixed agonists/antagonists are agonists at some receptors and antagonists at others and can reverse the effect of pure *mu* agonists, and therefore should not be administered with these unless the main objective is to reverse the *mu* agonists' effects; butorphanol is an

example of this class of opioids. Antagonists can reverse the effect of *mu (μ)* and *kappa* agonists due to their high affinity and low intrinsic activity (Kerr 2010).

The μ-opioid receptors act on the K^+ and Ca^{2+} channels and increase intracellular concentrations of Ca^{2+} by increasing the cellular Ca^{2+} uptake. These receptors can also restore the intracellular K^+ concentration, through the activation of the K^+ channels. Furthermore, as all opioid receptors, they act over the Ca^{2+} voltage-dependent channels. The changes in intracellular K^+ and Ca^{2+} will result in a decrease in the synaptic transmission and neurotransmitter release, which is very likely an imperative mechanism of the opioid-induced analgesia (Wandless et al. 1996).

Remifentanil is a potent μ-opioid receptor pure agonist with very specific characteristics, which has a rapid clearance rate, being easily titrated and suitable for intra and postoperative analgesia. Furthermore, the rapid onset of action and elimination makes it an excellent opioid to be used for total intravenous anaesthesia, due to its safe performance (Glass et al. 1999; F. S. Servin & Billard 2008). Remifentanil is a 3-(4-methoxycarbonyl-4-[(L-oxopropyl)-phenylamino]-L-piperidine propanoic acid, methyl ester and is supplied a white lyophilized powder - remifentanil hydrochloride. The incorporation of a propanoic methyl ester on the piperidine nitrogen was made in the 4-anilidopiperidine-like structure of remifentanil, making it able of being degraded by blood and tissue esterases (E. Servin & Billard 2008).

1.7.2.1. Metabolism, Elimination and Pharmacokinetics

Remifentanil is eliminated by blood esterases after intravenous administration, by de-esterification of the propanoic methyl ester on the piperidine nitrogen, thus having a metabolism which is independent of hepatic transformation and renal excretion, in contrast to what occurs with other anilidopiperidine opioids, as fentanyl, alfentanil and sufentanil (Beers & Camporesi 2004). For this reason, the clearance of remifentanil is substantially higher when compared with other anilidopiperidine opioids. A major metabolic acid (GI90291) and a minor remifentanil metabolite (GI94219) are originated from the esterase activity (Westmoreland et al. 1993). Although remifentanil seems to be greatly excreted in the urine in the form of the major acid metabolite it was suggested that the remifentanil and the GI90291 pharmacokinetics and pharmacodynamics are not influenced by impairment of the renal function (Hoke et al. 1997; Pitsiu et al. 2004). The total clearance of remifentanil is approximately 41 ml/min/kg, being independent of

the dose used, and is three to four times greater than the normal hepatic blood flow (Westmoreland et al. 1993) with the liver having no active role in the pharmacokinetics of remifentanil (Dershwitz et al. 1996). The pharmacokinetics of remifentanil are not significantly different in obese when compared with lean patients, being more related to lean body mass and not to total body weight (Minto, Schnider & Shafer 1997; Minto, Schnider, Egan, et al. 1997; Egan et al. 1998). The pharmacokinetics of remifentanil are affected by the patients' age (Westmoreland et al. 1993; Minto, Schnider & Shafer 1997; Minto, Schnider, Egan, et al. 1997).

1.7.2.2. Systemic and Cardiopulmonary Effects

Remifentanil decreases mean arterial pressure and heart rate in a dose dependent way (Kazmaier et al. 2000; Joshi et al. 2002). Remifentanil also causes direct vasodilatation, which may be due to the decrease in arterial elastance induced by this drug (Pittarello et al. 2004) and to the endothelium mediated vasorelaxation (Unlügenç et al. 2003), although this last factor seems to differ between species, in vitro (Duman et al. 2004). The decrease in arterial elastance and the activation of the sympathetic adrenergic nervous system tone in awake patients (Guarracino et al. 2004) may justify the fact that remifentanil decreases diastolic blood pressure, but with no significant changes in the systolic blood pressure and heart rate (Jhaveri et al. 1997). Remifentanil also decreases the ventricular elastance in a dose dependent way (Pittarello et al. 2004), leading to a decrease in the myocardial contractility (Guarracino et al. 2004). A 25% decrease in cardiac index accompanied by a concomitant decrease in stroke volume index by 14% and in heart rate by 13%, is described with administration of high doses of remifentanil (Kazmaier et al. 2000). A dose-dependent decrease in heart rate of approximately 35% associated to the remifentanil administration during volatile anaesthesia was also found in animals (Michelsen 1996). This opioid also induces bradycardia, by vagal activity (Reitan et al. 1978). At a pulmonary level, high remifentanil doses have no influence in the pulmonary mean arterial pressure, vascular resistance and capillary wedge pressure (Kazmaier et al. 2000).

1.8. Evaluation of intestinal histological lesions caused by hypoperfusion/ischaemia-reperfusion.

1.8.1. Light microscopy - Haematoxylin and eosin staining

Low-flow states can cause several morphological changes in the intestinal wall, which are possible to evaluate using light microscopy. Chiu and colleagues (Chiu et al. 1970), proposed a grading system to more objectively characterize the histological intestinal lesions (Table 2).

Table 2. Chiu and colleagues grading system for intestinal mucosa lesion in low-flow states (Chiu et al. 1970).

Grade	Morphological lesions
0	Normal mucosal villi
1	Development of subepithelial space at the apex of the villi
2	Extension of subepithelial space with moderate lifting of the epithelial layer from the lamina propria
3	Massive epithelial lifting, down the sides of the villi with loss of epithelial surface of few villi
4	Denuded villi with exposure of the lamina propria and dilated capillaries
5	Disintegration of the lamina propria with haemorrhage and ulceration

Later, in the eighties, Parks and colleagues (Parks et al. 1982; Parks & Granger 1986) in a study on ischaemia and reperfusion lesions in the intestine, evaluated, apart from the morphological changes previously reported by Chiu and colleagues, also the presence inflammation and necrosis on both villi and crypts, and assessed villus height, crypt depth and mucosa thickness. The measurement of the villus height, crypt depth and mucosa thickness was performed in order to objectively quantify the epithelium and mucosal loss (Table 4).

In the early nineties, Sonnino and colleagues (Sonnino et al. 1992) developed a grading system to evaluate the intestinal lesions of ischaemia and ischaemia-reperfusion (IR) seen in rat small bowel transplants. This grading system evaluated separately changes in the villi, epithelium, connective tissue, lacteals, crypts, lamina propria, muscularis mucosa and the presence or absence of bacteria (Table 3).

In 2000, Quaedackers and colleagues (Quaedackers et al. 2000) performed a study

to examine the validity, reliability and ease to use of the published grading systems for morphological changes in the intestine due to ischaemia/IR. In this study the three grading systems mentioned previously were included, and Chiu and colleagues grading system was evaluated as a combination with Parks and colleagues system (Table 3). Their results suggested that the combined grading system of Chiu and colleagues and Parks and colleagues was the most suitable as a standard scoring system (Quaedackers et al. 2000).

Table 3. Description of the main scoring systems for the intestinal morphological changes in ischaemia and ischaemia-reperfusion (Chiu et al. 1970; Parks et al. 1982; Granger, Höllwarth, et al. 1986; Sonnino et al. 1992). (MMN – monomorphonuclear; PMN – polymorphonuclear).(J. Quaedackers et al. 2000).

Parks and colleagues	**Sonnino and colleagues**	**Parks et al., and Chiu et al.**
Villus epithelial cell inflammation and necrosis	0 for normal, 1 for abnormal findings:	Chiu
0. No damage	**Villi**	0. Normal mucosa
1. Occasional tips affected	Present/absent	1. Subepithelial space at villus tips
2. Majority of tips affected	Normal/abnormal: long, short, narrow, wide	2. Extension of subepithelial space with moderate lifting
3. Majority of tips and some villi affected	**Epithelium**	3. Massive lifting down sides of villi, some denuded tips
4. Tips, mid- and lower portions of majority of villi affected	Normal/abnormal cell loss, complete denudation, edema	4. Denuded villi, dilated capillaries
	Connective tissue Normal/abnormal	5. Disintegration of lamina propria
Crypt epithelial cell inflammation and necrosis:	edema, intra- or extravascular infiltrate	Parks
0. No damage	**Lacteals**	6. Crypt layer injury
1. Occasional crypts affected	Present/absent	7. Transmucosal infarction
2. Scattered crypts affected	Normal/abnormal dilated, occluded	8. Transmural infarction
3. Many crypts affected	**Crypts**	
4. Majority of crypts affected	Present/absent	
	Normal/abnormal degeneration disorganization Regeneration: present/absent	
	Lamina propria	
	Present/absent	
	Normal/abnormal edema, MMN or PMN infiltrate, intravascular stasis, hemorrhage	
	Muscularis mucosae Present/absent	
	Normal/abnormal intravascular stasis, hemorrhage	
	Bacteria	
	Present/absent	

In this study, the grading system developed by Chiu and colleagues was used; the combined system with the additional criteria from Parks and colleagues' grading system was elected as severe lesions, such as crypt layer injury, transmucosal infarction and transmural infarction were not expected, considering the level and duration of the overall hypoperfusion achieved.

1.8.2. Methods for detection of apoptosis - Basics and fundaments of the methods

Several different methods to detect cell apoptosis have been developed along the years, together with the increasing in knowledge on apoptosis. Different types of methods can be used, based on 1) morphology, 2) immunohistochemistry, 3) biochemistry, 4) immunology and 5) array - based techniques.

Morphology based methods can be performed using just light microscopy with standard haematoxylin and eosin staining, looking for the morphological features of apoptosis - cellular shrinkage, chromatin condensation and margination, pyknosis and nuclear fragmentation. Giemsa staining can be also used. Fluorescence microscopy can also be performed (Zhang et al. 1997; Vashishtha et al. 1998), using substances like Hoechst dye 33342, DAPI or Propidium Iodide, that will help differentiating live from death cells and also allow a better visualization of the nuclear morphology, for specific features of apoptosis (Zhang et al. 1997; Ulukaya et al. 2011). Among morphology-based methods, electron microscopy is considered an excellent technique to detect apoptotic cells, due to the possibility of observing in detail all the apoptotic features, allowing also the evaluation of subcellular details as mitochondria, integrity of the plasma cellular membrane, etc. (Wyllie et al. 1980; Yasuhara et al. 2003). Still among morphology-based methods, phase contrast microscopy can also be used (Ulukaya et al. 2011; Huh et al. 2012).

Regarding immunohistochemistry based methods, Annexin V-FITC Assay, TUNEL method, M30 antigen detection and detection of active caspase-3 are the most commonly used (Duan et al. 2003; Ulukaya et al. 2011).

The most commonly used biochemistry-based methods are a) Agarose Gel Electrophoresis for detection of DNA fragmentation (Matassov et al. 2004), b) Western Blotting, which can be used with different proposes, as to determine the expression of proteins specific for apoptosis; it is frequently used to detect cytochrome c release from

the mitochondria to the cytoplasm, which is considered a hallmark of apoptosis (Li et al. 1999), c) Flow Cytometry (Schmid et al. 1994; Vermes et al. 1995), which is often used to determine any protein whose expression change in apoptosis, as is also the case of cytochrome c (Campos et al. 2006; King et al. 2007).

ELISA and Fluorometric method are the most frequently used immunology-based methods. ELISA can be used to detect DNA fragmentation, and also the levels of cleaved cytokeratin 18, and the Fluorometric method is used to evaluate caspases' activity in cultured cells (Davis 2002; Gonzalez-Quintela et al. 2009; Lavallard et al. 2011).

The methods for detection of apoptosis used in the present study were the detection of cytosolic cytochrome c, M30 antigen detection and also the TUNEL method that will be described in detail in the following topics.

1.8.2.1. Cytochrome C oxidase

Cytochrome c is an electron carrier that in normal conditions is generally located in the intermembrane space of mitochondria. Cytochrome c plays and important role in the electron transport chain, being, when reduced, the substrate of the last chain reaction, which is the reduction of oxygen by cytochrome c oxidase (van Beek-Harmsen & van der Laarse 2005; Ow et al. 2008). Cytochrome c can be released from the mitochondria into the cytosol during ischaemia, and it plays an important role in disease and in reperfusion injury.

In the initial pre-apoptotic process, there are changes in the mitochondrial membrane, which lead to the release of cytochrome c, among other important molecules, such as apoptosis-inducing factor (AIF) and endonuclease G (Yang 1997; Antonsson 2000; Ow et al. 2008). The cytochrome c released to the cytosol will activate the caspase-dependent apoptotic pathway. Its capacity to induce the apoptotic cascade is related with its interaction with Apaf-1 (Zou et al. 1997), which will enable Apaf-1 to recruit caspase-9 and stimulate its activation. After caspase-9 is in its active form, the apoptotic cascade starts (Slee et al. 1999).

Being an important step in the induction of apoptosis, the release of cytochrome-c from the mitochondria to the cytosol is assessed to characterize mitochondrial dependent apoptosis. Cytochrome c can be released from the mitochondria into the cytosol during ischaemia and it plays an important role in disease and in reperfusion

injury. The release of cytochrome c from mitochondria can be investigated in different ways: in tissue homogenates using Western blotting, ELISA, or HPLC, on fixed cultured cells or paraffin embedded tissues via immunocytochemistry (Liu et al. 2012), and in live cells by using green fluorescent protein-labelled cytochrome c (Lim et al. 2002; Waterhouse & Trapani 2003). Flow cytometry may also be used (Campos et al. 2006; King et al. 2007).

As previously mentioned, the detection of different subunits of cytochrome c is possible by immunohistochemistry, in paraffin embedded tissues. Although the interpretation may be more difficult as results are not an absolute number, which would allow the quantification of cytochrome c in the cytosol, the location of cytochrome c is given by a different pattern of staining, which allows determining if there was release of this molecule into the cytosol or if it remains within the mitochondrial membrane. In normal non stressed mitochondria, the stain is expected at the level of the mitochondrion inner membrane, originating a punctate pattern, and, a diffuse pattern within the cytosol is expected after cytochrome c release into the cytosol (Liu et al. 2012).

1.8.2.2. M30 antibody

In epithelial cells, during caspase-dependent apoptosis, the cytokeratin 18 (CK18) is cleaved, leading to the formation of a neo-epitope (Caulín et al. 1997), CK18-Asp396, which is specific and is recognized by the M30 antibody (Leers et al. 1999). CK18 is an intermediate filament protein that is found in most simple epithelia in mammals, including the gastrointestinal epithelia (Bosch et al. 1988; Caulín et al. 1997). The antibody M30 detects a 10-residue epitope of CK18, in aminoacid positions 387-396 located at the liberated C terminus, after cleavage at the site DALD-S (Leers et al. 1999).

After the release of cytochrome c into the cytosol, there is activation of caspase-9 in epithelial cells, which has its subunits (p35 and p37) on cytokeratin fibrils, procaspase-9 and caspase-3. The activated caspase-3 also cleaves cytokeratin 18 (Dinsdale et al. 2004). Procaspase 9 is cleaved and activated. Cleaved caspase-9 will cleave and activate procaspase 3, originating cleaved-caspase-3, and procaspase 7, originating cleaved-caspase-7 (Slee et al. 1999). After, procaspase-6 is also cleaved by cleaved-caspase-3, and cleaved-caspase 3, cleaved-caspase 6 and cleaved-caspase 7 will

cleave CK18, forming cleaved-cytokeratin-18 (cCK18) (Caulín et al. 1997). There are also further apoptotic ways with cleavage of CK18.

The M30 antibody can be used to detect cCK18 in both frozen and paraffin-embedded sections (Grassi et al. 2004). In one study on apoptosis in the colonic epithelium, M30 identified apoptotic cells at a higher frequency when compared with an antibody for cleaved-caspase-3 and was almost as useful as cellular morphology in detecting apoptotic cells in healthy/non-neoplastic colon (Holubec et al. 2005). In addition, it is specific for epithelial cells that contain CK18.

1.8.2.3.TUNEL

The detection of intracellular DNA fragments in the early 90's was seen as a reliable method for investigation of apoptosis. This is achieved using the Terminal deoxynucleotidyl transferase (TdT) – mediated dUTP-digoxigenin nick end labelling (TUNEL) assay (Gavrieli et al. 1992). This method relies on the binding TdT to the DNA, at the 3' – OH ends, with the synthesis of a polydeoxynucleotide polymer. TdT is used to incorporate biotinylated deoxyuridine at the sites with DNA breaks, after the exposure of nuclear DNA on the tissue sections, which is achieved by proteolytic treatment. Amplification of the signal is made using avidin-peroxidase, which enables identification by light microscopy (Gavrieli et al. 1992).

Several points have been raised about the applicability of the TUNEL assay in the study of apoptosis: frequent false positive reactions, with limited reproducibility (Yasuda et al. 1995; Labat-Moleur et al. 1998; Huppertz et al. 1999); presence of a positive reaction also in necrotic cells (Grasl-Kraupp et al. 1995; Charriaut-Marlangue et al. 1996); absence of reaction in cells within the apoptosis cascade, as endonuclease activation occurs late in the cascade (Huppertz et al. 1999) and the fact that endonuclease activation may be bypassed during apoptosis, with absence of positive TUNEL reaction (Robertson et al. 2000).

Although it does detect the end stage of apoptosis, after endonuclease activation and consequent DNA fragmentation, TUNEL assay is also able to show a positive reaction in necrotic cells (Grasl-Kraupp et al. 1995; Charriaut-Marlangue et al. 1996), and in cells that suffer autolysis (Grasl-Kraupp et al. 1995).

As DNA fragmentation may occur in different types of cell death, TUNEL has to be carefully interpreted when used for identification of apoptotic cells. For this reason,

combining the TUNEL method with other earlier apoptotic markers, as markers for cleaved-caspase-3 and for cleaved-cytokeratin 18, will allow more unequivocal results (Leers et al. 1999; Duan et al. 2003).

Aims and hypothesis of the thesis

We hypothesize that:

1- HES 130/0.4 is associated with less severe mucosal injury in the small intestine, when used for volume replacement after severe controlled haemorrhage under total intravenous anaesthesia with propofol and remifentanil in pigs, when compared with Ringer's Lactate solution in the same conditions;

2- Hypoperfusion without achieving a hypovolaemic shock status, followed by reperfusion would cause quantifiable changes in the small intestine mucosa.

A hypoperfusion model, instead of a ischaemia-reperfusion model was used, to more realistically resemble clinical cases of severe blood loss that can occur under surgical procedures, both in human and veterinary medicine.

The objectives of this study are:

1- to investigate the morphological effect on the small intestine mucosa in a pig model of the intravenous administration of HES 130/0.4 and Ringer Lactate, during a short term initial resuscitation phase after acute controlled haemorrhage under total intravenous anaesthesia with propofol and remifentanil, followed by a short maintenance phase with Ringer lactate.

2- to investigate the presence of early apoptotic indicators in the cells of intestinal mucosa, that would provide us information about the possible occurrence of post anaesthetic intestinal necrosis.

Additionally, during the study, it will also be analysed:

1- the haemodynamic variables in pigs during total intravenous anaesthesia with propofol and remifentanil during acute bleeding and volume reposition with HES 130/0.4 and Ringer Lactate;

2- the electroencephalographic and cerebral blood oxygen saturation variables in pigs in the same conditions referred in the previous point.

CHAPTER 2

2.1. Experimental Methodology

2.1.1. Animals and anaesthetic protocols

The study enrolled 28 healthy, three months old, commercial Large White pigs from a certified pig farm (Unidade de Suinicultura da Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal). These animals were randomly selected thirty days previously to the beginning of the study, housed separately and provided *ad libitum* access to food and water and subjected to gradual interaction with humans for familiarization and reduction of stress; the pigs were also dewormed at this stage. The day before being submitted to general anaesthesia, the animals were fasted overnight but were allowed free access to water.

Ethical approval for this study (Direcção Geral de Veterinária, Portugal; approval number DGV000228) was provided by the Ethical Committee of the national regulatory office Direcção Geral de Veterinária from Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Lisbon, Portugal on the 6th of June 2011. All procedures were carried out under personal and project licenses, approved by the Direcção Geral de Veterinária.

The pigs, with three months of age, were submitted to acute haemorrhage under total intravenous anaesthesia (TIVA) with propofol (Propofol 1% Fresenius Kabi, Bad Homburg, Germany) and remifentanil (Ultiva®, GSK, Midlessex, UK), followed by volume replacement with Ringer's solution (Lactato Ringer Braun®, B. Braun Medical SA, Barcelona, Spain) (group 1 (n=11)), or HES 130/0.4 solution (Voluven®, Fresenius Kabi, Bad Homburg, Germany) (group 2 (n=11)). Pigs in group 3 (n=6), the control group, were submitted to the same instrumentation and anaesthetic procedures as those in Groups 1 and 2, except for bleeding, and did not receive any fluids, besides the Ringer Lactate for maintenance purposes described latter in this this section. Pigs were randomly allocated to three groups, according to the solution used for volume replacement.

All pigs were premedicated with 4mg/kg azaperone (Stresnil®, Janssen Animal Health, Belgium), IM, 30 min prior to induction of anaesthesia. After premedication, a

22G catheter was inserted in the right auricular vein for drug and fluid administration. A three-way stopcock was used to connect the intravenous catheter to the maintenance Ringer's delivery line, and to the lines delivering 1% propofol, and 20 μg/ml remifentanil. An infusion pump (Braun, Melsungen, Germany) was used for the administration of Ringer Lactate, at a constant infusion rate of 6ml/kg/h + 1ml/kg/h for each kg above 20 kg of weight (Johnson et al. 2004), during the entire study period. Propofol and remifentanil were delivered using two perfusion pumps (Asena GH, Alaris Medical Systems, San Diego, CA), controlled by the RugLoop II Waves software (developed by Tom De Smet, Demed Engineering, Temse, Belgium, and Michel Struys, Ghent University, Gent, Belgium), running on a personal computer.

Anaesthesia was induced with a propofol bolus of 4 mg/kg, with pigs breathing 100% oxygen, via a face mask. This was followed by tracheal intubation with a 6.5 mm endotracheal tube (Mallinckrodt, Dublin, Ireland). The pigs were mechanically ventilated with air + O_2 using a Datex Carestation (Datex-Ohmeda S/5, Helsinki, Finland) ventilator, with a tidal volume of 10 ml/kg, respiratory rate of 12 to 14 and with an expiration ratio of 1:3, with adjustments according to the observed $EtCO_2$, in order to obtain a $PaCO_2$ of 40±4mmHg.

After induction of anaesthesia, a propofol constant infusion was started at a rate of 15mg/kg and remained unaltered during the entire study period. Simultaneously, a remifentanil constant infusion was initiated at a rate of 0.3μg/kg/min. After ending all the monitoring procedures, the remifentanil infusion rate was decreased to 0.2μg/kg/min and was maintained unaltered during the study period. Peripheral oxygen saturation (SPO_2) (Datex-Ohmeda, Helsinki, Finland) was recorded by placing the probe on the pig's tongue, and heart rate was monitored using three ECG electrodes, placed according to the Academy of Veterinary Cardiology Committee.

2.1.2. Haemodynamic Monitoring

After reaching stable anaesthesia, an arterial catheter (Leadercath, Vygon Corporation, PA) was introduced in the left femoral artery for continuous monitoring of the arterial blood pressure; a 16 gauge catheter (Abbot Animal Health, IL) was introduced in the right femoral artery for passive bleeding; a surgical approach to the ventral cervical region was used in all pigs for introducing two 7F Swan-Ganz optic catheters (Edwards, Life Sciences, Irvine, CA): one in the internal jugular vein with its

optic tip in the *sinus petrosus ventralis*, for collecting data from venous blood oxygen saturation (SvjO2), and the other in the pulmonary artery, via left external jugular vein, for collecting data from central venous pressure, pulmonary artery pressure and cardiac output by the thermodilution method (at specific points during the study), and pulmonary pressure.

A multiparametric haemodynamic monitor (Datex-Ohmeda S/5, Helsinki, Finland) was used to collect all haemodynamic (systolic, diastolic and mean arterial blood pressure, central venous pressure, pulmonary artery pressure, cardiac output) and ventilatory data. All data were recorded every 5 seconds and stored in a personal computer running the RugLoop II Waves software, via a RS-232 interface.

2.1.3. Brain monitoring

The hair overlying the fronto-occipital region was clipped, and the skin grasped with fine sandpaper and cleaned with acetone, and a BIS adhesive electrode (Zipprep, Aspect Medical Systems, Natick, MA) was placed in the left side of the head as follows: number 1 electrode was placed over the external occipital protuberance; number 2 and number 4 electrodes were placed over the left hemisphere; number 3 electrode was placed over the rostral left portion of the frontal bone, at the level of the left eye. The EEG was recorded at 256 Hz using a BIS XP monitor (Aspect Medical Systems, Natick, MA). The same monitor also recorded the bispectral index (BIS), electromyographic activity (EMG), signal quality index, spectral edge frequency 95% (SEF) suppression ratio (SR), and total power (TP). Data from the BIS monitor were recorded every second in the personal computer running the RugLoop II Waves software.

To continuously monitor changes in peripheral regional oxygen saturation (rSO2), the near-infrared spectroscopy INVOS monitor 4100 with the software version 11.16.16 (Somanetics Corporation, Troy, MI) was used, with monitoring being performed using its non-invasive electrodes (SomaSensors, Somanetics Corporation, Troy, MI), which were placed in the left side of the head. Data from cerebral oxygen saturation given by the INVOS was recorded continuously using ASYS software (Bressan 2011) running in a second personal computer, with the clock synchronized with that in the computer running the RugLoop II Waves software.

An Oxymetrix 3 monitor (Abbott Laboratories, North Chicago, LL, USA) with a 7F Swan-Ganz optic catheter (Edwards, Life Sciences, Irvine, CA), with its optic tip

placed in the *sinus petrosus ventralis*, was used for collecting data from venous blood oxygen saturation (SvjO2), for monitoring the overall brain oxygenation.

2.1.4. Haemorrhage and volume replacement

After completing all the necessary instrumentation procedures, pigs in Groups 1 (n=11) and 2 (n=11) were submitted to an acute severe haemorrhage, by passively removing 30 ml/kg of blood from each pig, via the right femoral artery, over 20 min. Twenty minutes after the bleeding ceased, the volume replacement was started at 999 ml/h, using Ringer's solution (Lactato Ringer Braun, B. Braun Medical SA, Barcelona, Spain) in Group 1, and HES 130/0.4 solution (Voluven, Fresenius Kabi, Bad Homburg, Germany) in Group 2. The volume of HES 130/0.4 administered was 20 ml/kg (Madjdpour et al. 2005) and the volume of Ringer's solution administered for blood replacement was 25 mg/kg. The 25mg/kg Ringer's solution dose used was the minimum dose necessary to obtain similar haemodynamic response to HES 130/0.4 administration during volume replacement after bleeding and was obtained during pre-trial testing in pigs. These volumes were administered via the catheter placed in the cranial vena cava. After the end of the volume replacement, pigs in Groups 1 and 2 were maintained under general anaesthesia for one extra hour. Pigs in Group 3 (n=6) were submitted to the same instrumentation and anaesthetic procedures as those in Groups 1 and 2, except for bleeding and volume replacement; pigs in Group 3 just received Ringer´s solution for maintenance purposes, at the same rate as the pigs in groups 1 and 2, and were anaesthetised for similar time as pigs in the other groups (Figure 5).

Arterial blood samples were collected from the left femoral artery for blood gas analyses, before bleeding was started, at the end of the waiting period (20 minutes after the end of the bleeding), at the end of the volume replacement period (time needed to infuse the total amount of replacement fluids) and at the end of the study, in Groups 1 and 2. In Group 3, the gas analyses were performed before bleeding and at the end of the study. Blood gas analyses were performed using a Gem Premier 3000 analyser (Instrumentation Laboratory, Massachusetts, USA).

Euthanasia was performed by intravenous administration of potassium chloride 4 mEq/ml, in the cranial vena cava, one hour after the end of volume replacement (in Groups 1 and 2) or after similar anaesthetic time (in Group 3). Euthanasia was

immediately followed by necropsy and collection of samples from the small intestine, among samples from other organs for different studies.

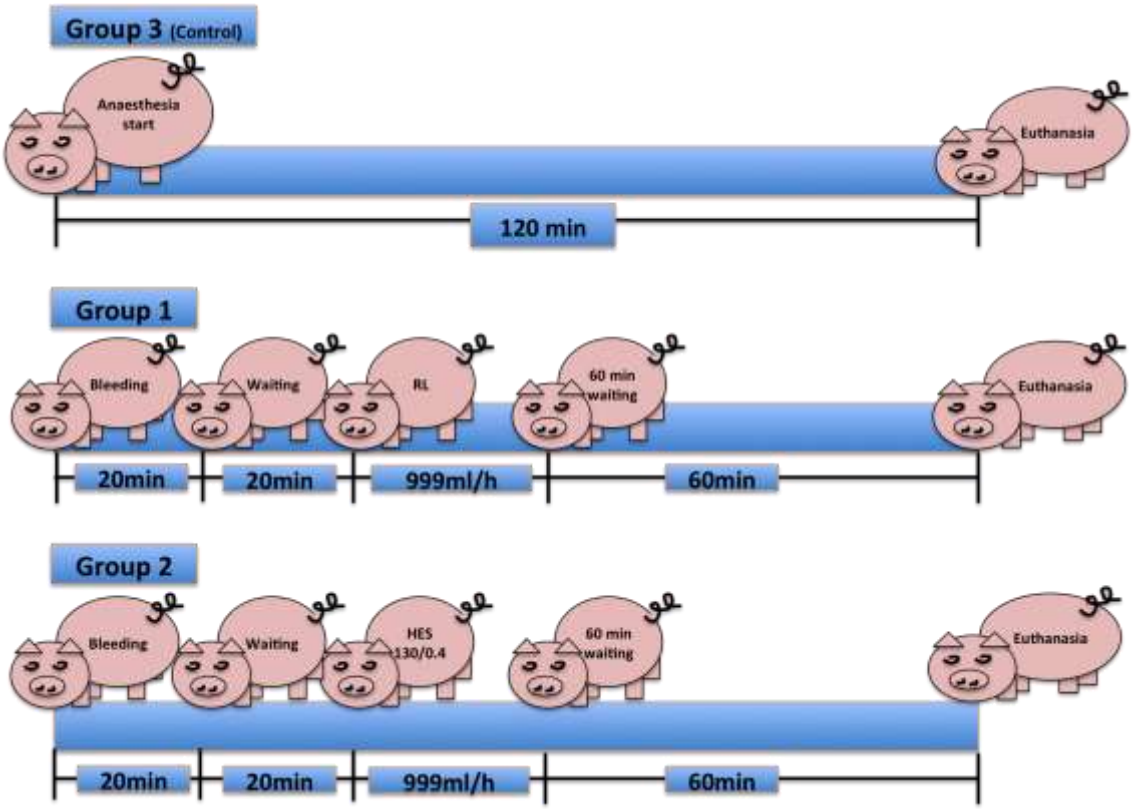

Figure 5. Main set up procedures performed in each group.

2.1.5. Necropsy and samples fixation

After opening the abdominal cavity, samples were collected from the descending portion of the duodenum, middle jejunum, and ileum, three centimetres proximal to the ileo-caecal opening. The mesentery was removed from the samples and each segment was opened along its length, gently rinsed in a solution of 0.9% sodium chloride and immediately fixed in 10% neutral-buffered formalin, for a maximum of 24 h.

2.2. Laboratorial Methodology - Histopathology

2.2.1. Haematoxylin & Eosin

The intestinal samples collected at necropsy were dehydrated through graded ethanol series (2 baths of 96% alcohol followed by 2 alcohol baths of 100% for periods of 5 minutes each), were collected on glass slides, submitted to deparaffinization, by

placing the slides in xylene twice for five minutes, rehydrated through graded ethanol series and stained for routine histopathological diagnosis with haematoxylin and eosin (HE). Additional three micrometres sections were mounted on charged glass slides for immunohistochemistry (IHC) staining. All samples were examined by light or fluorescence microscopy, using a Microscope Zeiss Mod. Axioplan 2, in a double-blinded fashion by the pathologists.

2.2.1.1. Qualitative and semiquantitative analysis

The qualitative and semiquantitative parameters were evaluated in all the pigs used in the study (group 1, n=11; group 2, n=11; group 3, n=6). The semiquantitative parameters passive congestion (blood accumulation in dilated capillaries and venules), active congestion or hyperaemia (dilatation of arterioles as a result of the increased blood influx into the tissues), haemorrhage (numbers of extravasated erythrocytes), cellular degeneration and necrosis, were evaluated and classified from 0 to 3, as absent, mild, moderate and severe, respectively, according to Çetin and colleagues (Çetin et al. 1995).

Regarding the inflammatory infiltrate, a general grading of the infiltrate considering all inflammatory cells present, was attributed, as being absent, mild, moderate or severe. In addition, the numbers of neutrophils, macrophages, lymphocytes, plasma cells and eosinophils present within the lamina propria, were evaluated separately as well, as being small numbers, moderate numbers or large numbers, in order to better characterize the type of infiltrate and recognise the predominant cell type(s). The numbers observed in the control group were considered the normal expected number for the other two groups and differences were assumed by comparison to the controls.

Careful examination of the sections was performed in order to correctly distinguish between passive congestion and hyperaemia (active congestion), due to the different and important meanings of these two processes in this specific clinical situation. As mentioned previously, the presence of passive congestion was registered when erythrocytes were significantly accumulated within the lumen of capillaries and venules and hyperaemia was registered when erythrocytes were accumulated within the lumen of arterioles.

For the grading of oedema, the following specific alterations were considered to characterize each grade, from grade 0 to grade 3 according to Ortiz and colleagues (Ortiz et al. 2013):

- Grade 0, normal – normal central lacteal (central lacteal up to 10% of the width of villous longitudinal section);
- Grade 1, mild – lacteal dilatation (central lacteal up to 30% of width of the villous longitudinal section);
- Grade 2, moderate - lacteal dilatation (central lacteal up to approximately 50% of the width of villous longitudinal section) and/or oedema of lamina propria;
- Grade 3, marked – lacteal dilatation (central lacteal up to 70% of the width of villous longitudinal section), oedema of lamina propria and/or presence of submucosal oedema.

The epithelial detachment was evaluated and registered on a specific scale from 0 to 5, according to Chiu and colleagues (Chiu et al. 1970):

- Grade 0 – Normal mucosa;
- Grade 1 - Development of subepithelial space at the apex of the villi;
- Grade 2 – Extension of subepithelial space with moderate separation of mucosa;
- Grade 3 - Separation with extensive loss of epithelial surface of some villi;
- Grade 4 - Loss of the surface of the villi with exposure of dilated capillaries;
- Grade 5 - Disintegration of lamina propria with haemorrhagic ulceration.

2.2.1.2. Quantitative analysis

As for the qualitative and semiquantitative parameters, the quantitative analysis was also performed in all the pigs used in the study (group 1, n=11; group 2, n=11; group 3, n=6). The mucosal loss percentage (%ML) and crypt:interstitium (C:I) ratio were assessed by randomly selecting one crypt and one villus from one, also randomly selected, microscopic field, in a total of 10 fields for each intestinal section, using a 10x objective. An ocular 1000 micrometric reticule division (Pyser – SGi Limited, UK) was placed over each selected villus or crypt and an average of the measurements was calculated for each intestinal section.

2.2.1.2.1. Percentage of mucosal loss

For obtaining the %ML, the height of 10 randomly selected intact villi (distance in µm between the muscularis mucosa and the lumen, at the level of the tip of the villi) and 10 randomly selected villi with morphologically visible mucosal loss (distance in µm between the muscularis mucosa and the position where mucosa was lost) was measured, per intestinal segment and per animal. The average height for the intact villi and for the villi with mucosal injury was calculated, and the average height of intact villi was then subtracted from the average height of the damaged villi. This value was divided by the average height of the intact mucosa and multiplied by 100, to obtain the %ML (Faleiros et al. 2001).

2.2.1.2.2. Crypt:Interstitium ratio

The crypt:interstitium (C:I) ratio was assessed, in order to better characterise any possible change within the interstitium that would change its width, namely oedema, inflammation and haemorrhage. This method quantifies the relationship between the average crypt depth, measured in microns, next to the muscularis mucosa, at the crypt base, and the average width of the interstitium of the lamina propria, also measured in microns, obtained at the central portion of the chorion of the villi, and provides us information about a possible expansion of the lamina propria. This method was adapted from Faleiros and colleagues (Faleiros et al. 2001) in their study on ischemia and reperfusion in equine small colon, and had also been adopted by our group for use in pigs (Oliveira et al. 2010) in the absence of specific studies in the pig and once none species-specific incompatibility was to be expected. All measurements were obtained using an ocular with 25-point grid (KPL 25points 10x; Zeiss).

2.2.2. Immunohistochemistry – Apoptosis study

2.2.2.1. Cytochrome c Antibody

The detection of cytosolic cytochrome c was assessed by immunohistochemistry in six pigs from each group, in the duodenum, jejunum and ileum. This technique was performed using the Avidin-Biotin indirect method, via the streptavidin-biotin complex (ImmunoCruz TM goat LSAB Staining System: sc-2053). It was performed using a primary antibody, polyclonal goat Cytochrome c (C20): sc-8385 (Santa Cruz Biotechnology, Inc.) applied against the desired antigen. It was followed by the introduction of a biotinylated secondary antibody, directed against the primary antibody

and, subsequently, by the application of streptavidin labelled with peroxidase, which allows viewing.

The treatment for antigen retrieval was performed in microwave, with citrate buffer solution pH 6.0. Three cycles of 5 minutes were performed at a radiation of 850 watts, interspersed with brief cooling. A small amount of distilled water was added, between each cycle, when the citrate buffer solution was seen to be boiling and/or evaporating, to avoid the blades to dry. After three cycles of microwave radiation, the preparations were left to cool, in a cool place, for a period of approximately 25 minutes, after which three washes in TBS buffer (Tris-buffered saline, pH 7.4) were performed. The preparations were placed in a vertical humidity chamber and 2 drops of serum blocking solution were applied, for a period of 10 minutes. The primary antibody incubation (Cytochrome c (C20): sc-8385) was then performed overnight, at a dilution of 1: 1500, at room temperature. The dilution was performed using a diluting solution for antibodies (Antibody Diluent, Zytomed). Thereafter, three washes were performed in TBS buffer solution, of 5 minutes each, followed by incubation with the biotinylated secondary antibody for 30 minutes, at room temperature. The removal of the secondary antibody was performed by three repeated washes, with TBS buffer, for 5 minutes each, followed by incubation of the streptavidin-biotin enzyme complex, for a period of 10 minutes. This was followed by three washes in TBS again, for 5 minutes each.

The revelation was made with the DAB substrate for 5 minutes, at room temperature, and the reaction was stopped by immersion in buffer solution and subsequent thorough washing in warm water. Contrast staining was then performed by Harris's haematoxylin, for a period of 5 minutes, at room temperature, followed by washing with water, for 10 minutes.

Finally, the dehydration of the slides was performed through baths in graded ethanol series (2 baths of 96% alcohol, followed by 2 baths of 100% ethanol, for periods of 5 minutes each), and finalized with a 5 minutes xylene immersion bath. The assembly of the preparations between slide/coverslip was performed with mounting medium (Entellan®).

Tissue from rabbit thymus was used as positive control and underwent identical procedures to the described above. Incubation of the primary antibody was not performed for the negative control, and only diluting solution was applied. The final response was evaluated by observation, using a Zeiss microscope, model Axioplan2.

Images were obtained through a microscopic digital camera Leica DFC450 and software LAS Advanced Analysis Bundle, using 20x and 40x magnifications. Immunoreactivity was scored in accordance to staining intensity and staining pattern - diffuse or punctate (Liu et al. 2012). Staining intensity was evaluated as 0, undetectable; 1, weak staining; 2, moderate staining, 3, intense staining and 4, very intense staining.

2.2.2.2. M30 Antibody

The M30 Cytodeath® Fluorescein (Roche, Manheim, Germany) immunofluorescence assay was used in in six pigs from each group, in the duodenum, jejunum and in ileum as a complementary method for detection of apoptosis. It consists of a mouse monoclonal antibody (Monoclonal antibody, clone M30, IgG2b, mouse) for the detection of a caspase cleavage product of cytokeratin 18, was used. The M30 CytoDEATH® kit was used to detect apoptosis in six pigs from each group, in the duodenum, jejunum and ileum, according to the manufacturer's instructions. Pretreatment for antigen retrieval was performed by microwave irradiation at 850 W until boiling, in 0.01 M citrate buffer, at pH 6.0. When the solution was boiling, the power of the microwave oven was decreased to the "keep warm" mode (100 W) and slides incubated, at this power, for 15 minutes. The slides were then allowed to cool down for 5 min, at room temperature, rinsed three times in PBS and incubated for 2 minutes, in a separate jar of PBS. The blocking solution PBS + 1% BSA was applied for 10 min, at room temperature and removed after that period of time.

The M30 CytoDEATH working solution was then added and the slides left for 1 hour at room temperature, being washed three times in PBS, immediately after. Anti-Mouse-Biotin was applied and slides left at room temperature for 30 minutes, with a following wash in PBS for 3 times, and labelling with streptavidin-POD for 30 minutes, at room temperature. Slides were, again, washed three times in PBS and sections were disclosed with diaminobenzidine. Counterstaining with Harris's haematoxylin and mounting of the slides with a specific solution for immunofluorescence was then performed. Normal canine duodenum was used as a positive control and germinal centres of lymphoid follicles served as negative controls.

Immunoreactivity was scored in accordance to staining intensity and percentage of positive cells. The sections were examined by fluorescence microscopy using a Zeiss

Axioplan 2 microscope, and the images were obtained with the digital camera Axiocam MRM REV 3. Image processing was performed with LAS Advanced Analysis software.

2.2.2.3. TUNEL Method

The TUNEL method detects the fragmentation of cellular DNA that occurs in late stages of apoptosis (Gavrieli et al. 1992), hours after the occurrence of the eventual apoptotic stimulus. In our study, the intestinal tissue was collected approximately 90 minutes after the stimulus occurred. Thus, the possibility of the occurrence of DNA fragmentation during this study period is limited. Nevertheless, two pigs from each group were enrolled in this analysis to the duodenum, jejunum and ileum in order to verify if intense cellular lesions have occurred in this period.

The detection of apoptosis based on the enzymatic capacity of the terminal deoxynucleotidyl transferase (TdT) to catalyse a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double-stranded or single-stranded DNA was performed in two pigs from each group in the duodenum, jejunum and ileum, using the In Situ Cell Death Kit Detection, POD (Cat. No.11684817910, Roche, Mannheim, Germany), according to the protocol recommended by the manufacturer. According to the product datasheet, several options for the method of antigenic recovery were described. In the present study, two of the recommended possibilities were tested, including treatment with proteinase K and enzymatic treatment, in microwave, with the buffer solution citrate pH 6.0. The latter option was chosen as the treatment of choice, as better results were obtained in the control sample (canine thymus) when compared to the alternative method. To perform the chosen method, the slides underwent two cycles of microwave radiation at 800 watts, for two periods of 5 minutes each, with intermediate and subsequent cooling. To prevent drying of the blades during this process, a small amount of distilled water was added between each cycle. This was followed by three washes in TBS buffer, drying of the area around the sample and delimitation of the tissue with a hydrophobic barrier, to prevent the leakage of the solution to be applied later. The TUNEL mixture was carried out according to guidelines specified by the manufacturer, and was obtained by the addition of the Label Solution and enzymatic Enzyme Solution in the recommended amounts. The ideal dilution for this enzymatic mixture was determined, which was seen to be of 1:2.5. The

slides were incubated with 100 µl of enzyme solution (TdT), in a horizontal moist chamber, at 37 degrees Celsius, for 1 hour. The incubation step with TdT mixture was not performed for the negative control, which only underwent treatment with Label Solution.

Canine thymus was used as positive control tissue, requiring previous treatment with recombinant DNase I grade I (3000-3 U / ml, Roche, Mannheim, Germany), for approximately 10 minutes (to induce DNA breaks) and the reaction was stopped by buffer solution washing. The incubation conditions for the TdT enzyme mixture used in the positive control were similar to the previously described for the other tissues (37 degrees Celsius for 1 hour). Subsequently, the preparations were submitted to three cycles of 5 minutes washes in buffer solution. For observation of the immunoperoxidase reaction by light microscopy, it was necessary to convert the fluorescence signal obtained. This was performed adding 100 µl of Converter-POD solution (anti-fluorescein antibody, Fab fragment from sheep), conjugated with horseradish peroxidase, following the manufacture's instructions.

This incubation was performed for 30 minutes, at a temperature of 37 degrees Celsius, in a humid atmosphere, followed by three successive washes, in phosphate buffered saline, for 5 minutes each. Disclosure of the slides was performed by applying the DAB substrate, for a period of 5 minutes, at room temperature. Buffer solution washing, followed by washing with abundant warm water, stopped the reaction.

The nuclear staining with Harris haematoxylin contrast was then performed, for a period of 5 minutes, at room temperature, followed by water washing for 10 minutes. Finally, dehydration of the slides was performed through graded ethanol series (2 baths of 96% alcohol, followed by 2 baths of 100% ethanol, for periods of 5 minutes each), with the process ending by immersion in xylene bath (5 minutes). Subsequently, assembly of the slides with mounting medium (Entellan®) was performed.

The TUNEL reaction was assessed by observation using a Zeiss microscope model Axioplan2, and images were acquired using a digital camera (Olympus C5060 Zoom), with 20x and 40x magnifications. Immunoreactivity was scored in accordance to staining intensity and percentage of positive cells. Staining intensity was evaluated as 0, undetectable; 1, weak staining; 2, moderate staining, 3, intensive staining and 4, very intense staining.

2.2.2.4. Level of staining intensity and apoptotic cell counting – TUNEL Method and M30 antibody

For counting of the apoptotic cells, for both the TUNEL method and M30 antibody, an accurate manual counting system was adapted from Detre and colleagues and Bologna-Molina and colleagues (Detre et al. 1995; Bologna-Molina et al. 2011). Ten representative fields, at a magnification of 400x were selected for each section, in which the cells that exhibited positive (from weak to very intense) and negative staining reaction were recorded, in order to calculate the percentage of cells exhibiting each level of staining intensity, so the H-score could be calculated, using the following formula:

High-score = (% labelled cells intensity 1 x 1) + (% cells labelled with intensity 2 x 2) + (% labelled cells intensity of 3 x 3) + (% cells labelled with intensity 4 x 4).

The High-score values obtained vary between 0 and 400, where 0 corresponds to a negative sample and 400 to a reaction with a staining intensity of 4 of all the cells, in the microscopic field. The value for each pig was determined by the average scores of 10 fields, and the value for the group, calculating the average of the High-scores of all the pigs, in each group.

The percentage of apoptotic cells was also calculated, which is considered the apoptotic index; for the percentage of apoptotic cells/apoptotic index, all the positive cells were counted, regardless of the intensity of the staining reaction, as well as all the cells present in each field. The percentage of apoptotic cells was then calculated using the formula:

% Apoptotic cells = Total number of positive cells / Total number of cells * 100.

A further calculation was made of the number of apoptotic cells per mm^2. Each picture has an area of 0.0951 mm^2, in the case of the M30 marker, and of 0.047003 mm^2, in the case of the TUNEL method. Therefore, the total number of apoptotic cells, per square millimetre, is obtained as follows:
For the M30 marker,
Positive cells per mm^2 = Total number of positive cells / (0.0951 * 10 fields),

For the TUNEL staining,

Positive cells per mm^2 = Total number of positive cells / (0.047003 * 10 fields).

2.3. Statistical Analysis

All data was analysed for normality using D'Agostino Pearson omnibus normality test, or, when the sample was too small, normality was analysed using a graphical representation by histograms. All data was also analysed for variance using Levene's test. The Kruskal-Wallis test was used to compare data from the grades of oedema, hyperaemia, haemorrhage, congestion, inflammatory infiltrate and cellular degeneration between groups. Dunn's post hoc test was used to compare groups 1, 2 and 3, for %ML, C:I ratio, cytochrome c, M30 staining and TUNEL method for each small intestinal segment and whole small intestine.

Haemodynamic, ventilatory data and blood analysis, including blood gases values were analysed by repeated measurements analysis of variance (ANOVA), using Bonferroni corrections for pairwise comparisons. $P<0.05$ was considered to be statistically significant. Analysis was performed using GraphPad Prism ver. 5.01 software (GraphPad Software, San Diego, CA) and Hmisc package in R software (R 2.15.1) (R Development Core Team, 2012).

Data are presented as means +/- standard deviation (SD), in grading or in percentage (%) as appropriate.

CHAPTER 3

RESULTS

3.1. Demographic data

Twenty-eight healthy, three months old, commercial Large White pigs, with a body weight of 26.82±3.28 Kg were enrolled in the study.

3.1.1. Haemodynamic parameters, blood analysis and cerebral monitoring

In the analysis of haemodynamic parameters, blood analysis and cerebral monitoring, the following animals have been enrolled:

Group 1 - Eleven healthy pigs weighing 27±3 kg;

Group 2 - Eleven healthy pigs, weighing 27±1 Kg;

Group 3 – Six healthy pigs, weighing 26±5 Kg.

3.1.2. Histopathology analysis

In the histopathology analysis (intestinal mucosal loss, crypt:interstitium ratium and qualitative histopathological parameters) the following animals have been enrolled:

Group 1 - Eleven healthy pigs weighing 27±3 kg;

Group 2 - Eleven healthy pigs, weighing 27±1 Kg;

Group 3 – Six healthy pigs, weighing 26±5 Kg.

3.1.3. Immunohistochemical analysis

In the immunohistochemical analysis the following animals have been enrolled:

Cytochrome c and M30 Cytodeath assay:

Group 1 - Six healthy pigs weighing 27±3 kg;

Group 2 - Six healthy pigs, weighing 27±4 Kg;

Group 3 – Six healthy pigs, weighing 26±5 Kg.

TUNEL Method

Group 1 - Six healthy pigs weighing 27±6 kg;

Group 2 - Six healthy pigs, weighing 28±4 Kg;

Group 3 – Six healthy pigs, weighing 22±1 Kg.

3.2. Blood analysis

The values of the blood analysis performed during the different study periods are shown in tables 4 and 5. At the end of the study period, haematocrit and total proteins were lower in groups 1 and 2, when compared to group 3 ($p<0.001$ and $p<0.05$, respectively). Also at the end of the study, red blood cells were significantly lower in group 2 when compared to group 3 ($p<0.05$), and the haemoglobin was significantly lower in both groups 1 and 2, when compared with group 3 ($p<0.05$ and $p<0.01$, respectively). The values of albumin were also significantly lower in groups 1 and 2 when compared with group 3, at the end of the study ($p<0.01$). No significant differences were observed in any of the other parameters.

3.3. Haemodynamic data

Group 1

The bleeding period lasted 21±1 minutes, the waiting period lasted 24±3 minutes, and the volume replacement period lasted 46±7 minutes. The time between the commencement of the bleeding and euthanasia was 156±6 minutes. The blood temperature was 37±1.5 degrees Celsius (ºC) during the study period. Heart rate (HR) was 79±22 beats per minute (bpm), mean arterial pressure (MAP) was 60±12.8 millimetres of mercury (mmHg) (Figure 9) and peripheral oxygen saturation (SPO_2) was 96±7.8 % during the study period (Figure 10).

In the bleeding period, a 50.79% decrease in SAP, a 49.82% decrease in MAP and a 46.59% decrease in DAP were observed at T10 when compared to baseline. There was also a 7.73% decrease in the cardiac output and a 13.2% decrease in the central venous pressure at T10 when compared to baseline. No significant changes were observed in HR or SPO_2 (Figures 6 to 12).

In the waiting period, there was a 29.93% increase in SAP, a 30.50% increase in MAP and a 35.31% increase in DAP at T10 when compared to baseline. A 8.34% decrease in cardiac output and a 33.19% decrease in the central venous pressure at T10 when compared to baseline were also observed. No significant changes were observed in HR or SPO_2 (Figures 6 to 12).

Table 4. Results from the blood analysis performed before the beginning of bleeding and at the end of the study for all groups. Results are mean ± standard deviation.

	Before bleeding			**End of study**		
	Group1	Group2	Group3	Group1	Group2	Group3
RBC (10^12/L)	5.83±0.26	5,60±0.74	5,62±0.43	4,35±0.54	4,03±0.64	5,40±0.49*
HGB (g/dL)	7.80±0.77	8,05±0.83	8,16±1.21	5,95±0.90	5,70±0.69	7,86±0.60*/**
MCV (fL)	49.08±6.16	50,95±1.79	51,36±6.36	48,48±6.43	49,40±1.73	50,14±5.78
MCHC (g/dL)	27.35±0.64	29,15±2.42	28,38±1.09	28,08±0.53	28,80±0.50	30,16±2.17
PLT (10^9/L)	708.75±263.53	459±107.37	470,80±129.98	476,75±178.07	344,67±87.90	364,00±217.31
MPV (10^9/L)	12,28±7.41	9,53±1.60	10,58±1.96	9,03±2.08	8,43±0.49	9,74±1.23
WBC (10^9/L)	14,40±4.25	17,85±2.70	15,06±4.62	16,03±3.19	21,03±9.62	14,16±3.54
NEU (10^9/L)	7,65±2.46	12,78±2.64	9,36±3.87	9,80±3.11	16,60±9.14	8,62±3.03
LYM (10^9/L)	6,20±1.71	4,53±0.81	5,12±0.80	5,70±2.17	3,93±0.86	4,92±0.87
MONO (10^9/L)	0,33±0.13	0,2±0.08	0,30±0.07	0,25±0.06	0,17±0.12	0,28±0.11
EOS (10^9/L)	0,18±0.10	0,28±0.10	0,22±0.13	0,18±0.15	0,30±0.17	0,20±0.07
BAS (10^9/L)	0,03±0.05	0,03±0.05	0,04±0.05	0,03±0.05	0,00±0.00	0,00±0.00
AST (UI/L)	31,25±23.82	51.00±10.34	52,80±21.57	69,25±65.31	39,20±4.66	44,00±14.46
ALT (UI/L)	33,75±12.63	39.00±12.10	44,00±14.56	36,75±10.28	27,20±7.73	38,80±8.04
ALKP (UI/L)	89,75±66.67	126.00±63.08	146,60±31.54	74,50±53.27	96,80±44.60	139,80±38.44
GGT (UI/L)	16,50±12.07	21,60±15.50	15,60±8.59	13,25±4.03	13,40±6.15	15,80±6.30
BUN (mg/dL)	19,50±8.06	19,20±2.77	20,40±6.66	20,50±6.81	18,20±2.95	22,40±7.27
CREAT (mg/dL)	0,88±0.15	0,90±0.10	0,88±0.11	0,85±0.13	0,86±0.09	0,84±0.09
ALB (g/dL)	1,60±0.16	1,78±0.13	1,96±0.24	1,28±0.10	1,32±0.15	1,82±0.30**
α-1 GLOB (g/dL)	0,10±0.00	0,08±0.04	0,10±0.00	0,08±0.05	0,08±0.04	0,10±0.00
α-2 GLOB (g/dL)	0,70±0.42	0,82±0.25	0,76±0.11	0,55±0.31	0,56±0.09	0,78±0.11
β-GLOB (g/dL)	0,75±0.21	0,96±0.19	0,98±0.38	0,58±0.21	0,56±0.19	0,80±0.20
γ-GLOB (g/dL)	1,68±1.82	0,78±0.28	0,66±0.11	1,00±0.80	0,52±0-18	0,66±0.17
ALB/GLOB Ratio	0,55±0.21	0.70±0.19	0,82±0.22	0,60±0.14	0,82±0.25	0,82±0.26

RBC – red blood cells; HGB – haemoglobin; MCV - mean corpuscular volume; MCHC - mean corpuscular haemoglobin concentration; PLT – platelets; MPV - mean platelet volume; WBC – white blood cells; NEU – neutrophils; LYM – lymphocytes; MONO – monocytes; EOS – eosinophils; BASO – basophils; AST - aspartate aminotransferase; ALT – alanine aminotransferase; ALKP - alkaline phosphatase; GGT - gamma-glutamyl transpeptidase; BUN – Blood urea nitrogen; CREAT – creatinine; ALB – albumin; GLOB - globulin. Significant differences between groups: *P<0.05, **P<0.01. Haematocrit was lower in groups 1 and 2, when compared to group 3 ($p<0.001$ and $p<0.05$, respectively) in the end of the study period. The red blood cells were significantly lower in group 2 when compared to group 3 ($p<0.05$), and the haemoglobin was significantly lower in both groups 1 and 2, when compared to group 3 ($p<0.05$ and $p<0.01$, respectively). Albumin was also significantly lower in groups 1 and 2 when compared to group 3 ($p<0.01$).

Table 5. Results from arterial blood gas analysis performed before the beginning of bleeding, at the end of the waiting period, at the end of the volume replacement and at the end of the study, one hour after the end of the volume replacement. Results are mean ± standard deviation.

	Before bleeding			**End waiting**		**End volume replacement**		**End of study**		
	Group1	Group2	Group3	Group1	Group2	Group1	Group2	Group1	Group2	Group3
PH	7.45±0.06	7.44±0.03	7.44±0.03	7.4±0.08	7.4±0.08	7.5±0.07	7.5±0.05	7.5±0.06	7.46±0.04	7.49±0.01
PCO_2 (mmHg)	46.2±5.8	47.5±4.1	47.75±2.33	48.5±6.9	48.9±9.1	44.3±6.4	43.0±3.9	44.8±5.6	45.9±3.5	43.93±3.64
PO_2(mmHg)	217.4±73.9	188±48.8	210.25±40.15	230.5±62.0	195.5±38.0	233.5±49.9	221.0±65.0	225.0±40.9	201.8±50.6	233.00±68.89
Na^+ (mEq/L)	134.1±5.1	136±2.0	137.25±1.26	133.5±4.8	137.4±2.2	133.7±5.0	137.1±1.3	134.2±5.0	137.9±1.64	136.25±1.89
K^+(mEq/L)	5.8±1.7	5.1±1.2	4.13±0.92	5.3±1.0	4.6±0.73	5.13±1.0	4.0±0.5	4.9±1.2	4.40±0.52	4.18±0.46
Ca^{++}(mg/dL)	1.1±0.22	1.2±0.2	0.94±0.22	1.1±0.1	1.1±0.10	1.1±0.04	0.98±0.21	1.2±0.3	1.18±0.22	1.05±0.28
Gluc (mg/dL)	60.9±22.6	57.9±13.5	4.80±1.27	63.3±22.6	80.2±25.7	60.3±28.2	81.5±15.9	68.6±27.8	68.3±13.3	5.10±1.98
Lact (mmol/L)	2.1±1.1	2.0±1.0	1.80±0.71	2.8±1.0	2.2±0.68	2.9±0.7	1.7±0.5	1.8±1.0	1.11±0.34	0.90±0.28
HTC (%)	26.1±4.6	26.4±5.6	28.82±5.11	17.1±2.5	20.9±4.2	15.9±1.13	14.5±2.1	18.2±4.2	17.5±3.5	27.30±2.72**/*
HCO3- (mEq/L)	31.6±2.5	32.4±2.0	31.82±1.25	31.0±1.6	30.9±0.9	30.5±1.4	30.0±2.73	31.6±1.1	32.6±1.39	33.68±1.97
TP (g/dL)	4.3±0.8	4.1±0.25	4.46±0.42	-	-	-	-	3.2±0.6	2.78±0.19	4.16±0.11**/*

PCO2 - Partial Pressure of Carbon Dioxide; Na+ - Sodium; K+ - potassium; Ca++ - Cacium; Gluc – glucose; Lact – Lactate; HTC – haematocrit; HCO3- - bicarbonate; TP – total protein. Significant differences between groups: *P<0.05, **P<0.001. At the end of the study period, total proteins were lower in groups 1 and 2, when compared to group 3 ($p<0.001$ and $p<0.05$, respectively).

In the volume replacement period there was a 55.77% increase in SAP, a 50.61% increase in MAP and a 36.43% increase in DAP at T10 when compared to baseline. A 2.48% decrease in cardiac output and a 65.76% increase in central venous pressure at T10 when compared to baseline were also seen. There were no significant changes in HR or SPO_2 (Figures 6 to 12).

In the final period, a 1.54% decrease in SAP, a 0.89% increase in MAP and a 4.07% increase in DAP at T10 when compared to baseline were observed. There was also a 23.72% increase in cardiac output and a 25.51% decrease in central venous pressure at T10 when compared to baseline. No significant changes have been observed in HR or SPO_2 (Figures 6 to 12).

Group 2

The bleeding period lasted 21±4 minutes, the waiting period lasted 22±1 minutes, and the volume reposition period lasted 35±2 minutes. The time between the commencement of the bleeding and euthanasia was 141±5 minutes. The blood temperature was 38±0.6 ºC during the study period. HR was 86±25 bpm, MAP was 63±11.45 mmHg (Figure 9) and SPO_2 was 98±2.3 % during the study period (Figure 10).

In the bleeding period, a 59.99% decrease in SAP, a 56.16% decrease in MAP and a 50.46% decrease in DAP were observed at T10 when compared to baseline. There was also a 4.65% decrease in the cardiac output and a 50.27% increase in the central venous pressure at T10 when compared to baseline. No significant changes have been observed in HR or SPO_2 (Figures 6 to 12).

In the waiting period, there was a 41.36% increase in SAP, a 35.69% increase in MAP and a 30.58% increase in DAP at T10 when compared to baseline. A 18.24% decrease in the cardiac output and a 75.01% decrease in central venous pressure at T10 when compared to baseline were also observed. No significant changes have been observed in HR or SPO_2 (Figures 6 to 12).

In the volume replacement period there was a 63.65% increase in SAP, a 69.65% increase in MAP and a 57.07% increase in DAP at T10 when compared to baseline. A 2.27% increase in cardiac output and a 356.62% increase in central venous pressure at T10 when compared to baseline were also seen. There were no significant changes in HR or SPO_2 (Figures 6 to 12).

In the final period, a 0.96% decrease in SAP, a 1.12% increase in MAP and a 7.16% increase in DAP at T10 when compared to baseline were observed. A 72.85% increase in cardiac output and a 35.47% decrease in the central venous pressure at T10 when compared to baseline also occurred. There were no significant changes in HR or SPO_2 (Figures 6 to 12).

No statistically significant differences were observed between groups 1 and 2 in demographics, body temperature or haemodynamic variables during the entire study period.

Group 3

The time between the commencement of the study period until euthanasia was 172±29 minutes. The blood temperature was 37±0.6 ºC during the study period. HR was 80±9 bpm, MAP was 71.8±5.72 mmHg and SPO_2 was 99±1.7 % during the study period.

No significant differences were observed between groups 1, 2 and 3 in demographics and body temperature.

Statistically significant differences between groups 1 and 3 and 2 and 3 were observed in SAP, MAP, DAP, cardiac output and central venous pressure, when compared to the bleeding period ($p<0.05$).

The mean baseline values (values at T0 of the initial period) for heart rate, systolic, diastolic and mean arterial blood pressure, cardiac output, and central venous pressure are showed in table 6. Statistically significant differences were observed between groups 1 and 3 in heart rate at baseline (T0 of initial period; $p<0.05$) and systolic arterial blood pressure ($p<0.05$) and also in central venous pressure between groups 2 and 3 ($p<0.05$) at the same point. No statistically significant differences were observed between groups in the remaining variables.

Table 6. Mean values of heart rate (HR-bpm), systolic arterial blood pressure (SAP-mmHg), diastolic arterial blood pressure (DAP-mmHg), mean arterial blood pressure (MAP-mmHg), peripheral oxygen saturation (SPO2/%), cardiac output (CO-L/min) and central venous pressure (CVP-mmHg) at the beginning of the study, in all groups (between-group comparisons: *P<0.05).

Baseline values	HR	SAP	DAP	MAP	SPO2	CO	CVP
Group 1	87.10±3.80	107.97±2.41	48.20±1.40	70.0±1.83	98.67±0.46	0.50±0.02	9.60±3.89
Group 2	76.35±2.74	108.18±3.49	50.11±1.58	70.95±1.65	96.69±0.31	0.38±0.01	5.40±1.35*
Group 3	73.95±6.62*	94.42±3.13*	53.23±1.77	68.33±0.70	99.03±0.08	0.51±0.08	9.22±0.03

Heart rate, systolic, diastolic and mean arterial blood pressure, peripheral oxygen saturation, cardiac output and central venous pressure during the entire study for all groups are represented in Figures 6, 7, 8, 9, 10, 11 and 12, respectively.

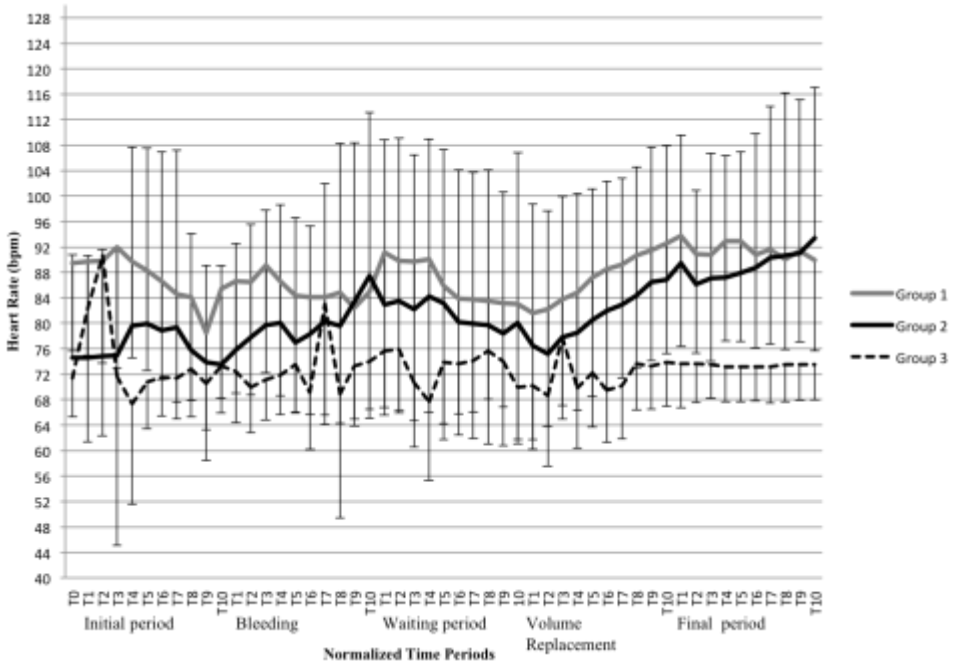

Figure 6. Heart rate (HR) trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Data are mean±SD. No statistically significant differences were observed between groups.

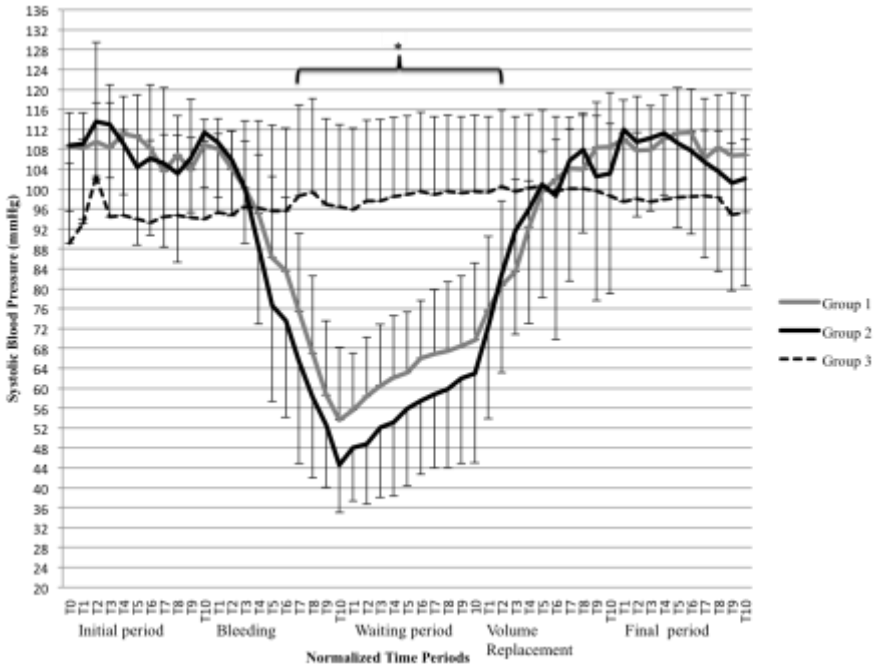

Figure 7. Systolic blood pressure trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Systolic blood pressure was significantly lower in groups 1 and 2 when compared to group 3 (control group) between the normalized time periods T7 of the bleeding period and T1 of the volume replacement period. Data are mean±SD. Between-group comparisons: *P<0.05.

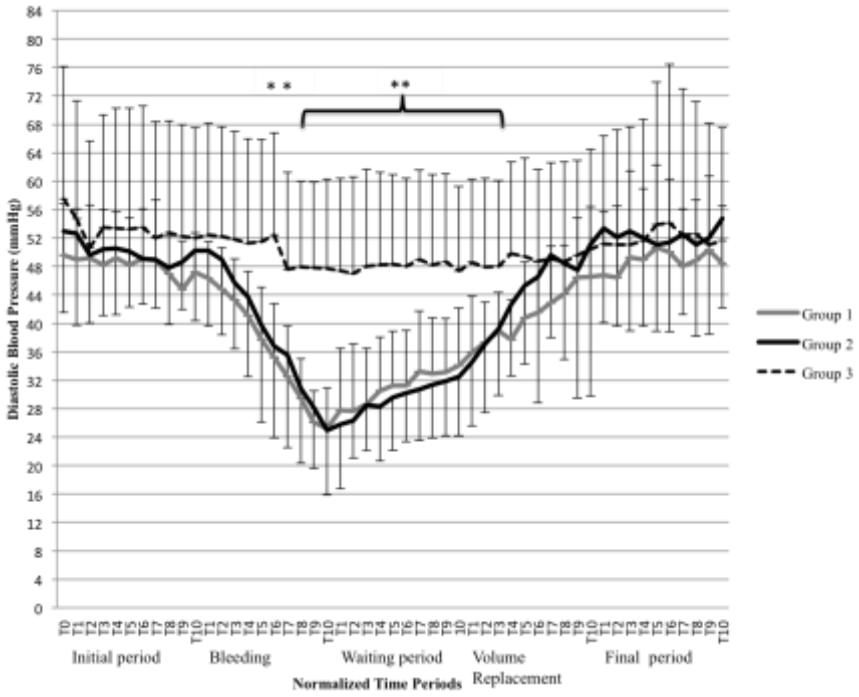

Figure 8. Diastolic blood pressure trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is

divided in 10% normalized time periods, T0 to T10. Diastolic blood pressure was significantly lower in groups 1 and 2 when compared to group 3 (control group) in the normalized time periods T6 and T7 of the bleeding period and between the normalized time periods T8 of the bleeding period and T1 of the volume replacement period. Data are mean±SD. Between-group comparisons: *P<0.05, **P<0.01.

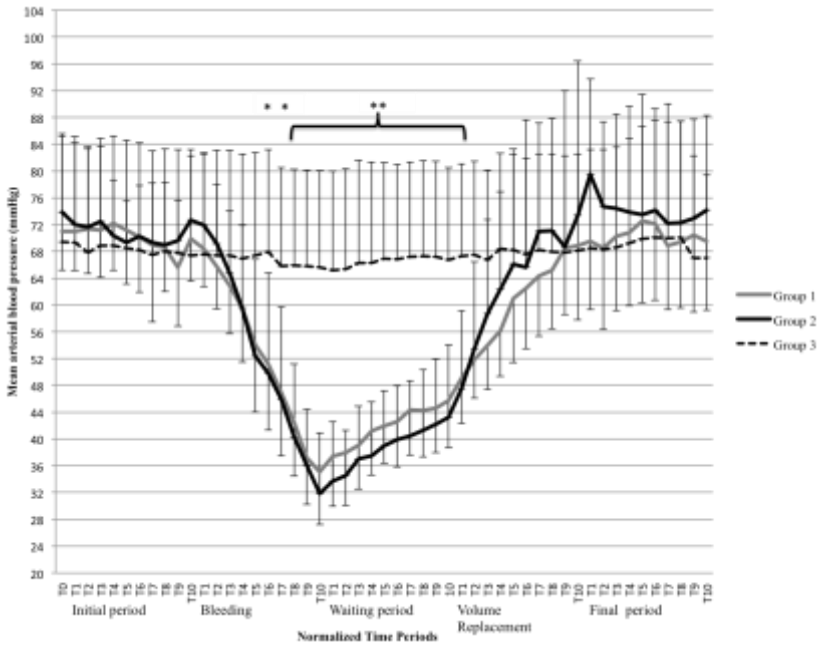

Figure 9. Mean arterial blood pressure trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Mean arterial blood pressure was significantly lower in group 2 when compared to group 3 (control group) in the normalized time period T6 of the bleeding period and significantly lower in both groups 1 and 2 in the normalized time period T7 of the bleeding period and between the normalized time periods T8 of the bleeding period and T1 of the volume replacement period. Data are mean±SD. Between-group comparisons: *P<0.05, **P<0.01.

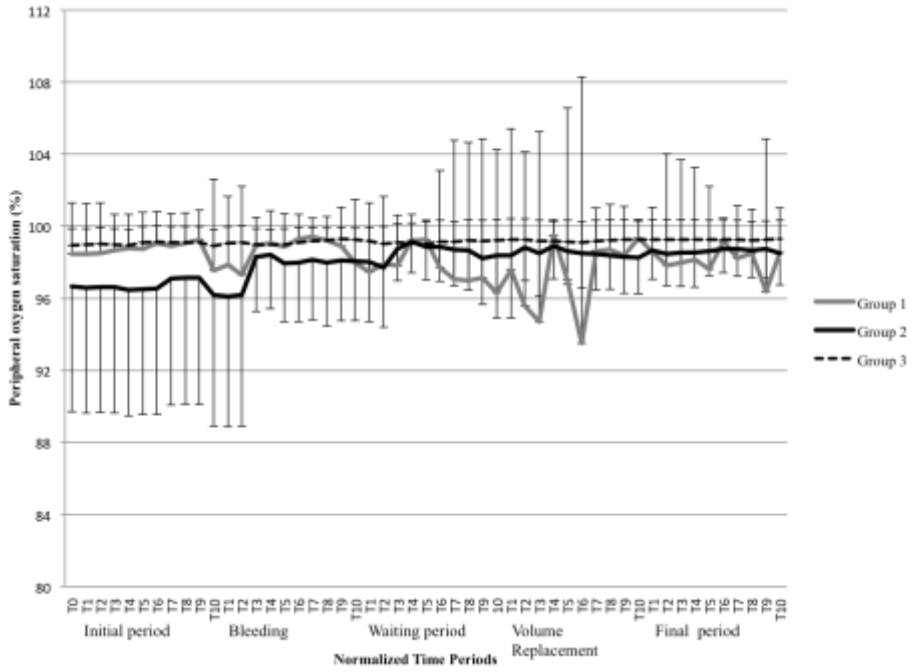

Figure 10. Peripheral oxygen saturation (SPO_2) pressure trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Data are mean±SD. No statistically significant differences were observed between groups.

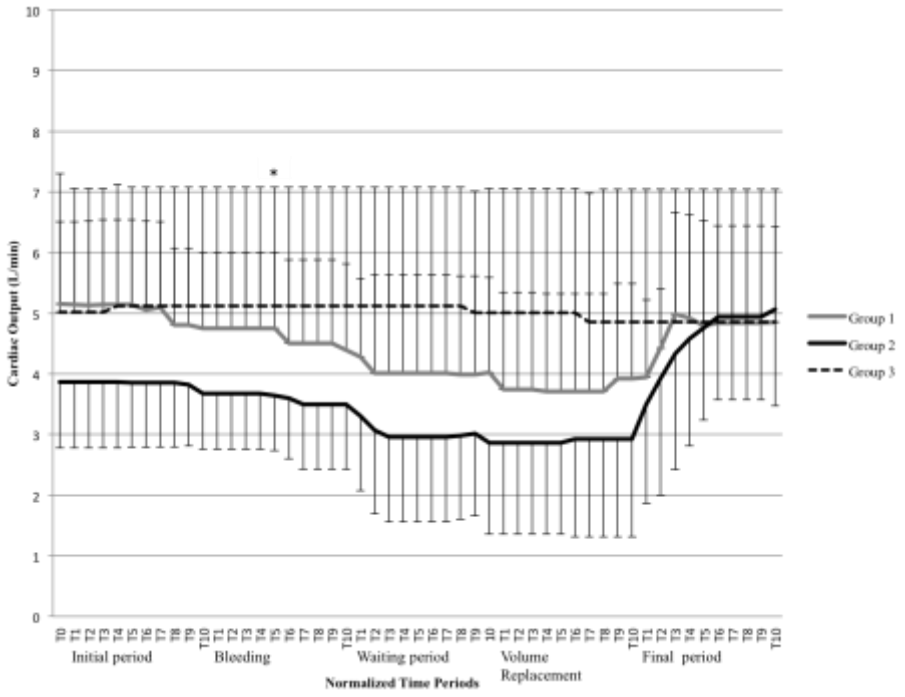

Figure 11. Cardiac Output (CO) trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Cardiac output was significantly lower in group 2 when

compared to group 3 (control group) in the normalized time period T5 of the bleeding period Data are mean±SD. Between-group comparisons: *P<0.05.

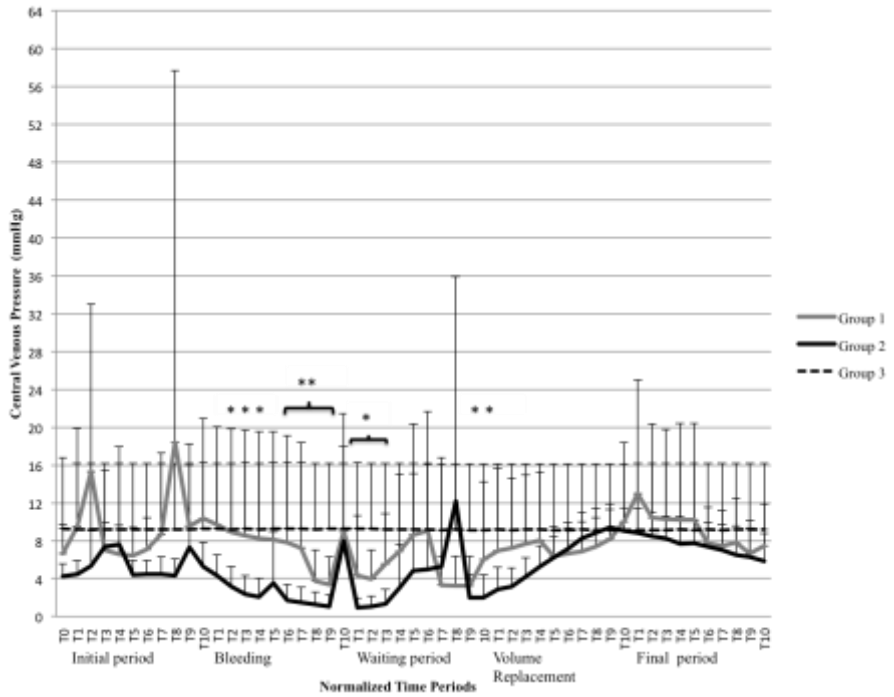

Figure 12. Central venous pressure (CVP) trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Central venous pressure was significantly lower in group 2 when compared to group 3 (control group) in the normalized time periods T2 to T4 and T6 to T9 of the bleeding period and also in the normalized time periods T1 to T3 of the waiting period. It was significantly lower in group 1 when compared to group 3 in the normalized periods T9 and T10 of the waiting period. Data are mean±SD. Between-group comparisons: *P<0.05, **P<0.01.

3.4. Brain monitoring (INVOS, BIS, SvjO2)

Brain monitoring data are presented in Figures 13, 14 and 15. For the BIS and INVOS monitoring, no statistically significant differences were observed between groups 1 and 2 during the study period. However, significant differences in these parameters were seen between group 3 and group 2, in the bleeding period (BIS: T10 of the bleeding period, T1 of the volume replacement period and T5 of the final period, $p<0.05$; INVOS: T8 of the bleeding period to T2 of the volume replacement period, $p<0.05$) (Figures 13 and 14, respectively).

No statistically significant differences have been observed in $SvjO_2$ between groups during the entire study period (Figure 15).

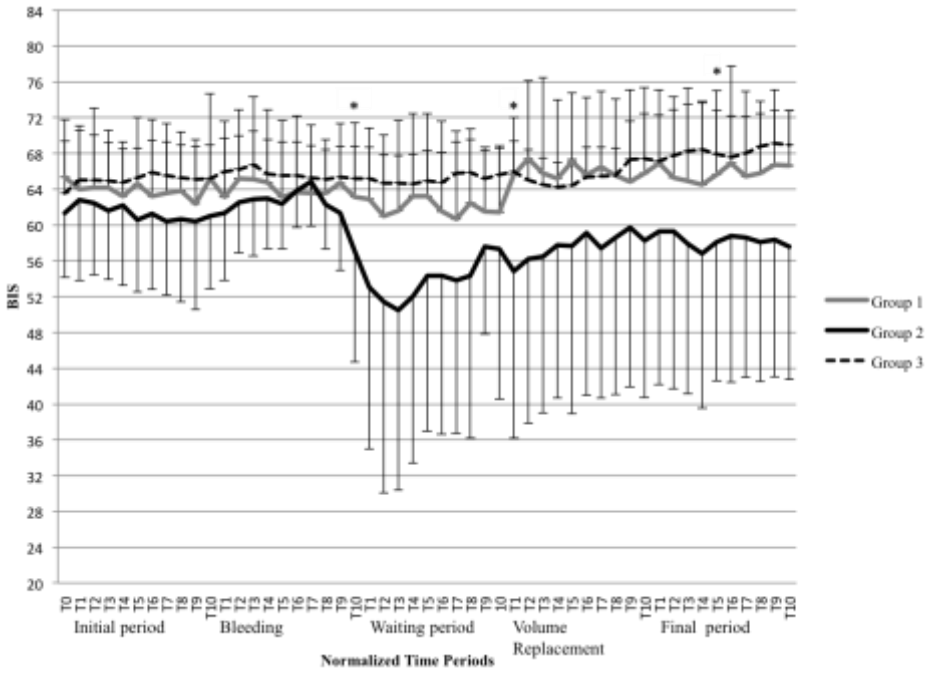

Figure 13. BIS trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. BIS was significantly lower in group 2 when compared to group 3 (control group) in the normalized time periods T10 of the bleeding period, T1 of the volume replacement period and T5 of the final period. Data are mean±SD. Between-group comparisons: *P<0.05.

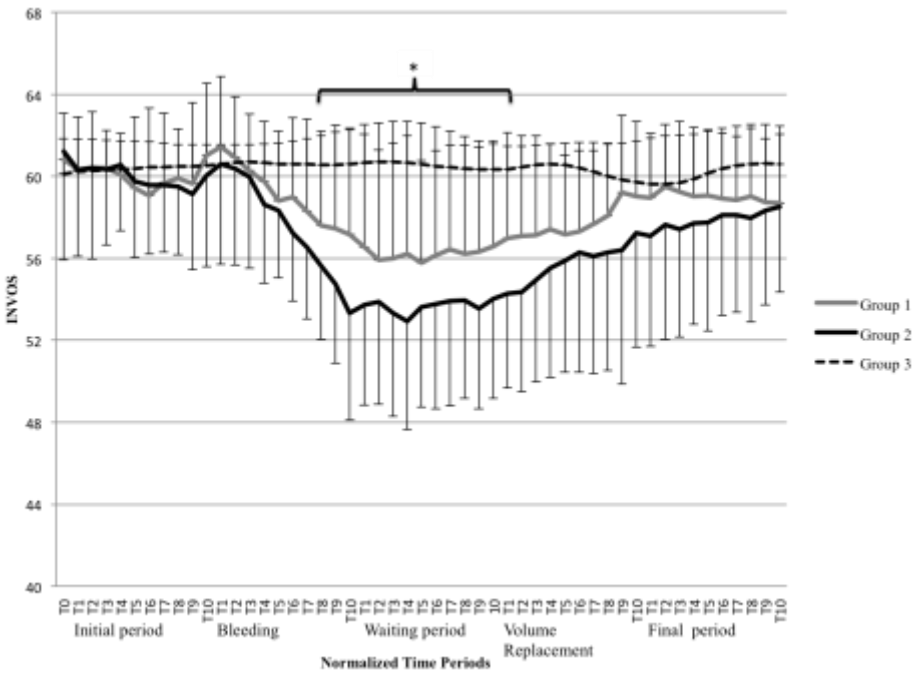

Figure 14. INVOS trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. INVOS was significantly lower in group 2 when compared to group 3 in the

normalized time periods T8 of the bleeding period to T2 of the volume replacement period. Data are mean±SD. Between-group comparisons: *P<0.05.

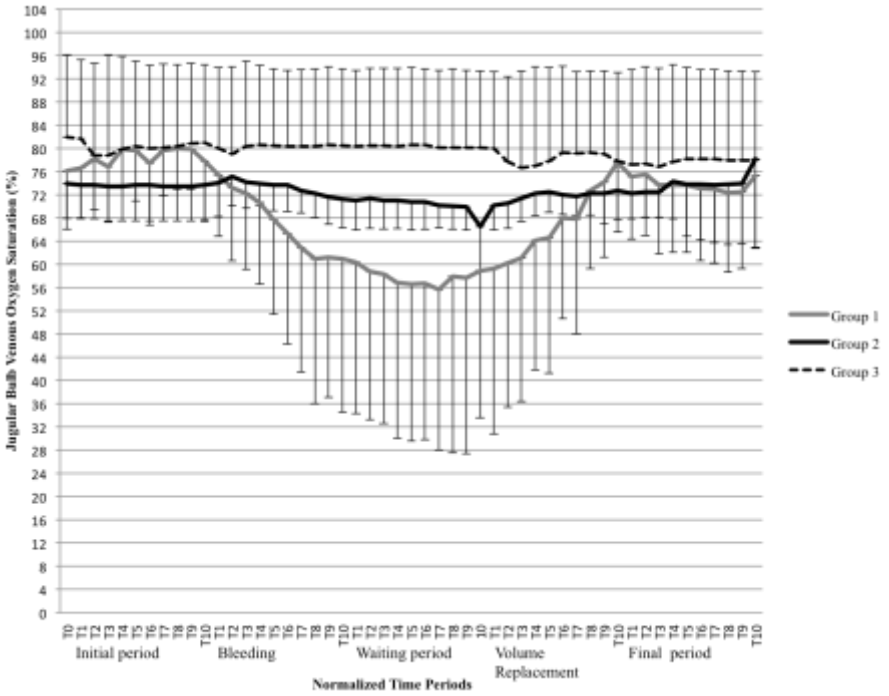

Figure 15. SvjO2 trend in groups 1 (Ringer solution), 2 (HES 130/0.4 solution) and 3 (control) in all the periods of the study - initial period, bleeding, waiting, volume replacement and final period; each period is divided in 10% normalized time periods, T0 to T10. Data are mean±SD. No statistically significant differences were observed between groups.

3.5. Histopathology data

3.5.1. Intestinal mucosal loss

The intestinal mucosal loss was evaluated in groups 1 (n=11), 2 (n=11) and 3 (n=6). The morphological changes observed in the epithelium and mucosa of the small intestine consisted in the development of a subepithelial space at the apex of the villi, the detachment of a variable number of epithelial cells as the subepithelial space was increasing in size, with loss of the surface of the villi occurring with relative frequency, especially in group 1, with consequent exposure of dilated capillaries (Figure 16).

The median percentage of mucosal loss was a) for the duodenum 2.73% in group 1, 1.03% in group 2 and 1.05% in group 3, b) for the jejunum 1.85% in group

1, 0% in group 2 and 0.41% in group 3, and c) for the ileum 1.19% in group 1, 0% in group 2 and 0.41% in group 3. The percentage of mucosal loss in the duodenum was significantly higher in Group 1, comparing with Groups 2 and 3 ($P<0.05$). In the jejunum and ileum, the percentage of mucosal loss was significantly higher in Group 1 comparing with Group 2 ($P<0.05$). For the whole small intestine, the median levels of mucosal loss percentage were 1.65% in Group 1, 0.00% in Group 2 and 0.48% in Group 3. Values were significantly higher in Group 1 than in groups 2 and 3 ($P< 0.05$) (Figure 17). Detailed data can be found in Appendix A (Tables 1, 2 and 3).

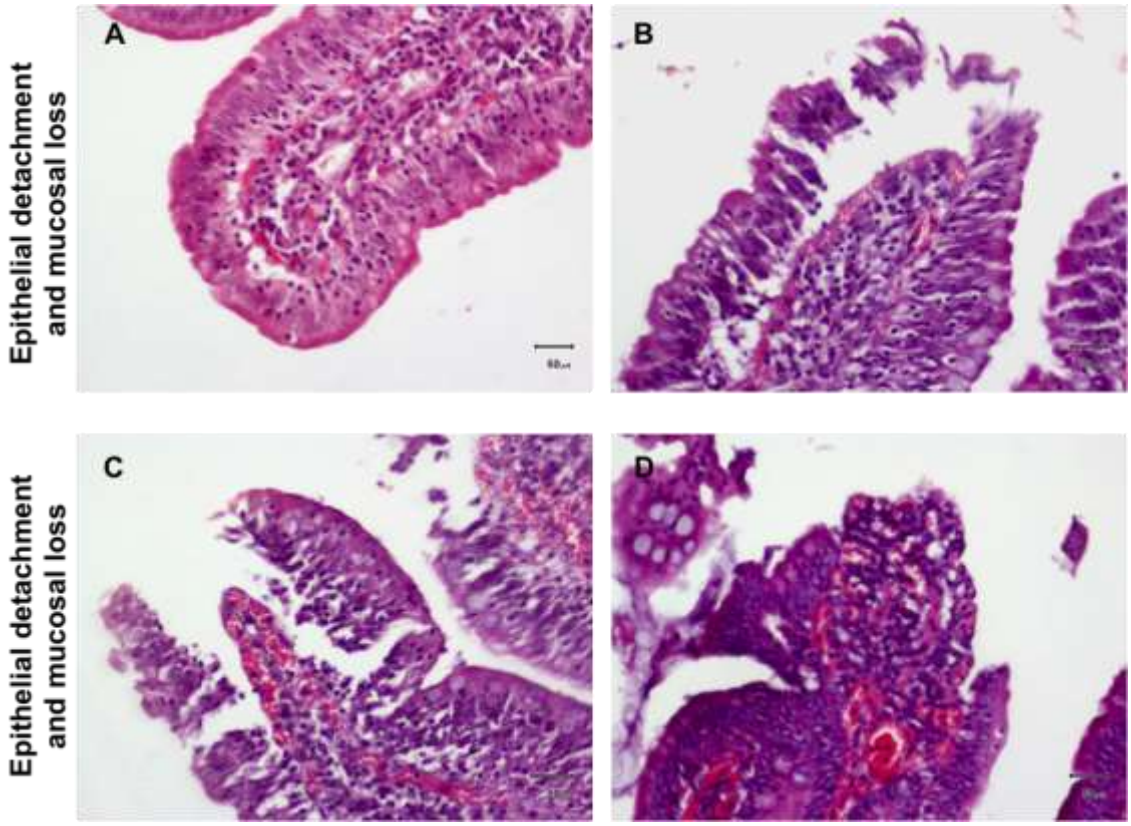

Figure 16. Mucosal loss. Note the increasing severity, from the development of a small subepithelial space, which corresponds to a grade 1 of the mucosal loss grading (A), in the duodenum of one pig from group 2 (pig 8) and extension of the subepithelial space and moderate mucosal detachment also in the duodenum of the same pig (B); separation of the superficial epithelium with extensive loss of the epithelium of the villus (C), in the duodenum of one pig from group 1 (pig 10), and loss of the surface of the villus and exposure of dilated capillaries of the lamina propria, which corresponds to a grade 4 of mucosal loss (D), also in one pig from group 1 (pig 7). Note also the marked (grade 3) congestion of the capillaries within the lamina propria. Disintegration of the lamina propria with haemorrhagic ulceration, which would be considered a grade 5, was not observed in this study.

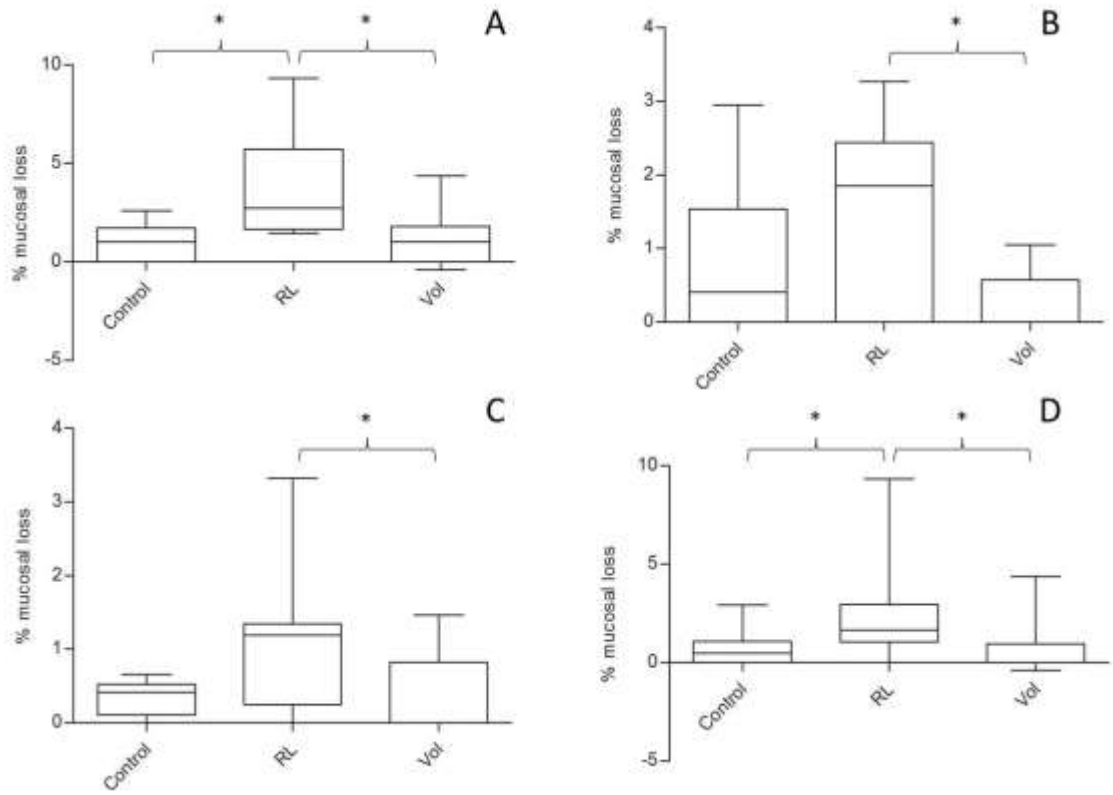

Figure 17. Box and whisker plots showing the percentage of mucosal loss (% ML) per group, for each intestinal segment and entire small intestine (A, duodenum; B, jejunum; C, ileum; D, total small intestine; *, statistically significant differences between groups – $p<0.05$). The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. The percentage of mucosal loss in the duodenum was significantly higher in Group 1, comparing with groups 2 and 3. In the jejunum and ileum, the percentage of mucosal loss was significantly higher in group 1 comparing with group 2. For the whole small intestine, the median levels of mucosal loss percentage were significantly higher in group 1 than in groups 2 and 3.

3.5.2. Crypt:Interstitium ratio

The crypt:interstitium ratio was evaluated in groups 1 (n=11), 2 (n=11) and 3 (n=6). Median C:I ratio was for the duodenum, 0.45 in Group 1, 0.53 in Group 2 and 0.39 in Group 3, for the jejunum 0.48 in Group 1, 0.44 in Group 2 and 0.45 in Group 3, and for the ileum 0.51 in Group 1, 0.51 in Group 2 and 0.47 in Group 3. For the whole intestine, median C:I ratio was 0.48 in Group 1, 0.48 in Group 2 and 0.46 in Group 3. No significant differences were observed between groups (table 7).

Table 7. Median and 95% confidence interval for C:I Ratio, in each intestinal segment and entire small intestine, for all groups.

	C:I ratio					
	Group 3 (Control)		Group 2 (HES 130/0.4)		Group 1 (RL)	
Small Intestine	Median	95% CI	Median	95% CI	Median	95% CI
Total small intestine	0.4500	0.41-0.49	0.4800	0.43-0.53	0.4800	0.45-0.51
Duodenum	0.3900	0.30-0-47	0.4500	0.28-0.78	0.5300	0.39-0.51
Jejunum	0.4550	0.34-0.57	0.4800	0.40-0.49	0.4400	0.41-0.55
Ileum	0.4700	0.42-0.53	0.5100	0.44-0.58	0.5100	0.44-0.59

HES 130/0.4 - Hydroxyethyl Starch 130/0.4; RL – Ringer Lactate solution-

3.5.3. Qualitative histopathological parameters

The qualitative histopathological parameters congestion, hyperaemia, haemorrhage, cellular degeneration, inflammation, oedema, epithelial detachment grading and necrosis were evaluated in groups 1 (n=11), 2 (n=11) and 3 (n=6). Mucosal inflammation, in all groups and segments, varied between moderate to marked. Duodenal general mucosal inflammation was significantly elevated in Groups 1 and 2, when compared to Group 3 ($P<0.05$). Jejunal inflammation was significantly higher in Group 2 compared with Group 3 ($P<0.05$). For the whole small intestine, inflammation was significantly higher in Group 2 when compared with Group 3 ($P<0.05$). In all groups, the animals showed a mixed infiltrate within the lamina propria, with variable numbers of lymphocytes, plasma cells, macrophages and eosinophils. Neutrophils were occasionally present within the lamina propria in the three groups. In Group 3 (control), neutrophils were present in small numbers in three pigs in the duodenum and in one pig in the ileum. In Group 1 (Ringer Lactate), in the duodenum, small numbers were also present in three pigs, moderate numbers in one pig, and large numbers in two pigs; in the jejunum, small numbers of neutrophils were present in one pig, and moderate numbers also in one pig; in the ileum, there were small numbers of neutrophils in two pigs. For Group 2 (HES 130/0.4), in the duodenum, small numbers of neutrophils were present in two pigs and moderate numbers in three pigs; in the jejunum, moderate numbers of neutrophils were present within the lamina propria in one pig, in the ileum in small numbers in one pig, and in moderate numbers in another pig. Detailed information on the inflammatory infiltrate in each intestinal segment is in Appendix A (Appendix tables 4, 5 and 6).

The number of neutrophils per 10 high power fields (HPFs, 40x) was determined at the level of the villi for the duodenum in all the animals from groups 1, 2 and 3. Although no statistically significant differences were found in the neutrophil count between groups, the number of neutrophils was higher in groups 1 and 2, in comparison with the control group, with a mean of 7,09 (median 5, range 1-18) neutrophils per 10 HPFs in group 1, 5,36 (median 4, range 2-12) neutrophils per 10 HPFs in group 2 and 2 (median 1.5, range 0-5) neutrophils per 10 HPFs in group 3 (control) (Figure 18).

Within the submucosa, a minimal to mild infiltrate with minimal to small numbers of plasma cells and, in some pigs, occasional eosinophils and rare neutrophils, was present independently of the group (Figure 19).

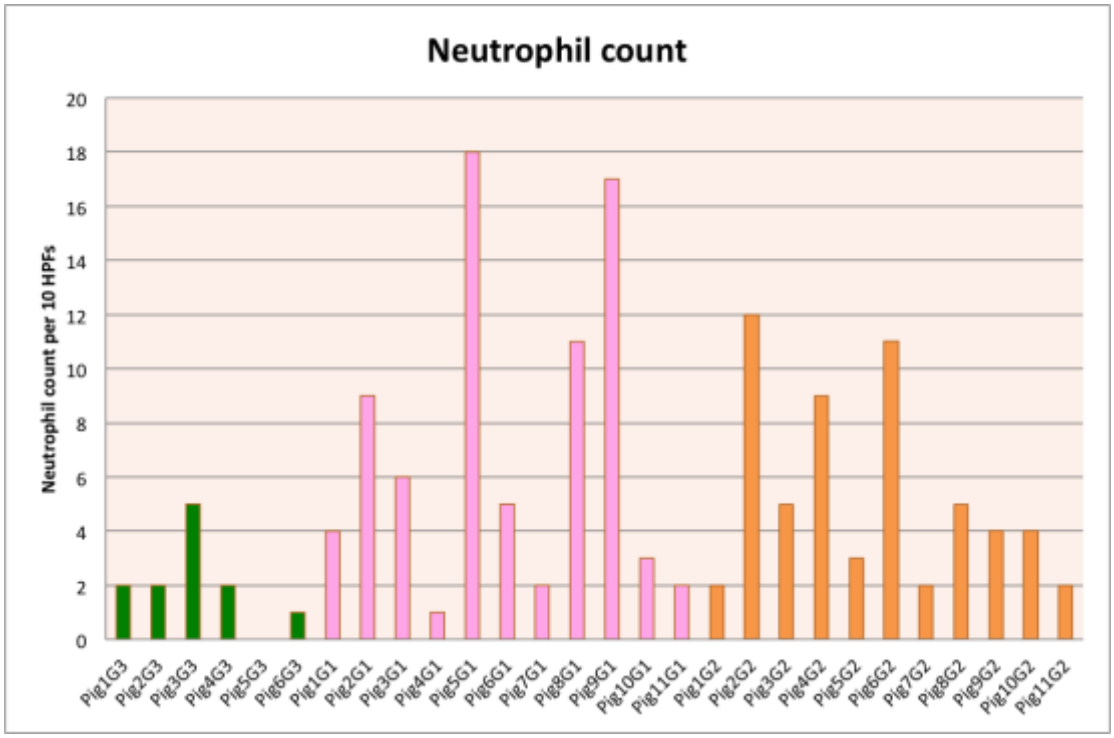

Figure 18. Number of neutrophils per 10 high power fields (HPFs) in the lamina propria of the villi, in all the pigs from groups 1 (G1), 2 (G2) and 3 (G3; control group). A higher number of neutrophils per 10 HPFs was observed in the animals from groups 1 and 2, when compared with the animals belonging to the control group, however this difference was not statistically significant.

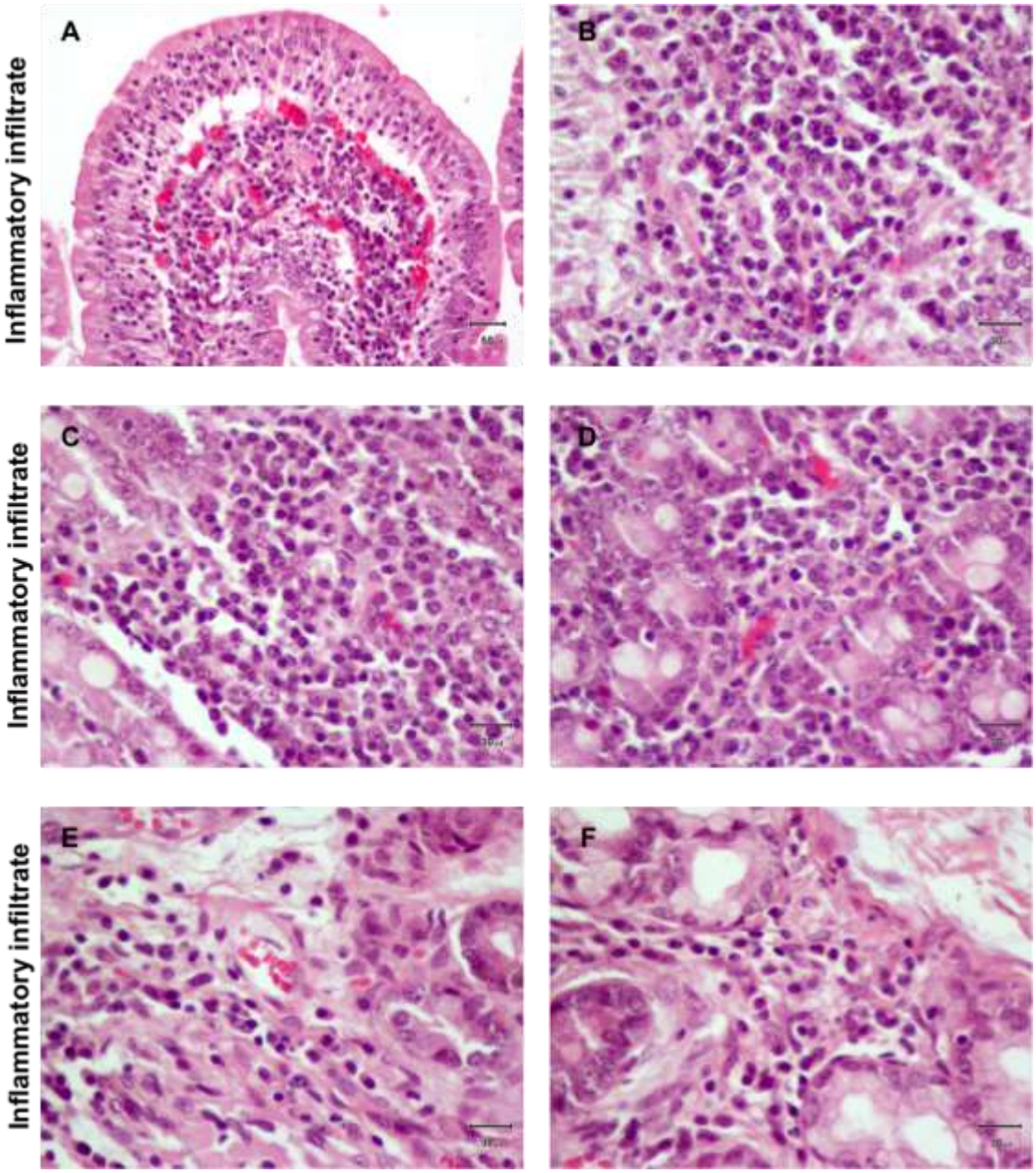

Figure 19. Inflammatory infiltrate (grade 3) composed by lymphocytes, plasma cells and occasional neutrophils observed within the lamina propria at the level of the tip of the villi (note also the moderate, grade 2, congestion of the capillaries present) (A & B), middle of the villi (C) and glands within the mucosa (D) in the jejunum of one pig from group 1 (pig 8). Note the presence of a mild infiltrate composed by small numbers of lymphocytes, plasma cells and also rare neutrophils within the submucosa in the same pig (E & F).

The oedema present in the sections evaluated was represented by a variable degree of distension of the lymphatics, occasionally containing pale eosinophilic material, and by the presence of clear spaces between collagen strands within the lamina propria and submucosa. A variable amount of pale eosinophilic material was also present, occasionally, within the lamina propria (Figure 20). The oedema was significantly higher in the duodenum in Group 1, when compared with Groups 2 and 3 ($P<0.01$). When considering the whole small intestine, mean levels of oedema were significantly higher in Groups 1 and 2 when compared with Group 3 ($P<0.05$).

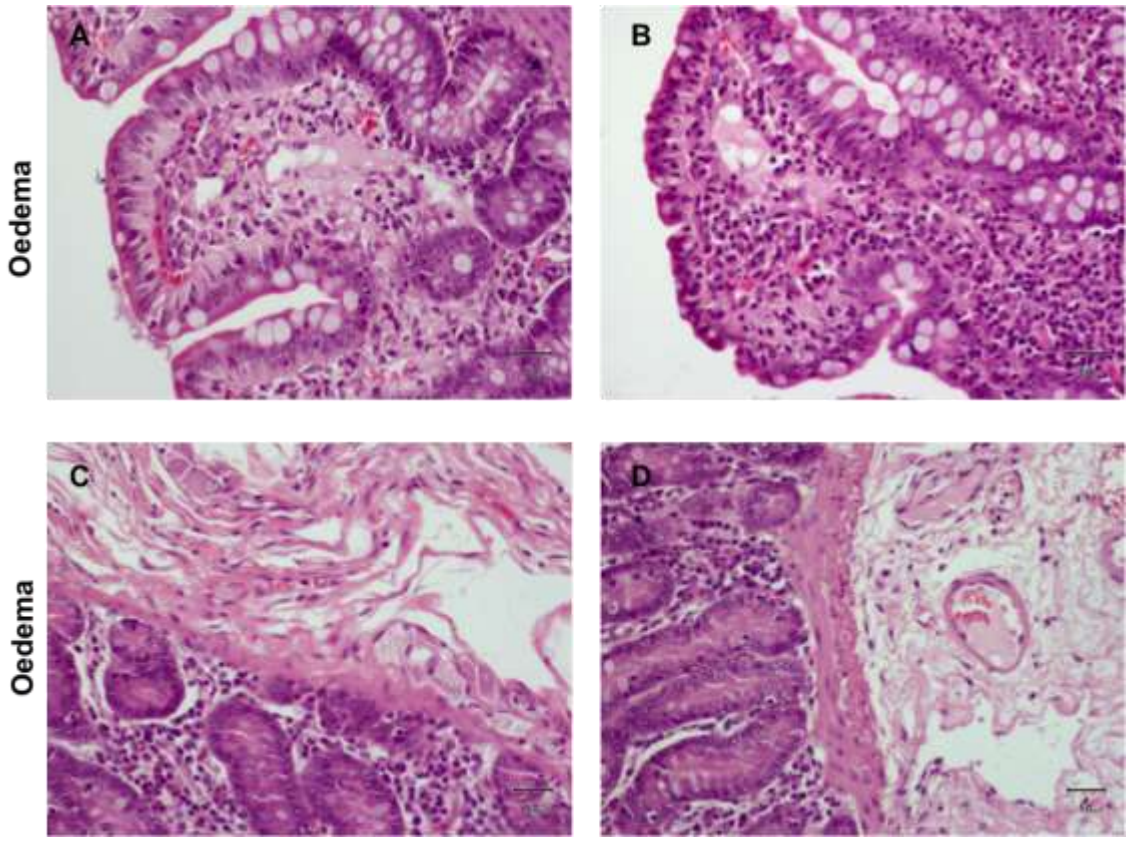

Figure 20. Oedema. Grade 2 oedema in the lamina propria of the jejunum in one pig from group 1 (pig 5) (A & B). Grade 3 oedema of the submucosa and grade 2 oedema of the lamina propria, adjacent to the muscularis mucosa, in the jejunum of a pig belonging to the same group (pig 6) (C & D).

When assessing the congestion levels in the whole small intestine, median levels were significantly lower in Groups 1 and 2 than in Group 3 ($P<0.05$).

Regarding hyperaemia of the small intestine, the median level in the pigs in which volume replacement was done with HES 130/0.4 was significantly higher than in pigs treated with Ringer Lactate ($P<0.05$), but the same could not be confirmed when comparing these with the Group 3. No significant differences in haemorrhage, necrosis, cell degeneration or epithelial detachment grading were observed in the small intestinal segments or whole small intestine, between the three groups. Focal coagulative necrosis at the tip of one villus was observed in the duodenum of one pig from Group 1 (Ringer Lactate). Grading data for each parameter is presented in tables 7 and 8 of Appendix A.

3.5.4. Cytochrome c Antibody

The immunoreactivity to the cytochrome c antibody was assessed in six animals from each group for the duodenum, jejunum and ileum.

The intensity of cytoplasmic immunoreactivity to the cytochrome c antibody varied between 1 (weak) to 4 (very intense), in all the intestinal segments, for all groups, with a predominant punctate staining pattern in the crypts and glands of the lamina propria and submucosa, independently of the intensity of staining (Figure 21A).

In group 1, the predominant staining pattern in the epithelium of the villi in the duodenum was punctate in 1 pig and diffuse in 5 pigs; in the jejunum, it was punctate in all 6 animals and in the ileum it was punctate in 5 pigs and diffuse in 1 pig.

In group 2, in the duodenum, the predominant pattern of staining was punctate in 2 pigs and diffuse in 4 pigs; in the jejunum and ileum it was predominantly punctate in 4 pigs and diffuse in 2 pigs.

In group 3, in the duodenum and jejunum, the predominant staining pattern in the epithelium of the villi was punctate in all 6 pigs; in the ileum it was predominantly punctate in 4 pigs and diffuse in 2 pigs.

Overall a diffuse pattern was predominant in the duodenum in groups 1 and 2 (Figure 21B) when compared to group 3 (control group), in which there was a predominant punctate pattern (Figures 21C and 21D). However, and although

statistical significance has been observed with the Kruskal Wallis test ($p<0.05$), no statistical significance was observed with post-hoc comparisons.

For the crypts, the staining pattern observed was punctate in all the animals in groups 1, 2 and 3.

A punctate staining pattern was observed in the muscularis mucosa and in the muscularis propria in all groups (Figures 21E and 21F).

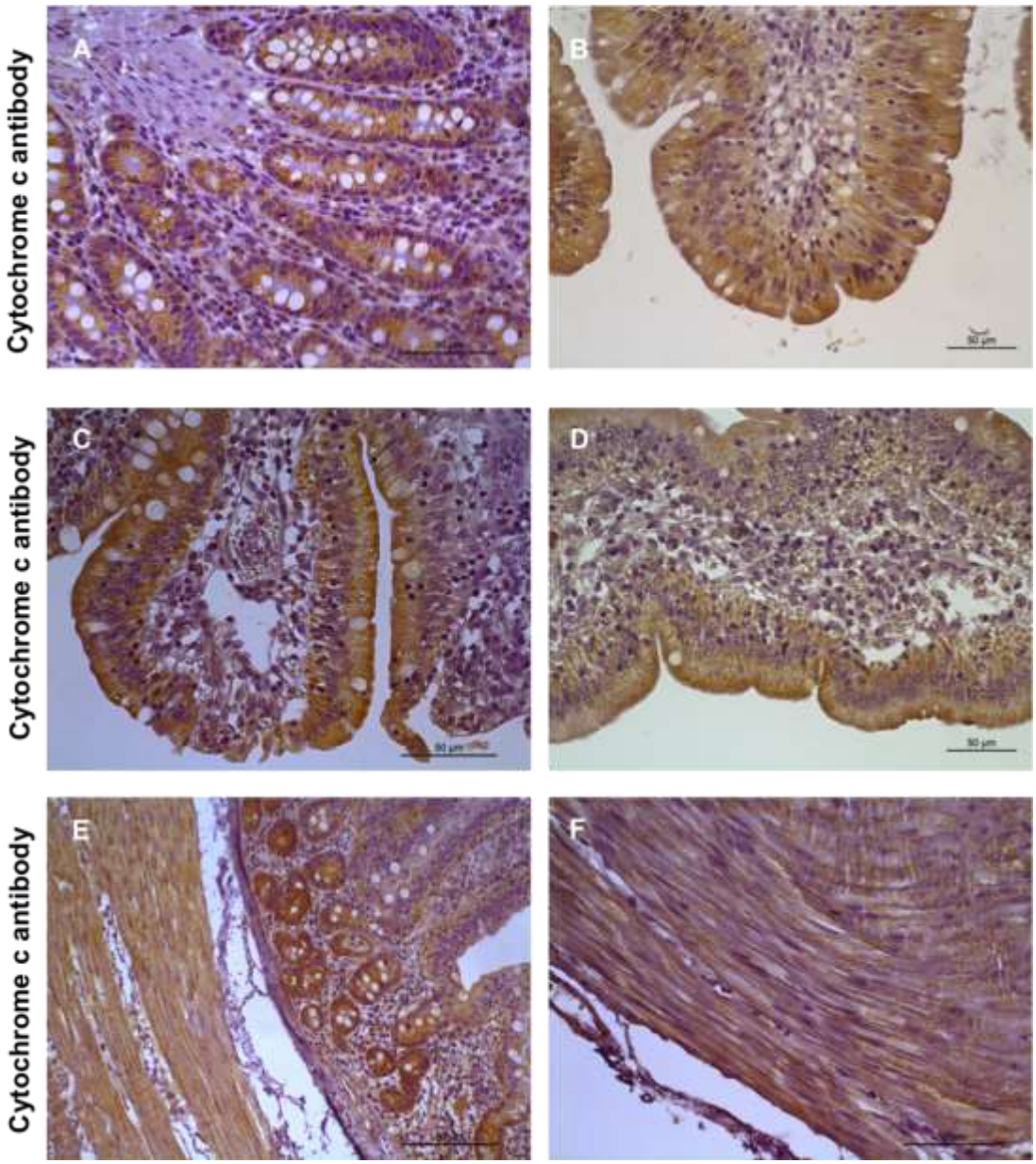

Figure 21. Cytochrome c antibody immunoreaction. Moderate positive cytoplasmic reaction in the glands of the jejunum, with a predominant punctate pattern (A) and intense positive cytoplasmic reaction in the tip of the duodenal villus, with a predominant diffuse pattern in one pig from Group 1 (pig 2) (B). Moderate positive reaction in the villus epithelium, with a predominant punctate pattern in two animals from the control group (Group 3) (C&D; pigs 1 and 2, respectively). Positive reaction with a marked punctate pattern in the muscularis propria (E&F; Group 3, pig 3).

Regarding the intensity of staining observed in the epithelium of the villi, in group 1 the median intensity of staining with cytochrome c antibody was 3 (intense) for the duodenum, 2 (moderate) for the jejunum and 3 (intense) for the ileum. In group 2, the median intensity of staining was 3 (intense) for all the small intestinal segments. In group 3, the median intensity of staining was 2 (moderate) in the duodenum and jejunum and 3 (intense) in the ileum. No statistically significant differences were observed between groups.

The median intensity of staining for the crypts in groups 1 was 2 (moderate) in all the small intestinal segments. In group 2, it was 2 (moderate) in the duodenum and jejunum and 2.5 (moderate to intense) in the ileum. In group 3 the median intensity of staining for the crypts was 2 (moderate) in the duodenum, 1.5 (weak to moderate) in the jejunum and 3 (intense) in the ileum. No statistically significant differences have been observed between groups.

Cytoplasmic staining with variable intensity and pattern was also present within the cytoplasm of plasma cells and lymphocytes within the lamina propria. Macrophages were negative.

Detailed data for the staining pattern and intensity of staining for the cytochrome c antibody is shown in tables 9, 10 and 11 in Appendix A.

3.5.5. M30

Apoptosis detection using the M30 Cytodeath® Fluorescein (Roche, Manheim, Germany) immunofluorescence assay was performed in six pigs from each group, in the duodenum, jejunum and ileum.

Apoptotic small intestine epithelial cells exhibited different patterns of cytoplasmic positivity, from infrequent weak reactions confined to the periphery of the cell, to very strong reactions in the entire cytoplasm (Figures 22 to 25).

The median high score was a) for the duodenum 27.08 (4.89-52.8) in group 1, 42.16 (7.05-74.97) in group 2 and 33.11 (0.46-66.96) in group 3; b) for the jejunum 10.19 (1.33-69.7) in group 1, 16.03 (0.63-112.31) in group 2 and 5.34 (3.22-15.57) in group 3; and c) for the ileum 10.44 (3.99-23.12) in group 1, 11.37 (5.84-117.88) in group 2 and 5.73 (2.16-9.77) in group 3. No significant differences were found between groups.

For the entire small intestine, the median high score was 12.36 in group 1, 16.03 in group 2 and 5.645 in Group 3. Values were significantly higher in group 2 than in group 3 ($P< 0.05$).

The median of the total number of apoptotic cells positive to M30 per small intestine segment was a) for the duodenum 151.5 (65-381) in group 1, 181 (40-414) in group 2 and 192.5 (7-399) in group 3; b) for the jejunum 67 (17-451) in group 1, 75.50 (7-566) in group 2 and 45 (29-70) in group 3; and c) for the ileum 76.50 (56-120) in group 1, 92.50 (33-846) in group 2 and 37 cells (13-60) in group 3. The total number of apoptotic cells in the ileum was significantly higher in group 2, when compared to group 3 ($P<0.05$) (Figure 26). For the entire small intestine, the median numbers of apoptotic cells were 90 (17-451) in group 1, 88.5 (7-846) in group 2 and 40.50 (7-399) in group 3. No significant differences were seen between groups (Figure 27).

Considering the percentage of apoptotic cells positive for M30, the median value was a) for the duodenum 8.96% in group 1, 13.47% in group 2 and 13.04% in group 3; b) for the jejunum 5.33% in group 1, 6.285% in group 2 and 3.895% in group 3; and c) for the ileum 4.63% in group 1, 5.315% in group 2 and 2.145% in group 3. In the ileum, the percentage of apoptotic cells was significantly higher in group 1 and group 2 when compared to group 3 ($P<0.05$). For the entire small intestine, the median percentage of apoptotic cells was 5.935% in Group 1, 6.38% in Group 2 and 3.085% in Group 3. No significant differences were observed between groups.

The median number of apoptotic cells positive with M30 per mm^2 was a) for the duodenum 159.3 (68.35-400.6) in group 1, 190.3 (42.06-435.3) in group 2 and 202.4 (7.36-419.6) in group 3; b) for the jejunum 70.46 (17.88-474.2) in group 1, 78.34 (7.36-595.2) in group 2 and 47.32 (30.49-73.61) in group 3; and c) for the ileum 80.44 (58.89-126.2) in group 1, 97.27 (34.70-889.6) in group 2 and 38.91 (13.67-63.09) in group 3. The median numbers of apoptotic cells per mm^2 in the ileum was significantly higher in group 2, when compared to group 3 ($P<0.05$) (Figure 28). For the entire small intestine, the median number of apoptotic cells per mm^2 was 94.63 (17.88-474.2) in Group 1, 92.01 (7.36-889.6) in Group 2 and 42.59 (7.36-419) in Group 3. No significant differences were observed between groups.

The median number of detached cells per segment was a) for the duodenum, 11 in group 1 (0 -83), 31 in group 2 (0-57) and 0 in group 3 (0 - 36); b) for the jejunum, 0 in group1 (0-34), 0 in group 2 (0-49) and 15 in group 3 (0-234); c) for the ileum, 0 in group1 (0-72), 0 in group 2 and 0 in group 3. From these detached cells, in the duodenum, the median number of positive cells for M30 was 2.5 (0-46) in group 1, and 7 (0-27) in group 2; all detached cells were negative for M30 in group 3. In the jejunum, the median number of positive detached cells for M30 was 0 in all groups, however ranges were, in group 1, 0 to 3, in group 2, 0 to 21 and in group 3, 0 to 31. No positive detached cells for M30 were identified in the ileum for any of the groups.

The median percentage of detached cells positive for M30 was a) for the duodenum, 15.63% in group 1, 22.35% in group 2 and 0% in group 3; b) for the jejunum, it was 0 in all groups, with the ranges 0 to 8.82% in group 1, 0 to 60% in group 2, and 0 to 18.42% in group 3; c) for the ileum it was 0 in all groups, as no positive detached cells were detected.

The median number of detached cells for the entire small intestine was 0 in all groups, however ranges were, in group 1, 0 to 83, in group 2, 0 to 57 and in group 3, 0 to 234. In the entire small intestine, the median number of detached cells positive for M30 was 0 for all groups, however ranges were, in group 1, 0 to 46, in group 2, 0 to 27, and in group 3, 0 to 38.

The median number of detached cells negative to M30 was a) in the duodenum, in group 1, 8.5 (0-37), in group 2, 24 (0-35) and in group 3, 0 (0-36); b) in the jejunum, in group 1, 0 (0-31), in group 2, 0 (0-28) and in group 3, 15 (0-196); c) in the ileum, it was 0 for all groups, however in group 1, range was 0 to 72.

The median number of detached negative cells for the entire small intestine was 0 for all groups with ranges of 0 to 72 in group 1, 0 to 35 in group 2 and 0 to 196 in group 3.

No significant differences were observed between groups for detached cells per segment, detached cells for the entire small intestine, detached cells per segment positive for M30, detached cells in the entire small intestine positive for M30, detached cells per segment negative to M30, or negative detached cells in the whole small intestine.

The values of high score, total number of apoptotic cells, percentage of apoptotic cells, apoptotic cells per mm^2, detached cells, negative detached cells,

positive detached cells and percentage of positive detached cells for all groups are showed in tables 14 and 15 in Appendix A.

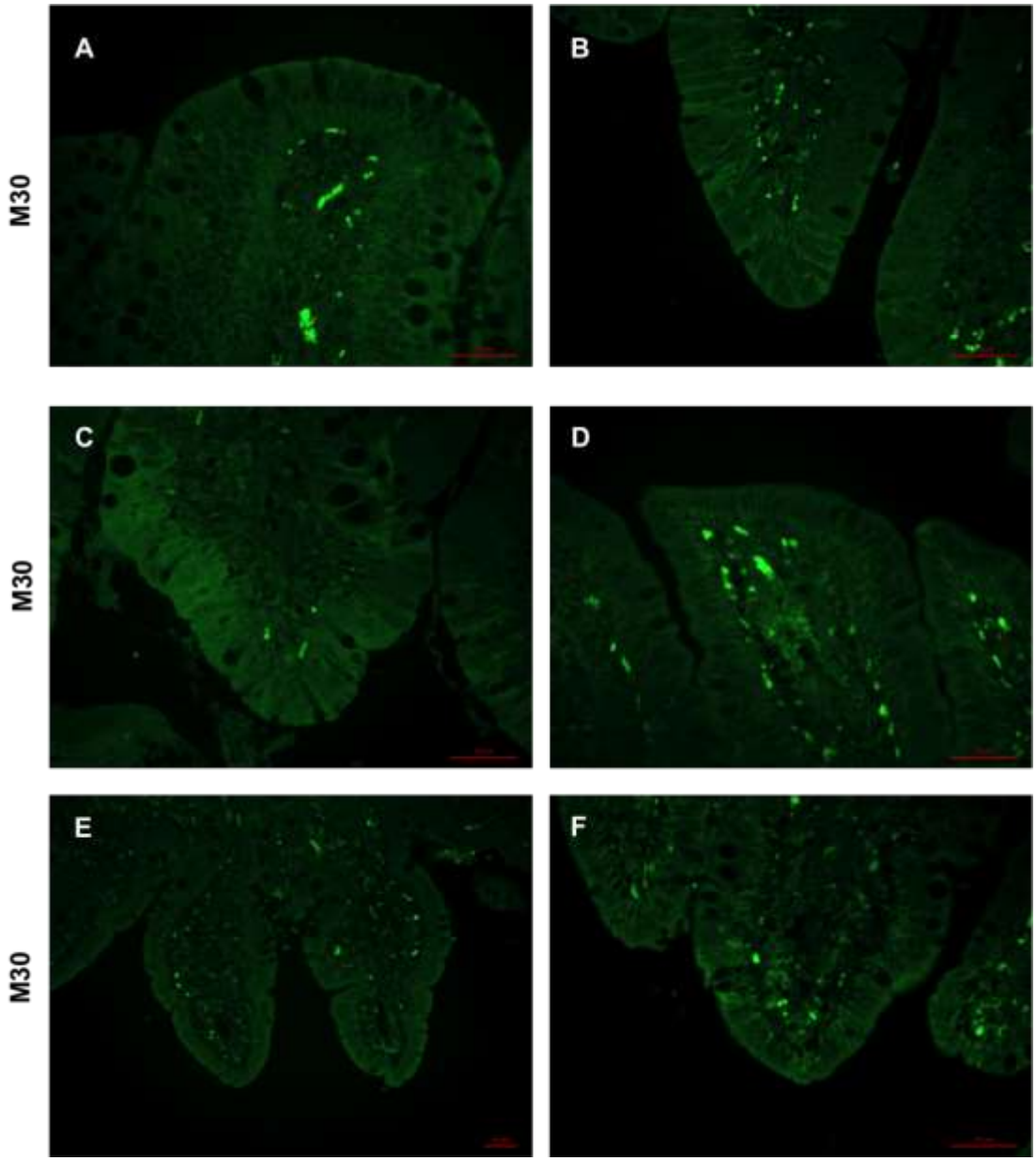

Figure 22. Immunofluorescence reaction observed for M30 in group 1 (Ringer Lactate). In the duodenum, weak, intense in individual cells and diffuse intense positive cytoplasmic reaction, in the epithelial cells of the tip villi (A, pig 4; B, pig 1 and C, pig 2, respectively). Weak positive cytoplasmic reaction at the apical surface and basal surface of the epithelial cells from the villi in the jejunum (D, pig 4) (these cells were not counted as apoptotic cells). In the ileum, weak positive cytoplasmic reaction in the apical surface of epithelial cells at the tip and body of the villi (E, pig 6), and intense positive cytoplasmic reaction in individual epithelial cells at the tip of the villi (F, pig 1).

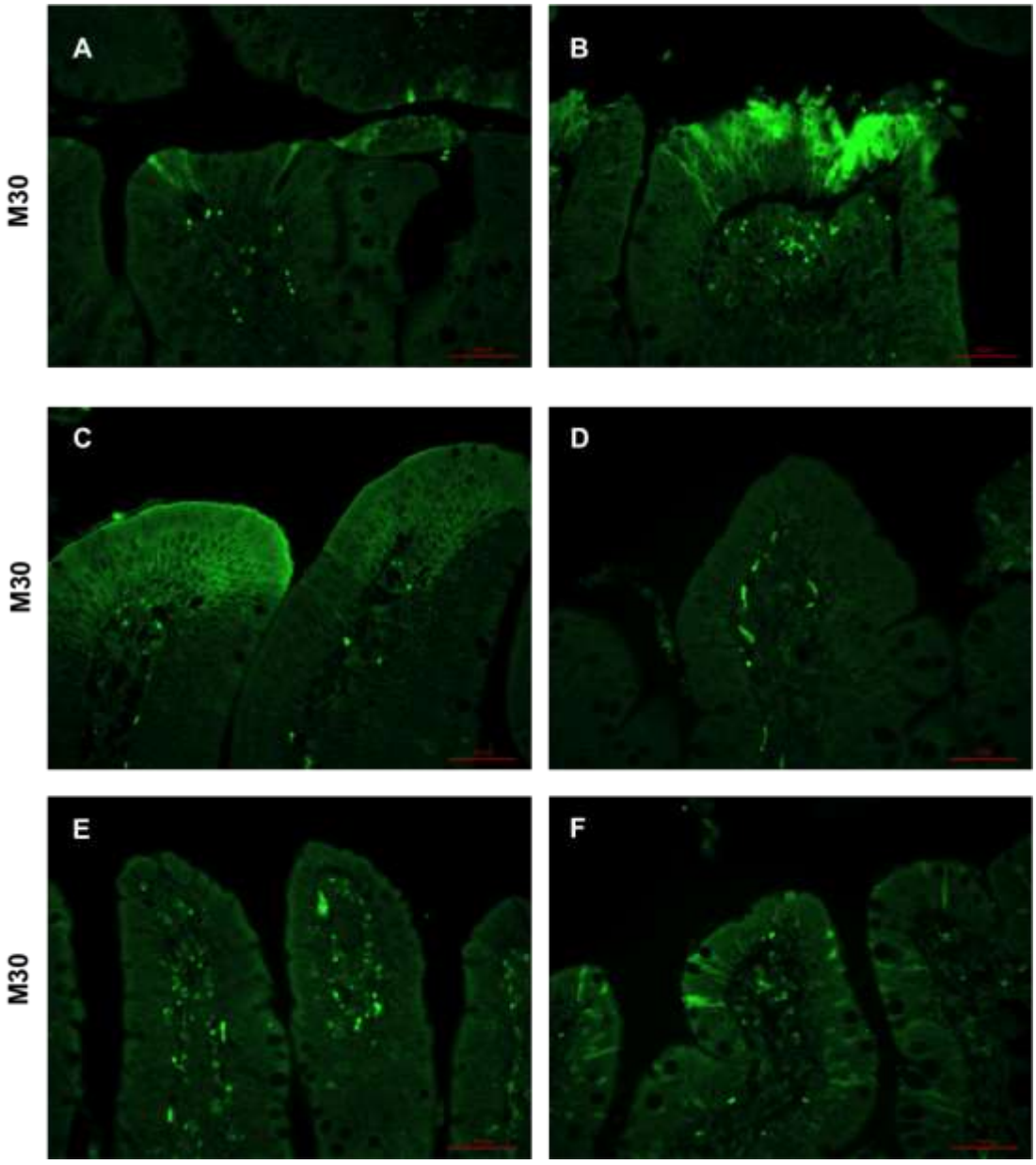

Figure 23. Immunofluorescence reaction observed for M30 in group 2 (HES 130/0.4). Intense positive cytoplasmic reaction in individual epithelial cells of the tip of the duodenal villi (pig 2) (A) and intense positive cytoplasmic reaction in one group of detaching epithelial cells from a duodenal villus (pig 2) (B). Very intense, diffuse, positive cytoplasmic reaction in the epithelium of duodenal villi (pig 5) (C). Weak to moderate positive cytoplasmic reaction at the basal surface of the epithelial cells from a jejunal villus (pig 2) (D) (these cells were not counted as apoptotic cells). Moderate positive cytoplasmic reaction at the apical surface of the epithelial cells from the villi in the ileum (pig 4) (E) (these cells were not counted as apoptotic cells). Also in the ileum, intense positive cytoplasmic reaction seen in individual epithelial cells at the tip and body of villi (pig 1) (F).

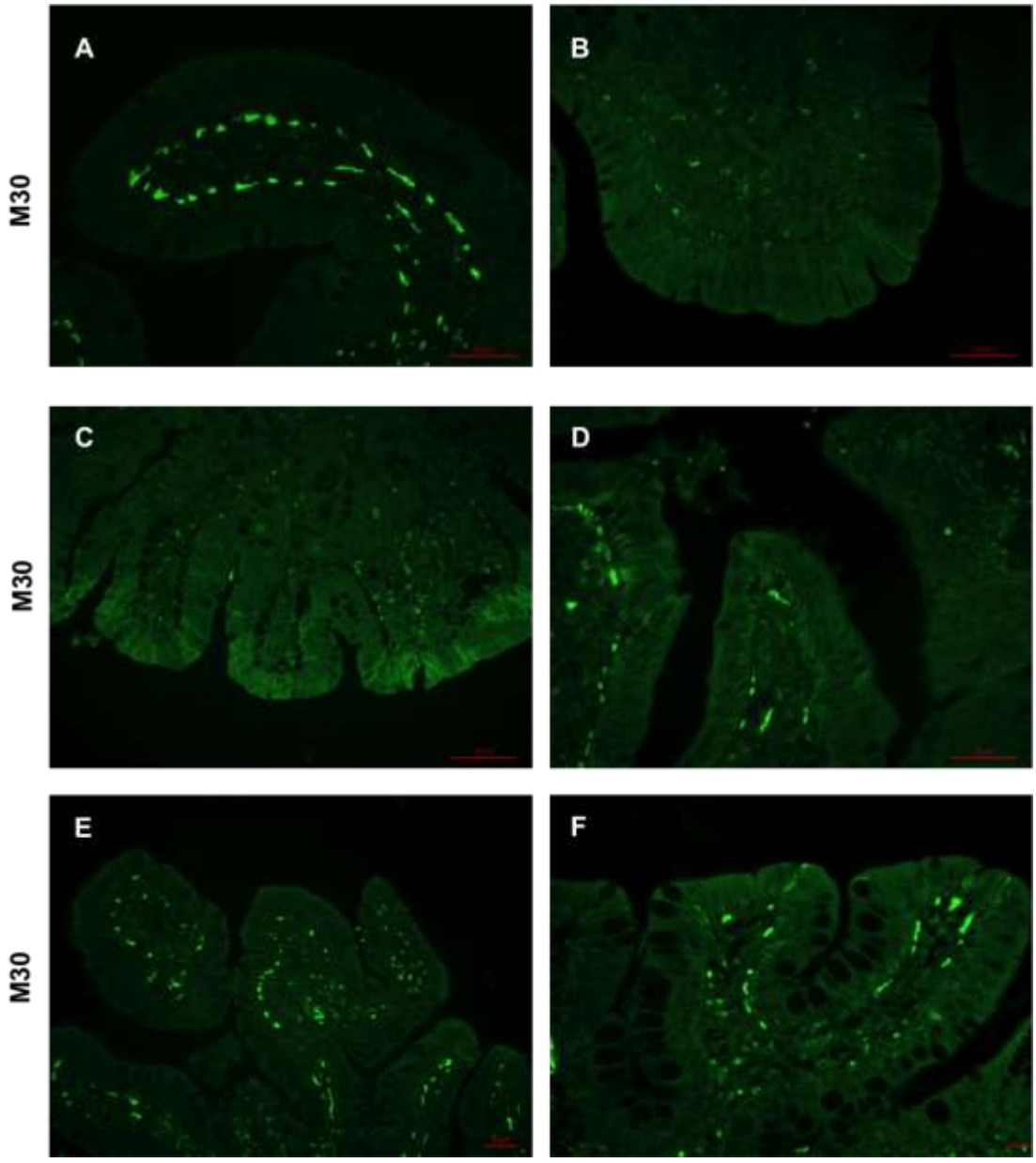

Figure 24. Immunofluorescence reaction observed for M30 in group 3 (Control). Negative reaction in one duodenal villus (pig 4) (A) and, also in the duodenum, weak positive cytoplasmic reaction in epithelial cells of the tip of one villus (pig 5) (B). Intense, diffuse, positive cytoplasmic reaction in the epithelium of duodenal villi (pig 3) (C). Moderate positive cytoplasmic reaction at the apical surface and intense at the basal surface of the epithelial cells from the villi in the jejunum (pig 1) (D) (these cells were not counted as positive apoptotic cells). In the ileum, weak positive cytoplasmic reaction in epithelial cells of the tip of the villi (pig 2) (E), and intense positive cytoplasmic reaction in individual epithelial cells at the tip of the villi (pig 3) (F).

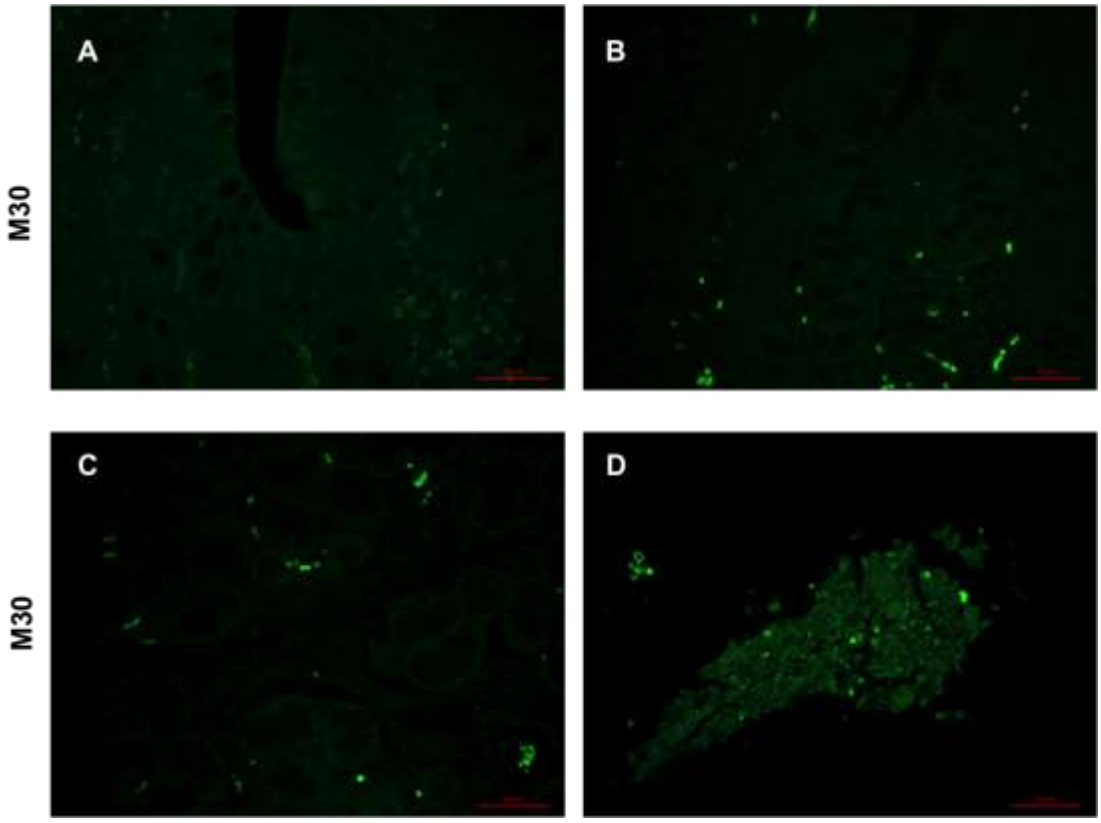

Figure 25. Immunofluorescence reaction observed for M30 in the crypts, Brunner glands and detached cells in all groups. Negative reaction in the epithelial cells of the crypts in the duodenum (A, pig 4, group1) and jejunum (B, pig 6, group 2), and in the Brunner glands in the duodenum (C, pig 4, group 3). Moderately intense to intense positive reaction in the detached cells within the intestinal lumen (D, pig 2, group 1).

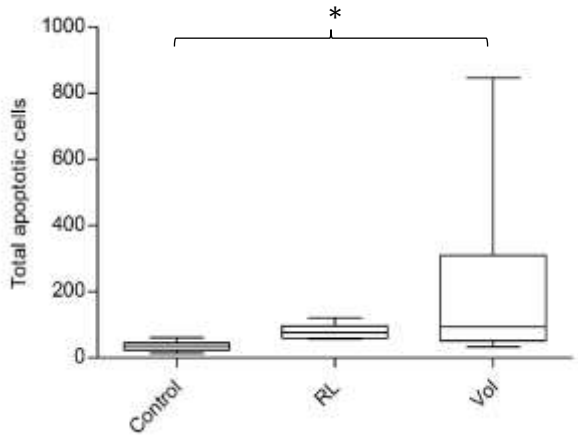

Figure 26. Box plot of the total number of apoptotic cells positive for M30, per group, in the ileum. The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. The total number of apoptotic cells in the ileum was significantly higher in group 2, when compared to group 3 (control group) (*, statistically significant differences between groups – $p<0.05$).

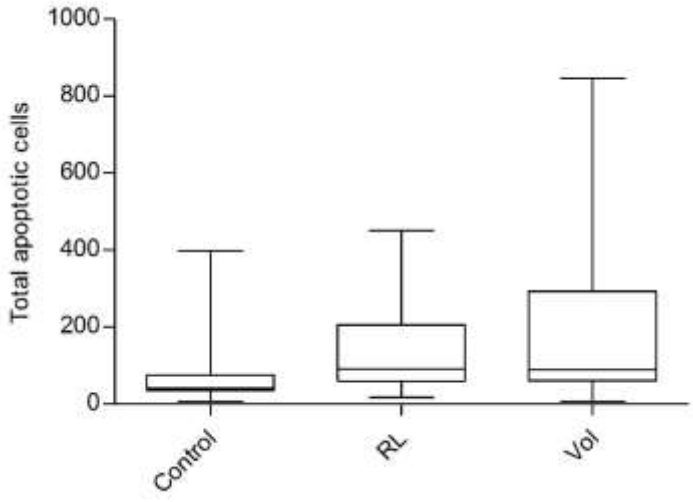

Figure 27. Box and whisker plot showing the total number of apoptotic cells positive for M30 per group, in the entire small intestine. The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. No significant differences were observed between groups.

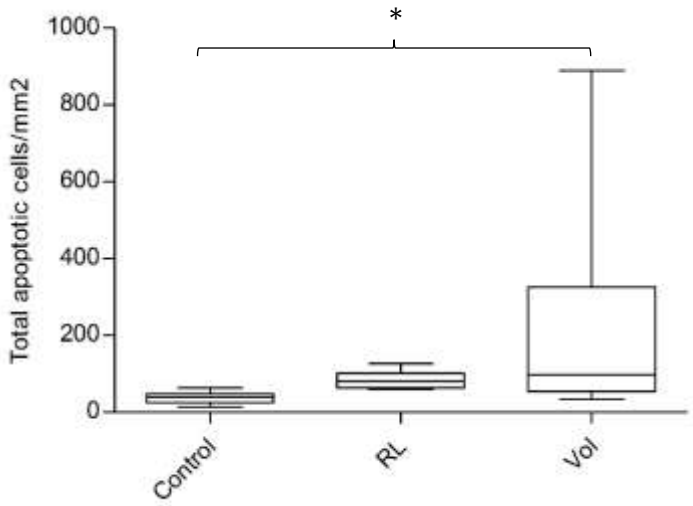

Figure 28. Box plot of the total number of apoptotic cells positive for M30 per mm^2, per group, in the ileum. The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. The total number of apoptotic cells per mm^2 was significantly higher in group 2 (HES130/04) when compared with the control group (group 3) (*, statistically significant differences between groups – $p<0.05$).

3.5.5 TUNEL Method

The detection of apoptosis using the TUNEL Method was performed in two pigs from each group in the duodenum, jejunum and ileum. The TUNEL method detects late stages of apoptosis, in which DNA fragmentation has already occurred. Thus, following a short period of time after hypoperfusion and volume replacement, such a late stage of apoptosis was not expected to be present in the analysed small intestinal tissues (Duan et al. 2003). Therefore, a small number of animals were analysed as a screening method, to rule out possible unexpected differences between groups.

The median high score obtained was a) for the duodenum 5.925 (1.25-10.6) in group 1, 10.71 (2.21-19.21) in group 2 and 58.37 (4.53-112.2) in group 3; b) for the jejunum 11.56 (3.83-19.29) in group 1, 12.72 (7.49-17.95) in group 2 and 11.20 (6.27-16.13) in group 3; and c) for the ileum 2.385 (0.11-4.66) in group 1, 9.09 (7.49-17.95) in group 2 and 2.40 (0.67-4.13) in group 3. No significant differences were found between groups. For the entire small intestine, the median high score was 4.245 in Group 1, 9.230 in Group 2 and 5.400 in Group 3. No significant differences were observed between groups.

The median of the total number of apoptotic cells with positive nuclear immunoreaction to the TUNEL staining per small intestine segment was a) for the duodenum 71 (15-127) in group 1, 75 (11-139) in group 2 and 294.5 (61-528) in group 3; b) for the jejunum 111 (43-179) in group 1, 107 (104-111) in group 2 and 196 (130-262) in group 3; and c) for the ileum 27 (2-52) in group 1, 153.5 (152-155) in group 2 and 23.5 cells (12-35) in group 3. For the entire small intestine, the median numbers of apoptotic cells positive to the TUNEL staining were 47.50 (2-179) in Group 1, 125 (11-155) in Group 2 and 92.5 (12-528) in Group 3. No significant differences were observed between groups (Figure 29).

Regarding the percentages of apoptotic cells with positive nuclear immunoreaction for TUNEL staining, median value was a) for the duodenum 4.665% in group 1, 4.91% in group 2 and 22.98% in group 3; b) for the jejunum 7.16% in group 1, 6.66% in group 2 and 9.035% in group 3; and c) for the ileum 1.275% in group 1, 8.19% in group 2 and 1.385% in group 3. For the entire small intestine, the

median percentage of apoptotic cells was 2.39% in Group 1, 7.26% in Group 2 and 4.375% in Group 3. No significant differences were observed between groups.

The median number of apoptotic cells exhibiting positive nuclear reaction for TUNEL staining per mm^2 was a) for the duodenum 151.1 (31.91-270.2) in group 1, 159.6 (23.40-295.7) in group 2 and 65.45 (1.12-129.8) in group 3; b) for the jejunum 236.2 (91.48-380.80) in group 1, 228.7 (221.3-236.2) in group 2 and 417 (276.6-557.4) in group 3; and c) for the ileum 57.45 (4.26-110.6) in group 1, 326.6 (323.4-329.8) in group 2 and 49.99 (25.52-74.46) in group 3. For the entire small intestine, the median number of apoptotic cells positive to TUNEL Staining per mm^2 was 101.1 (4.26-380.8) in Group 1, 265.9 (23.40-329.8) in Group 2 and 102.1 (1.120-557.4) in Group 3. No significant differences were observed between groups.

The median number of detached cells per segment was a) for the duodenum, 39 in group 1 (1-77), 104 in group 2 (56-152) and 17.50 in group 3 (0-35); b) for the jejunum, 40.50 in group1 (37-44), 122.5 in group 2 (44-201) and 72 in group 3 (13-131); c) for the ileum, 101.5 in group 1 (34-169), 9.5 in group 2 (0-19) and 11.50 (2-21) in group 3. From these detached cells, in the duodenum, the median number of cells with nuclear positive immunoreaction for TUNEL staining was 2.5 (1-4) in group 1, 46 (8-84) in group 2 and 13.50 (0-27) in group 3. In the jejunum, the median number of positive cells for TUNEL staining was 7 (2-12) in group 1, 18.50 (13-24) in group 2 and 9 (1-17) in group 3. In the ileum, the median number of positive cells for TUNEL staining was 11.50 (5-18) in group 1, 0 in group 2 and 2 (0-4) in group 3.

The median percentage of detached cells positive for TUNEL staining was a) for the duodenum, 52.60% in group 1, 34.78% in group 2 and 38.57% in group 3; b) for the jejunum, 16.34% in group 1, 30.51% in group 2 and 10.34% in group 3; c) for the ileum it was 12.68% in group 1, 0% in group 2 and 9.525% in group 3.

The median number of detached cells for the entire small intestine was 40.50 (1-169) in group 1, 50 (0-201) in group 2 and 17 (0-131) in group 3. In the entire small intestine, the median number of detached cells positive for TUNEL staining was 4.50 (1-18) in group 1, 10.50 (0-84) in group 2 and 2.5 (0-27) in group 3.

The median number of detached cells negative to TUNEL staining was a) in the duodenum, in group 1, 36.50 (0-73), in group 2, 58 (48-68) and in group 3, 4 (0-8); b) in the jejunum, in group 1, 33.50 (32-35), in group 2, 104 (20-188) and in group 3, 63 (12-114); c) in the ileum, in group 1, 90 (29-151), in group 2, 9.5 (0-19) and in group

3, 9.5 (2-17). The median number of detached negative cells for the entire small intestine was 35.50 (0-151) in group 1, 34 (0-188) in group 2, and 10 (0-114) in group 3.

No significant differences were observed between groups for detached cells per segment, detached cells for the entire small intestine, detached cells per segment positive for TUNEL staining, detached cells in the entire small intestine positive for TUNEL staining, detached cells per segment negative to TUNEL staining, or negative detached cells in the whole small intestine. The results obtained with the TUNEL method are showed in tables 12 and 13 in Appendix A.

Epithelial cells, both still attached and detached, in areas of evident epithelial detachment exhibited frequently positive nuclear immunoreactivity with TUNEL staining. However positivity was also seen in areas with no morphological evidence of detachment (Figure 30A and 30C).

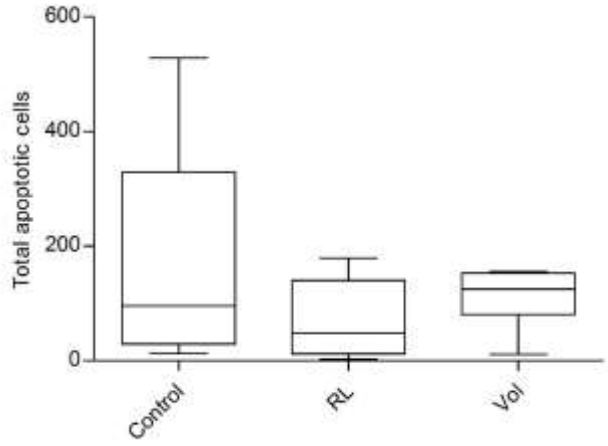

Figure 29. Box and whisker plot showing the total number of apoptotic cells positive for TUNEL staining per group, in the entire small intestine. The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. No statistically significant differences were observed between groups.

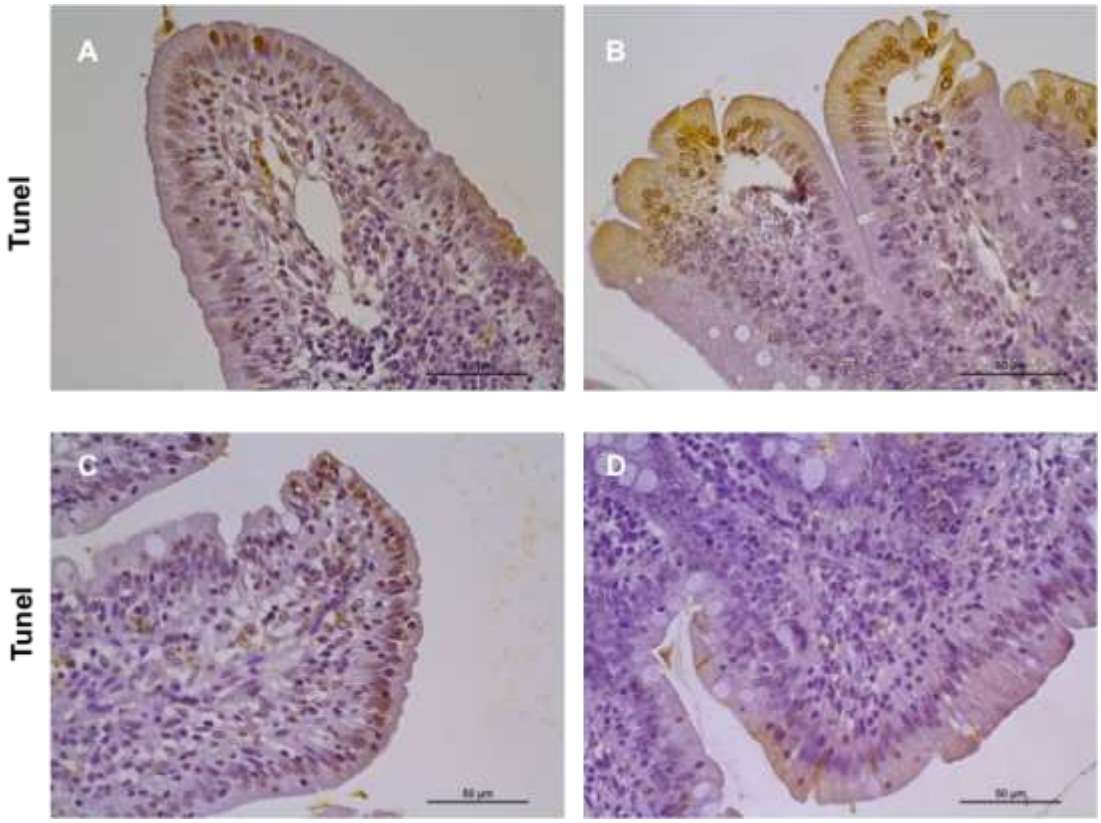

Figure 30. TUNEL method. Moderate positive nuclear reaction in the cells of the epithelium of the villus, in the duodenum, in one pig from Group 2 (pig 2) (HES 130/0.4) (A), and intense positive nuclear reaction at the tip of the duodenal villus, also in one pig from Group 2 (pig 1) (B). Note the enlargement of the subepithelial space, subjacent to the positive lifted epithelium. Moderate positive reaction in the epithelium of the villus in the jejunum of one animal from Group 1 (pig 2) (Ringer Lactate) (C). Negative reaction in a pig from the Group 3 (pig 1) (control) (D).

CHAPTER 4

Discussion and Conclusions

4.1. General findings

Haematology, biochemistry and blood gas analysis were performed during the different study periods in all groups and no significant differences have been observed between groups 1 and 2 at any stage. At the end of the study, and as expected, haematocrit, haemoglobin, albumin and total proteins were lower in groups 1 and 2 when compared to group 3 (control group). In addition, the red blood cell count was significantly lower in group 2 when compared to group 3.

Statistically significant differences in haemodynamic parameters such as systolic, mean and diastolic arterial blood pressures were observed between groups 1 and 3, and between groups 2 and 3, when comparing to the bleeding period. No differences have been observed between groups 1 and 2 in any of the haemodynamic parameters analysed during the study. At baseline, statistically significant differences were observed between groups 1 and 3 in heart rate, and in systolic arterial blood pressure, and between groups 2 and 3 in central venous pressure. No significant differences were present between groups 1 and 2.

Regarding cerebral monitoring, the only significant differences observed in the BIS and INVOS were between groups 2 and 3, when compared to the bleeding period. Groups 1 and 2 showed similar BIS and INVOS during the different periods of the study.

Overall, the differences observed between groups 1 and 3, and between groups 2 and 3 in the blood analysis, haemodynamic parameters and cerebral monitoring are considered to be related to the haemorrhage that occurred in groups 1 and 2, and did not occur in group 3 (control group). Thus, the differences observed in the intestinal mucosa in this study are attributed to the effect of bleeding and volume replacement in the different study groups, and not due to different haemodynamic or cerebral conditions that could influence the intestinal perfusion.

In this study, a significantly higher percentage of mucosal loss in the duodenum of the pigs from group 1, group in which the volume replacement was performed

using Ringer Lactate solution, when compared with group 2 (group in which the volume replacement was performed with HES 130/0.4 solution) and group 3 (control group) was observed. In the jejunum and ileum, the percentage of mucosal loss was also significantly higher in group 1 when compared to group 2 and the median levels of mucosal loss percentage in the whole small intestine were significantly higher in group 1 than in groups 2 and 3. The crypt:interstitium ratio was also assessed however no significant differences were observed between groups. Regarding the semi-quantitative and qualitative parameters evaluated in the small intestine, inflammation in the duodenum was significantly increased in groups 1 and 2, when compared to the control group and inflammation in the jejunum was significantly higher in group 2 also when compared to the control group. Overall, for the whole small intestine, inflammation was significantly higher in group 2 when compared to the control group. The inflammatory infiltrate observed within the lamina propria was composed by variable numbers of lymphocytes, plasma cells, macrophages and eosinophils and neutrophils were occasionally present in the three groups, being however in higher numbers in groups 1 and 2, although statistical significance was not achieved. Very few neutrophils were present in the lamina propria of animals belonging to the control group.

Oedema within both the lamina propria and submucosa was significantly increased in the duodenum in group 1, when compared to groups 2 and 3 and, in the whole small intestine, it was significantly higher in both groups 1 and 2 when compared to the control group (group 3).

Congestion in the whole small intestine was significantly lower in groups 1 and 2 than in the control group and hyperaemia was significantly higher in group 2, in which volume replacement was performed with HES 130/0.4, when compared to group 1, in which pigs received Ringer Lactate solution.

Regarding the immunohistochemical results obtained in the present study, and first for the immunoreactivity to the cytochrome c antibody, in the duodenum of groups 1 and 2, the staining pattern in the cells of the villi epithelium varied from punctate to diffuse, with a predominant and more pronounced diffuse pattern in the epithelium of the tip of the villi; and in the control group (group 3), for all segments, the punctate pattern was the predominant pattern, with this pattern of staining being also observed in the epithelium of the villi (including the tip of the villi). The

differences in staining pattern were not evident in the jejunum and ileum in the animals analysed. Overall no differences in the intensity reaction were seen between groups in any of the intestinal segments, with the main finding being the predominant punctate staining pattern observed in the animals from the control group when compared with a more diffuse cytoplasmic pattern in the animals form group 1 and 2, more noticeable in the duodenum and in the epithelium of the tip of the villi.

When investigating the immunoreactivity to the cytochrome c antibody in the duodenum of groups 1 and 2, the predominant staining pattern observed in the epithelium of the tip of the villi was a diffuse cytoplasmic pattern, while in the control group (group 3), for all intestinal segments, the punctate cytoplasmic pattern was the predominant pattern. Although the intensity of reaction has not been different between groups, the predominance of a diffuse cytoplasmic staining pattern in the epithelial cells of the tip of the villi in groups 1 and 2 suggest that cytochrome c has been released to from the mitochondrial membrane being present free within the cytosol. These observations are indicative that this pro-apoptotic event took place in the intestinal epithelium in the groups that suffered haemorrhage, although without obvious differences between the group in which Ringer Lactate solution has been administered for volume replacement (group1), and the group (group 2) that received HES 130/0.4. The predominance of this diffuse pattern was seen in the duodenum but not in the jejunum and ileum, supporting again a higher sensitivity of this intestinal segment to hypoperfusion.

For the detection of apoptosis using the M30 Cytodeath® Fluorescein, the staining intensity, evaluated through the calculation of the high score, was significantly higher for the whole small intestine in group 2 when compared to the control group. The total number of apoptotic cells in the ileum was significantly higher in group 2 when compared to group 1. Regarding the percentage of apoptotic cells, in the ileum, it was significantly higher in both groups 1 and 2 when compared to group 3. The number of apoptotic cells per mm^2 in the ileum was significantly higher in group 2, comparing to the control group. No significant differences were seen between groups in detached cells per segment positive for M30 or detached cells in the entire small intestine positive for M30.

For the evaluation of apoptosis in the small intestine with the TUNEL method, the number and percentage of apoptotic cells with positive nuclear immunoreaction

and detached cells positive for TUNEL staining did not differ between groups. Albeit the small number of animals used per group does not allow any significance to be attributed to these results, the relevant finding for the study is that the animals that were analysed showed no change that would justify continuing the TUNEL technique using a higher number of animals; this was as expected, as this method detects apoptosis at late stages of apoptosis, in which DNA fragmentation has already occurred. Considering this limitation, it would not be possible to draw objective conclusions and perform a clear, non-dubious, interpretation of the findings.

Regarding the blood analysis for the three groups, no significant differences were found between groups in the values of the parameters assessed in the beginning of the study. In the end of the study period, significant differences have been between groups 1 and 2 and the control group in red blood cell values, haematocrit, haemoglobin, total protein and albumin, with the values for these parameter being higher in the control group (group 3). These differences are likely to be related to the blood loss suffered by animals in groups 1 and 2.

The variations observed in the haemodynamic parameters monitored during the study, such as systolic, diastolic and mean blood pressure, heart rate, cardiac output, central venous pressure and SPO_2 were overall related to the bleeding. No significant differences were observed between groups 1, 2 and 3 in demographics, and body temperature. No statistically significant differences were observed in these parameters between groups 1 and 2. Significant differences between groups 1 and 3 and 2 and 3 were observed in systolic, diastolic and mean blood pressure, cardiac output and central venous pressure, when compared to the bleeding period, which was expected, given that animals in group 3 (control group) did not suffer haemorrhage.

Regarding brain monitoring, no statistically significant differences have been observed between groups 1 and 2 in BIS, INVOS or $SvjO_2$ during the entire study period. Differences have been observed in BIS and INVOS between group 3 and group 2, when compared to the bleeding period.

Overall, there were no significant differences in blood analysis, haemodynamic or cerebral activity between groups 1 and 2 and, therefore, the results from the histopathology and immunohistochemical analysis are considered to be related only to the effect of haemorrhage and reperfusion with different fluids, without any possible

effects of a distinct haemodynamic or cerebral behaviour between the groups, or of the anaesthetic protocol used, in the tissues examined.

In the present study, we hypothesized that the intravenous administration of HES 130/0.4 in the initial resuscitation phase in clinical situations where severe hypoperfusion occurs due do acute bleeding, followed by a short maintenance phase with Ringer Lactate under general anaesthesia, could yield a better intestinal perfusion reflected in the integrity of intestinal wall and mucosa, when compared to the infusion of Ringer Lactate. The percentage of mucosal loss observed in the small intestine, both in each small intestine segment and entire small intestine was higher in group 1, in which volume replacement was performed using Ringer Lactate solution, when compared to group 2, in which HES 130/0.4 was used. In both groups 1 and 2, the percentage of mucosal loss was higher in the duodenum than in the jejunum and ileum, suggesting that this segment is more sensitive to hypoperfusion.

Hyperaemia in the entire small intestine was higher in the pigs from group 2 when compared to those in group 1, suggesting that the administration of HES 130/0.4 solution for volume replacement may be associated with a compensatory hyperaemia and consequent increase in the intestinal blood flow and, likely, of oxygen transport to this organ.

Inflammation was significantly higher in groups 1 and 2, when compared with the control group (group 3), in the duodenum and whole small intestine, and significantly higher in group 2 when compared with the control group in the jejunum. These results indicate that a significant inflammatory response occurs following haemorrhage, which is still possible to assess histopathologically following volume replacement. Again, the duodenum was more consistently and similarly affected in both groups 1 and 2, which is agreement with the increased percentage of mucosal loss observed and supports once again an increased sensitivity of this segment to hypoperfusion.

Oedema of the intestinal wall was significantly higher in group 1 when compared to group 2 and control group (group 3), in the duodenum. For the whole small intestine, mean levels were significantly higher in both groups 1 and 2 when compared with the control group, and there were no significant differences between groups 1 and 2.

Congestion levels in the whole small intestine were lower in groups 1 and 2 when compared with the control group.

4.2. Histopathology discussion

The bleeding followed by reperfusion in groups 1 and 2, resulted in the development of subepithelial space at the apex of the villi, extension of the subepithelial space with moderate lifting of the epithelial layer and, less frequently, in congestion of the blood vessels at the tip of the villi. The development and extension of the subepithelial space was followed, in some cases, by the detachment and loss of epithelium of the tip of the villi, in variable degrees. These findings are in agreement with what was described by Chiu and colleagues in their study about intestinal mucosal lesions in hypoperfusion (Chiu et al. 1970). These authors, in the seventies, performed a series of experiments using a dog model in order to characterize thee morphological changes in the intestinal wall in low-flow states and correlating it to haemodynamic and metabolic alterations. Three groups were part of the study: one in which there was a blood flow to the superior mesenteric artery which was close to the normal flow; a second group with a decreased blood flow to the same artery and a group in which this artery was clumped. The histological lesions in the villi were characterized and sequence of development of these lesions was evaluated. The first change to be observed in the villi was the development of the subepithelial space (Gruenhagen's space), at the level of the tip, being often associated with congestion of the capillaries in the area. This space then extended and there was lifting of the intestinal epithelium from the lamina propria, which initially occurred at the tip of the villi and afterwards started then to extend to the sides of the villi, with epithelial detachment occurring also on the sides of the villi, and progressing to complete detachment, with the appearance of denuded villi; at this stage, complete exposure of the lamina propria and often dilated capillaries was observed. The most severe morphological change observed, considered by Chiu and colleagues to be the highest grade of the developed grading system, was the disintegration of the lamina propria, associated with haemorrhage and ulceration (Chiu et al. 1970). The percentage of intestinal mucosal loss mucosa was significantly higher when Ringer Lactate was used for volume replacement, when compared with using HES 130/0.4.

Intestinal mucosal loss is known to occur following ischaemia, starting with the detachment of the epithelial cells at the tip of the villi, due to a decreased level of oxygen available to these cells. If ischaemia is prolonged, the changes in the mucosa may affect the entire villi, up to its base (Chiu et al. 1970; Robinson et al. 1981; Juel et al. 2004; Derikx et al. 2008; Matthijsen et al. 2009; Kvietys 2010). The fact that less percentage of mucosal loss was observed in the animals in which volume replacement was performed using HES 130/0.4 solution is highly suggestive that this solution was capable of more efficiently restore the small intestine perfusion following severe controlled haemorrhage. In the present study the intestinal permeability was not evaluated, therefore it is not clear whether the degree of mucosal loss observed would be related with an increased permeability and impairment of the intestinal barrier function. However, the relation between intestinal damage and intestinal permeability in intestinal ischaemia and reperfusion injury has been reported to be directly correlated (Juel et al. 2004). In this same report, to investigate intestinal permeability, the transport across the intestinal wall of a molecule that has a similar size as endotoxins, polyethylene glycol-4000, was evaluated, and its increase in the urine and blood correlated with the increase in the percentage of denuded basement membrane at the tip of the villi, in the jejunum (Juel et al. 2004). This finding suggests that increased mucosal loss is very likely related with impairment of the intestinal barrier function, increasing the risk of endotoxin translocation. The results obtained in the present study indicate that HES130/0.4, when used for volume replacement in the clinical situation of controlled haemorrhage may decrease small intestine mucosal loss, decreasing as well the possibility of intestinal barrier function impairment and endotoxin translocation.

The duodenum was the small intestine segment more affected by haemorrhage followed by volume replacement, with a higher percentage of mucosal loss when compared with the jejunum and ileum. The blood supply of the duodenum is mainly assured by the cranial pancreaticoduodenal artery, which is a 4^{th} generation arterial branch from abdominal aorta, which is issued after the total hepatic and right gastric blood supply. The jejunum is irrigated by jejunal arteries, a direct 2^{nd} generation branch from abdominal aorta, and the ileum by three direct distinct 2^{nd} and 3^{rd} generation arterial branches from abdominal aorta (Barone 1996). Thus, in a situation of severe hypovolaemia, the duodenum is probably significantly more exposed to

hypoperfusion than jejunum and ileum. These anatomic characteristics may explain the increased duodenal mucosal loss observed in the pigs that have received Ringer Lactate when compared to HES 130/0.4 in our study.

Inflammation was significantly higher in the Ringer Lactate and HES groups, when compared with the control group, in the duodenum and whole small intestine, and significantly higher in the HES group when compared with the control group in the jejunum. Independently of the group and small intestine segment, the animals showed a mixed infiltrate within the lamina propria, with variable numbers of lymphocytes, plasma cells, macrophages, eosinophils and occasional neutrophils. Severe hypovolaemia and haemorrhagic shock are associated with decreased intravascular volume and, as a consequence, with decreased organ perfusion. Intestinal ischaemia followed by reperfusion is known to impair the intestinal barrier function, allowing bacterial translocation into the circulation, and promoting this way systemic inflammation (Collard & Gelman 2001; Fink & Delude 2005). In a recent study, Grootjans and colleagues reported that prolonged intestinal ischaemia followed by reperfusion is associated with a local and systemic inflammatory response, possibly due to loss of the intestinal barrier with exposure to intraluminal pathogens, and also to the leakage of intracellular components from damaged enterocytes (Grootjans et al. 2010). Although a state of hypovolaemic shock or complete ischaemia has not been reached in the present study, the increased inflammation observed in the Ringer Lactate and HES groups, when compared to the control group, may be related with the acute inflammatory response to intestinal hypoperfusion, as a consequence of the bleeding followed by volume replacement. The inflammatory response reported by Grootjans and colleagues is characterized by complement activation and neutrophil sequestration into damaged tissue (Grootjans et al. 2010).

Endothelial dysfunction is believed to be a key contributor to IR injury (Granger 1999; Massberg & Messmer 1998; D L Carden & Granger 2000). IR was seen to be associated with increased capillary permeability and that the permeable endothelium was important source of the xanthine oxidase-derived radicals. Xanthine oxidase-derived radicals in its turn can attract neutrophils to the intestinal mucosa (Grisham et al. 1986) and infiltrating neutrophils were seen to cause the increase in microvascular permeability in IR injury (Hernandez et al. 1987). IR is also believed to activate other interstitial or circulating inflammatory cells, which may result further generation of

inflammatory mediators and in the creation of a chemotactic gradient, potentially facilitating the migration of neutrophils into the damaged tissue (Kvietys 2010). In the present study, neutrophils were observed occasionally in the intestinal wall, and in small numbers in all groups. It is believed that, in IR injury, neutrophils adhere to the vascular endothelium and extravasate to reperfused areas, having an important role tissue damage (Grisham et al. 1986; Hernandez et al. 1987; Granger 1988; Kurtel et al. 1991; D L Carden & Granger 2000); these leukocytes seem to be involved in the injury of different organs in IR, as lungs and liver. However some studies on neutrophils' role in the local small intestine damage following intestinal IR report contradictory findings (Kurtel et al. 1991; Simpson et al. 1993; Matthijsen et al. 2009), and its role in intestinal IR is still unclear. In a recent study, depletion of circulating neutrophil attenuated the IR injury in the small intestine in wild-type mice (Watanabe et al. 2014). In our study, and although not statistically significant, the number of neutrophils present within the lamina propria at the level of the top of the villi, in the duodenum, was higher in groups 1 and 2 than in the control group (group 3). This is suggestive of an increase in the number of neutrophils being related with a period of decreased intestinal perfusion, with consequent decreased oxygen levels, followed by volume replacement. The increased duodenal inflammation observed in the Ringer Lactate and HES groups, when compared to the control group, may have been due to the acute inflammatory response caused by bleeding (Dzienis-Koronkiewicz et al. 1998) associated also with an improved intestinal microcirculation after the Ringer Lactate or HES 130/0.4 administration. The increase in inflammation observed also in the jejunum and in the entire small intestine in the HES 130/0.4 group, when compared to the control, may also be related with an improved intestinal microcirculation associated with HES 130/0.4 administration (Hiltebrand et al. 2009). Also, in the jejunum, HES 130/0.4 induced an increased inflammatory response in the small intestine mucosa, compared with Ringer Lactate (median inflammatory grade 3 for HES 130/0.4 group, and median inflammatory grade 2 for Ringer Lactate group), which was not statistically significant.

The Ringer Lactate solution is a crystalloid solution without intrinsic colloid osmotic pressure, and is expected to enter the interstitial space (Hillman et al. 1997) when used for volume replacement. Oedema was, in fact, significantly higher in the Ringer Lactate group when compared with HES group and control group in the

duodenum. However, for the whole small intestine, mean oedema levels were significantly higher in both Ringer Lactate and HES groups when compared with the control group, and there were no significant differences between Ringer Lactate and HES groups. This result may be related to the significant decrease in the total proteins level in both Ringer Lactate and HES groups, when compared to the control group that did not underwent blood loss. The development of oedema following ischaemia and reperfusion may also result from a vascular damage, with extravasation of intravascular fluid (Donna L Carden & Granger 2000; Eltzschig & Collard 2004). Being the duodenum the small intestinal segment that seemed more sensitive to hypoperfusion followed by reperfusion, with a with a higher percentage of mucosal loss in our study, and being also this segment the one showing a higher degree of oedema in the Ringer Lactate group, when compared with HES and control groups, it is possible that a higher degree of vascular compromise was present in this group in the duodenum, with consequent increased oedema.

The intestinal congestion of individual intestinal segments or of the entire small intestine, was not different between Ringer Lactate and HES 130/0.4 groups. However, congestion levels in the whole small intestine were significantly lower in Ringer Lactate and HES groups when compared with the control group. Animals in Ringer Lactate and HES groups suffered severe haemorrhage, while animals in the control group did not. Furthermore, propofol is known to cause vasodilatation, affecting both venous and arterial vessels, with effects on systemic circulation, probably due to smooth muscle relaxation (Bentley et al. 1989; Samain et al. 2000; Koch et al. 2008). The vasoactive effect of propofol, in association with the fact that bleeding was not performed, may justify the intestinal congestion observed in the animals in the control group.

Hyperaemia in the entire small intestine mucosa, was significantly higher in the group 2 (HES 130/0.4) than in group 1 (Ringer Lactate), with no significant differences when compared with the control group. A hyperaemic reperfusion reaction of the intestinal microcirculation has been reported after administration of hypertonic/hyperoncotic resuscitation fluids, following intestinal superior mesenteric artery occlusion, which induced the normalization of the pH and lactate. The presence of an osmotic gradient across vascular and cell membranes will cause the fluid to go from the interstitial space to the intravascular space, improving the blood flow (Jonas

et al. 2000). Tait and colleagues (Tait & Larson 1991) reported in a study on myocardial blood flow in a severe haemorrhage and resuscitation model in dogs that the use of 6% hetastarch resulted in a compensatory hyperaemia that increased myocardial blood flow and maintained oxygen transport, which was not observed with Ringer Lactate. By increasing the intestinal microcirculation, HES 130/0.4 may have caused a more pronounced hyperaemia in the HES group when compared to the Ringer Lactate group in our study. Hiltebrand and colleagues reported that goal-directed colloid administration using HES 130/0.4 increases microcirculatory blood flow in the small intestine, and intestinal tissue oxygen tension after abdominal surgery (Hiltebrand et al. 2009). Goal directed administration of HES 130/0.4 is also believed to improve the microcirculation of healthy and perianastomotic colon (Kimberger et al. 2009). Furthermore, microscopic in vivo observation of rabbit ear microcirculation showed that intravenous infusion of HES maintained the peripheral circulation, haemodynamics, and cardiac output more effectively in a model of acute severe haemorrhage, comparing with Ringer Lactate (Komori et al. 2005). The higher grade of intestinal hyperaemia observed in our study in the HES 130/0.4 group is in agreement with the increase in intestinal microcirculation reported to be caused by HES 130/0.4, and may have contributed to the low percentage of mucosal loss observed in the HES group when compared to the Ringer Lactate group. However, increased hyperaemia could also be related with an increased blood flow as part of an inflammatory response after hypoperfusion and volume replacement, which may simultaneously have a negative impact in the intestinal mucosa. That said, when the degree of inflammation was similar between the animals in groups 1 and 2, the grade of hyperaemia was still higher in group 2 (HES 130/0.4), which suggests that the hyperaemia is more likely related with an improved microcirculation.

In ischaemia and reperfusion injury, haemorrhage may occur following considerable vascular damage or following extensive damage of the mucosa, with disintegration (Chiu et al. 1970). Haemorrhage was absent in almost all the animals and intestinal segments for all the groups, and was detected within the lamina propria, in the ileum, in one animal form the Ringer Lactate group (moderate) and one animal from the HES 130/0.4 group (mild). It was considered most likely an incidental finding, as it was not associated with necrosis, disintegration of the lamina propria, ulceration or vasculitis.

Minimal, focal, coagulative necrosis of the tip of the villi was observed in the duodenum in one animal from the Ringer Lactate group. The fact that necrosis was a rare finding in the present study is most likely related with the levels of oxygen that were available to the tissues of the small intestine mucosa during the experiment; the partial reduction of the blood flow to the small intestine mucosa with no complete lack of blood circulation still allowed oxygenation of the tissues, although at a lower and abnormal level. Therefore necrosis is minimal when compared with what would be expected if a model of complete vascular occlusion with prolonged ischaemia, instead of a model of haemorrhage had been used. The fact that this focus of coagulative necrosis was seen in the Ringer Lactate group could suggest that the restoring of the intestinal perfusion was less efficient in this group, also supported by the fact that it was present in the duodenum, which was considered the most sensitive segment to hypoperfusion in the present study. However, as just one focal area was seen, these are just speculations.

No significant differences between groups for small intestine segments and entire small intestine were observed for the C:I ratio. C:I ratio was obtained to assess the presence of possible changes within the lamina propria, namely oedema, inflammation and haemorrhage. The reasons for the absence of differences between groups may be related with the fact that the degree of oedema, inflammation and haemorrhage in the intestinal mucosa did not differ in such a proportion between groups that expansion of the lamina propria would be significantly different. Therefore, C:I ratio may not be a reliable method to assess intestinal lesion in the lamina propria following hypoperfusion.

Regarding the mucosal damage grading, no differences were observed between groups. The mucosal damage grading, as developed by Chiu and colleagues, includes, for each grade, specific mucosal changes, from the development of subepithelial (Gruenhagen's) space at the apex of the villi (grade 1) to the loss of the surface of the villi, with exposure of dilated capillaries and disintegration of the lamina propria with haemorrhagic ulceration (grade 5) (Chiu et al. 1970), including more features than just the mucosal loss. In the present study, lesions included in grade 5 were not identified, which is most likely justified by the fact that there was not complete vascular occlusion and that the volume replacement was performed shortly after bleeding (30 minutes after). For the mucosal loss percentages, as previously explained

in the material and methods chapter of the present dissertation, results were obtained with objective measurements of the mucosa and mucosa loss calculation, without including the previous steps of the developing lesion. These two methods of evaluating mucosal damage were used because they were considered different and complementary methods, both with advantages and limitations. Regarding the measurement of the villi and calculation of the mucosal loss, it is important to consider that it is common to have processing artifacts in samples from intestinal mucosa and, for this reason, the observer needs to be extremely cautious not to consider these artifacts a true epithelial detachment when measuring the length of the villi. When performing the measurement of the villi with a manual grid there may be a small degree of subjectivity. Although, in the present study, a careful examination and measurement was performed, to try to overcome these limitations, they still need to be mentioned. In the mucosal damage grading, although Chiu and colleagues system is a very good system, there may still be some differences in lesions that are not accounted for, meaning that slightly severely affected mucosa is attributed the same grade as a mucosa that exhibits a slightly more pronounced injury; for example, considering grades 1, 2 and 3 (grade 1 - development of subepithelial space at the apex of the villi; grade 2 – extension of subepithelial space with moderate separation of mucosa; grade 3 - separation with extensive loss of epithelial surface of some villi), if in one case there was extension of the subepithelial space, with mild separation of the mucosa, this would be a milder lesion than if a moderate separation of the mucosa was present, and both of these lesions would be included in grade 2. The fact that the grading system may group in the same grade lesions that exhibit mild differences implies that these differences may be lost and statistical significance not achieved, especially when there is a relatively small number of animals per group.

Although no statistically significant differences were obtained between groups in this study for mucosal damage grading, grade 4 mucosal damage was seen in the duodenum of two pigs from group 1 (Ringer Lactate); in group 2 (HES130/0.4), mucosal damage grading varied between 0 and 3 in the duodenum (and 0 and 2 in the jejunum and ileum). These results are suggestive that, although no statistically significant differences were confirmed, the higher grade of mucosal damage occurred in animals belonging to Ringer Lactate group. Grade 4 of mucosal loss was scored for the duodenum in one animal form the control group, however, this was attributed to

processing artifact. It is important to reinforce that the mucosal damage grading considers a variety of features and not just the mucosal loss, therefore these two parameters may not be directly comparable.

4.3. Immunohistochemistry discussion

Cytochrome –c, under normal conditions, is located in the intermembrane space of the mitochondria and its release to the cytosol is induced by proapoptotic stimuli. Once in the cytosol, cytochrome-c, together with other released intermembrane proteins and cytosolic factors will activate the cascade of caspases, which are responsible for the progression of the apoptosis and cell death. Cytocrome –c labelling was performed in order to detect if the small intestine would exhibit any degree of mitochondrial disturbance, if there would be a possible and expected involvement of the mitochondrial related pathway in the apoptosis observed in this study, and if it would differ between groups. The cytochrome c can also be evaluated in paraffin embedded tissues using immunohistochemistry (Liu et al. 2012) and flow cytometry has been also more recently reported (Campos et al. 2006; Christensen et al. 2013). It was assumed that a predominant punctate pattern would be related to intermembrane space localization of the cytochrome c, while a diffuse staining reaction would mean that the cytochrome c would be diffusely located within the cytoplasm of the cells, and had been released due to proapoptotic stimuli (Liu et al. 2012). This interpretation was reinforced by the predominant punctate pattern observed in the muscularis propria, which was used as an internal negative control (normal) for each section. In the control group (group 3), the predominant pattern was punctate at the level of the villi epithelium and in the crypts in all small intestine segments, which was the expected, with cytochrome c being in its normal location at the mitochondrial membrane. In groups 1 and 2, in all the intestinal segments, the pattern of staining was punctate to diffuse at the tip of the villi in all the small intestinal segments, with predominance of a diffuse pattern, while the predominant pattern observed in the crypts was the punctuate staining. The diffuse pattern is interpreted as the cytochrome c being diffusely present within the cytosol, and not in the mitochondrial membrane, which indicates that proapoptotic stimuli had induced its release at the tip of the villi. This is suggestive that the epithelial cells at the tip of

the villi, in both groups 1 and 2, suffered proapototic stimuli. The release of cytochrome c into the cytosol occurs in a very early step in the apoptotic pathway, and is usually interpreted as being induced by a decreased level of available oxygen to the epithelial cells at the tip of the villi. Although a predominant diffuse staining pattern has been observed in the epithelium of the tip of the villi in groups 1 and 2, and a predominant punctate staining pattern had been seen in the same location in group 3, this difference was, in the end, not statistically significant. No differences were observed between groups 1 and 2, which is suggestive that likely independently of the fluid used for volume replacement, this early step in the apoptotic pathway was induced in the small intestine. It is important to mention that immunohistochemistry allows the detection of cytosolic cytochrome c but does not allow an objective quantification of the amount of cytosolic cytochrome c present. It could perhaps allow the identification of possible differences in the intensity of the staining that could be a somewhat qualitative indicative of a higher or decreased concentration of cytosolic cytochrome c in any of the groups. The existence of different concentrations of cytosolic cytochrome in the groups, even in the absence of obvious differences in intensity of staining cannot, therefore, be completely ruled out. Methods that would allow the quantification of cytochrome c release to the cytosol, such as Western blot, fluorescence microscopy of immunolabeled cells and flow cytometry, are being implemented for quantifying the cytosolic cytochrome c observed in the intestinal cells of the studied groups.

For the marker of apoptosis M30, duodenum, jejunum and ileum from six pigs from each group were analysed. The total number and percentage of apoptotic cells per intestinal segment and in the entire small intestine were similar for Ringer Lactate and for HES 130/0.4 groups, with the total number of epithelial cells positive for M30 being significantly higher in the HES 130/0.4 group (group 2) when compared with the control group (group 3) and the percentage of cells positive for M30 being significantly higher in both Ringer Lactate group (group 1) and HES 130/0.4 group (group 2) when compared to the control group (group 3), in the ileum. Regarding the number and percentage of apoptotic cells per mm^2, in the ileum it was significantly higher in the HES 130/0.4 group than in the control group. In the entire small intestine the median number of apoptotic cells was higher in the Ringer Lactate group (group 1) (90), but no statistical significance was observed when compared to the HES

130/0.4 group (88.5) or control group (40.5). Results were otherwise very similar between groups. The highest percentage of detached cells were counted in the HES 130/0.4 group, in the duodenum (13.7%), as well as the higher percentage of detached cells with positive reaction to M30 (22.35%). However, statistically significant differences were not observed when compared to Ringer Lactate group (percentage of apoptotic cells - 8.96%; and percentage of detached cells with positive reaction to M30 - 15.63%) or control group (percentage of apoptotic cells - 13.04%; and percentage of detached cells with positive reaction to M30 - 0%). As in the Ringer Lactate group the percentage of mucosal loss was significantly higher in the duodenum and entire small intestine when compared to the HES 130/0.4 group and control group, there was less intact epithelium at the villi tips, and, therefore, a smaller area of intact tissue was available to perform the counting of apoptotic cells, either per mm^2, total number or percentage. The mucosal loss having been higher in the Ringer Lactate group suggests that the apoptotic rate was also higher in this group; but as the epithelial cells were no longer present at the time the tissue was evaluated for apoptosis, this could not be confirmed with the apoptotic count.

Grootjans and colleagues (Grootjans et al. 2010) in their study of intestinal ischaemia-reperfusion induced inflammation and apoptosis, in human patients undergoing pancreaticoduodenotomy, also observed that apoptosis was observed in detaching enterocytes at 30 minutes of reperfusion, and in the luminal debris of damaged cells at two hours of reperfusion, while it was just sporadically present at villi tips after ischemia alone, suggesting that intestinal apoptosis in intestinal reperfusion is associated with reperfusion injury

In the present study, as animals were euthanized a short period of time after hypoperfusion and volume replacement, a late stage of apoptosis, with detectable DNA fragmentation, was not expected to be present in the small intestinal tissues at this stage. The caspase cleavage of CK18 can be detected at two hours after induction of apoptosis, reaching maximum levels after 4 – 6 hours, and occurs before disruption of membrane asymmetry and DNA fragmentation (Leers et al. 1999; Duan et al. 2003). TUNEL labeling has its peak much later, at 12 hours after induction of apoptosis (Duan et al. 2003), and, for this reason, just a small number of animals was included for analysis

with the TUNEL method, in order to allow a relatively quick screening of possible unexpected lesions reflected by DNA fragmentation in intestinal cells. As expected, the results obtained with this method for each individual small intestinal segment or for the whole small intestine did not differ between groups.

4.4 Major limitations of the study

1. The evaluation of the apoptosis just in one phase of the process, which is in a post reperfusion stage. This fact limited the conclusions regarding which changes are related to hypoperfusion and which changes are related to the volume replacement. This could also have been assessed if a control group that had suffered controlled haemorrhage not followed by volume replacement had been used. However, propofol infusion is associated with a marked decrease in splanchnic and renal perfusion (Piriou et al. 1999), which is a relevant fact to consider when evaluating the histological effects of intestinal hypoperfusion following more than four hours of anaesthesia with propofol, as occurred with the pigs in our study. Hence, the objective of the control group used in our study was to exclude the influence of the time of the anaesthesia with propofol, and possible intestinal lesions associated with an also possible marked splanchnic hypoperfusion associated with propofol administration.

2. The existence of a correlation between the intestinal histological changes observed in our study and the intestinal function/dysfunction was not assessed in the present study. It is, however, known that the mucosal injury following ischemia and reperfusion, in the intestine, is associated with impairment of the intestinal barrier function, facilitating bacterial translocation into the circulation, which may lead to systemic inflammation (Koziol et al. 1988; Rush 1992; Mythen & Webb 1994b; Biffl et al. 1996). The intestinal tissue injury is also aggravated by the reperfusion of the already damaged tissue, which also increases both local and systemic inflammation, and may lead to multiple organ failure (Grootjans et al. 2010). In the seventies, Chiu and colleagues tried to correlate the morphological lesions found in their study with the intestinal barrier function by injecting d-tubocurarine intraluminally, which was believed not to cross the mucosal barrier in normal circumstances,

at various stages of mucosal lesion; in animals in which the mucosa exhibited a grade 3 or higher of mucosal damage, death due to acute respiratory paralysis, a characteristic effect of curare poisoning, was seen (Chiu et al. 1970). It is important to mention that mucosal injury, with increase of the subepithelial space followed by mucosal loss, was observed in the present study without partial or complete obstruction of the blood supply to intestinal segments, which is very suggestive that even a smaller degree of hypoperfusion may cause morphologically significant mucosal injury in the small intestine. Furthermore, the intestinal mucosa did not fully recover after 60 minutes following volume replacement, with already re-established haemodynamic parameters. This suggests that there may actually be a degree of barrier function compromise following hypoperfusion due to severe haemorrhage followed by volume replacement.

3. The methods used in the immunohistochemical analysis, namely immunoreactivity to cytochrome c antibody, immunoreactivity to M30 antibody and TUNEL method may not allow the accurate evaluation of proapoptotic stimuli and apoptosis at such an early phase following injury and reperfusion and in the absence of complete ischaemia. In the intrinsic pathway of apoptosis, following the signalling phase, cytochrome c is released from the mitochondria into the cytosol and its release triggers caspase activation resulting in cleavage of cellular targets (execution phase). Therefore, from the methods used, the evaluation of cytosolic cytochrome c is considered the earliest method for detecting early phases of the apoptotic process, as its release to the cytosol is a critical step in this process. In rats, cytosolic localization of cytochrome c in the brain was detected immunohistochemically at four hours, after 90 minutes of ischaemia followed by reperfusion (Fujimura et al. 1998). In a different study also in rats, after transient global cerebral ischemia, cytosolic cytochrome c-positive cells were observed two hours after ischemia, while one day after ischemia, there were almost no TUNEL-positive cells, with a significant number of positive cells being just observed three and five days following ischaemia (Sugawara et al. 1999). In renal cells which underwent glucose-free hypoxia, cytocrome-c was observed free within the

cytosol after three hours, and its translocation form the mitochondrial membrane was complete by seven hours. Cells that leaked cytochrome c were seen to undergo apoptosis after reoxygenation (Saikumar et al. 1998). In the small intestine of mice, following 60 minutes of ischaemia and 60 minutes of reperfusion, the amount of cytochrome c determined from mitochondrial and cytosolic fractions using Western blotting was markedly increased in the cytosolic fractions of the intestinal mucosa after ischaemia or ischaemia and reperfusion (Wu et al. 2007). However no reports have been found in the literature regarding the release of cytochrome c to the cytosol in the small intestine after hypoperfusion followed by volume replacement and how long after injury this important step in the apoptotic process takes place in this organ.

Grootjans and colleagues reported that in the jejunum, following 45 minutes of ischaemia, and 30 minutes of reperfusion, apoptosis detected by immunohistochemistry using the M30 antibody was higher than in segments of jejunum that were just submitted to ischaemia for 45 minutes, suggesting that apoptotic cell death occurred particularly during reperfusion. However after 45 minutes of ischaemia without reperfusion, there was disruption of the epithelial lining and development of the Gruenhagen's space (Grootjans et al. 2010). After 45 minutes of ischaemia followed by 30 minutes of reperfusion, there was shed of injured enterocytes to the lumen (Grootjans et al. 2010). In this study, apoptosis evaluated through the immunohistochemical analysis of M30 was observed at the tips of the villi undergoing epithelial detachment and in the cellular debris seen within the lumen at a later stage (Grootjans et al. 2010). In the present study, although complete ischaemia was not induced, and with an overall period of injury of 20 minutes (bleeding) followed by a waiting period of another 20 minutes before reperfusion, M30 positive cells have been observed in the epithelium of the tip of the villi in the post reperfusion stage and positivity was also seen in detached cells within the lumen.

4.5. Conclusions

In conclusion, the general main findings in this study were:

1. The intravenous administration of HES 130/0.4 in the initial resuscitation phase when hypoperfusion occurred due do controlled arterial bleeding, followed by a short maintenance phase with Ringer Lactate under total intravenous anaesthesia with propofol and remifentanil, yielded a better intestinal perfusion reflected in the integrity of intestinal wall and mucosa, when compared to the infusion of Ringer Lactate alone, with the group of animals in which HES 130/0.4 was used for volume replacement exhibiting a smaller percentage of mucosal loss when compared with animals that received Ringer Lactate, which was statistically significant.
2. No differences in apoptotic events were observed between the groups in which HES 130/0.4 and Ringer Lactate were used for volume replacement following controlled severe arterial bleeding. However as in group 1 (Ringer Lactate) a higher percentage of mucosal loss occurred, the area available for evaluation was smaller and therefore, it is speculated that apoptosis was most likely higher in group 1, leading to higher percentage of mucosal loss. No statistically significant differences were observed regarding the inflammatory infiltrate present, in these groups.
3. Hypoperfusion followed by volume replacement, without complete vascular occlusion and without achieving hypovolaemic shock caused quantifiable changes in the small intestine mucosa, which were more severe when Ringer Lactate was used as volume replacement solution rather than when using HES 130/0.4.

In detail, this study showed us that:

1. There was a significantly higher percentage of mucosal loss in the duodenum of the pigs from group 1, group in which the volume replacement was performed using Ringer Lactate solution, when compared with group 2, in which the volume replacement was performed with HES 130/0.4 solution, and group 3, control group ($P<0.01$). In the jejunum and ileum, the percentage of

mucosal loss was also significantly higher in the Ringer Lactate group when compared to the HES 130/0.4 group ($P<0.05$) and the mucosal loss percentage in the whole small intestine was also significantly higher in the Ringer Lactate group than in HES 130/0.4 group and control ($P< 0.0001$).

2. No significant differences were observed in blood analysis, haemodynamic or cerebral activity between groups 1 and 2. This highly supports the fact that the results from the histopathology and immunohistochemical analysis are related only to the effect of haemorrhage and reperfusion with different fluids in the small intestinal mucosa, ruling out any possible effects of a distinct haemodynamic or cerebral behaviour between the groups, or of the anaesthetic protocol used, in the tissues examined.
3. There were no significant differences were observed between groups in the crypt:interstitium ratio.
4. There was significantly increased inflammation in the duodenum the Ringer Lactate group and HES 130/0.4 group, when compared to the control group ($P<0.05$) and inflammation in the jejunum was also significantly increased in the HES 130/0.4 group when compared to the control group ($P<0.05$). Overall, for the whole small intestine, inflammation was significantly higher in the HES 130/0.4 group when compared to the control group ($P<0.05$).
5. As part of the inflammatory infiltrate observed in the duodenum, neutrophils were present in higher numbers in the lamina propria of animals belonging to Ringer Lactate and HES 130/0.4 groups than in controls, in which just rare neutrophils were seen in the superficial lamina propria. However this difference was not statistically significant.
6. Oedema was significantly increased in the duodenum in the Ringer Lactate group, when compared to HES 130/0.4 group and controls ($P<0.01$); when considering the entire small intestine, it was significantly increased in both Ringer Lactate and HES 130/0.4 groups when compared to the control group ($P<0.05$).
7. Hyperaemia was significantly higher in the HES 130/0.4 group when compared to the Ringer Lactate group ($P<0.05$).

8. Significantly increased congestion in the whole small intestine was observed in Ringer Lactate and HES 130/0.4 groups when compared to the control group ($P<0.05$).
9. For the immunoreactivity to the cytochrome c antibody, the intensity of staining was seen not to be significantly different between groups. However, independently of the intensity of staining, in the duodenum of the animals belonging to the Ringer Lactate and HES 130/0.4 groups, a more pronounced diffuse staining pattern in the epithelium of the tip of the villi was observed, while in the control group a punctate pattern was observed at the same location (epithelium of the tip of the villi). Differences in staining pattern were not evident in the jejunum and ileum.
10. The staining intensity (high score) when using the M30 Cytodeath® Fluorescein for detection of apoptosis was significantly higher for the whole small intestine in HES 130/0.4 group when compared to the control group ($P< 0.05$).
11. The total number of apoptotic cells in the ileum positive for M30 was significantly higher in the HES 130/0.4 group when compared to Ringer Lactate ($P<0.05$). Regarding the percentage of apoptotic cells, in the ileum, it was significantly higher in both Ringer Lactate and HES 130/0.4 groups when compared to the control group ($P<0.05$). The number of apoptotic cells per mm^2 in the ileum was significantly higher in the HES 130/0.4 group, comparing to the control group ($P<0.05$). Apoptosis detection using the M30 Cytodeath® Fluorescein was performed in the, duodenum, jejunum and ileum of six pigs from each group.
12. The number and percentage of apoptotic cells with positive nuclear immunoreaction to the TUNEL staining did not differ between groups. In addition, detached cells positive for TUNEL staining did not differ significantly between groups.

4.6. Conclusiones

En conclusión, los hallazgos principales en este estudio fueron:

1. La administración intravenosa de HES 130/0.4 en la fase de resucitación inicial, cuando la hipoperfusión es debida a un sangrado arterial controlado, seguida por una fase de mantenimiento corta con solución de Lactato Ringer bajo anestesia total intravenosa con propofol y remifentanilo, produjo una perfusión intestinal mejor, como se refleja en la integridad de la mucosa y pared intestinal, en comparación con una infusión de solamente solución de Lactato Ringer, con el grupo de animales en los que HES 130/0.4 fue usado para reemplazo de volumen exhibiendo un menor porcentaje de perdida de mucosa en comparación con los animales que recibieron solución de Lactato Ringer; esta diferencia fue estadísticamente significativa.
2. No hubo diferencias observadas en el número de eventos apoptóticos cuando HES 130/0.4 y solución de Lactato Ringer fueron usados para reemplazo de volumen tras sangrado arterial severo. Sin embargo, como en el grupo 1 (solución de Lactato Ringer) ocurrió un porcentaje de perdida de mucosa mayor, el área disponible para evaluación fue menor y por lo tanto, se especula que la apoptosis pudo ser mayor en el grupo 1, llevando a un porcentaje mayor de pérdida de mucosa. No hubo diferencias estadísticas significativas en la cantidad de infiltrado inflamatorio presente en estos grupos.
3. La hipoperfusión, seguida por reemplazo de volumen, sin oclusión vascular completa y sin llegar a shock hipovolémico, causó cambios cuantificables en la mucosa del intestino delgado; estos fueron más severos cuando solución de Lactato Ringer fue usado para reemplazo de volumen en lugar de HES 130/0.4.

En detalle, este estudio nos mostró que:

1. Hubo un porcentaje de perdida de mucosa duodenal significativamente mayor en los cerdos del grupo 1, grupo en el cual se utilizó solución de Lactato Ringer para reemplazo de volumen, en comparación con el grupo 2 (en el cual para el reemplazo de volumen se utilizó solución HES 130/0.4) y con el grupo 3 (control) ($P<0.01$). En el yeyuno y íleon, el porcentaje de perdida de mucosa también fue significativamente mayor en el grupo

solución de Lactato Ringer en comparación con el grupo HES 130/0.4 ($P<0.05$) y el porcentaje de pérdida de mucosa en la totalidad del intestino delgado también fue significativamente mayor en el grupo solución de Lactato Ringer comparado con el grupo HES 130/0.4 y el grupo control ($P<0.01$).

2. No se observaron diferencias significativas en el análisis de sangre, hemodinámica o actividad cerebral entre los grupos 1 y 2, por lo que se considera que los resultados de la histopatología y análisis inmunohistoquímico se relacionan solamente con el efecto de la hemorragia y la reperfusión con diferentes fluidos en la mucosa del intestino delgado, descartando cualquier posible efecto de un comportamiento hemodinámico o cerebral distinto entre los grupos , o del protocolo anestésico utilizado , en los tejidos examinados.
3. No hubo diferencias estadísticamente significativas entre grupos en cuanto al ratio cripta:intersticio.
4. La inflamación fue significativamente mayor en el duodeno del grupo solución de Lactato Ringer y del grupo HES 130/0.4 en comparación con el grupo control ($P<0.05$) y la inflamación en el yeyuno también estuvo incrementada de forma significativa en el grupo HES 130/0.4, en comparación con el grupo control ($P<0.05$). En general, para la totalidad del intestino delgado, la inflamación fue significativamente mayor en el grupo HES 130/0.4 en comparación con el grupo control ($P<0.05$).
5. Como parte del infiltrado inflamatorio observado en el duodeno, los neutrófilos estuvieron presentes en números mayores en la lámina propia de los animales pertenecientes a los grupos solución de Lactato Ringer y HES 130/0.4 cuándo comparados con el grupo control, en el cual los neutrófilos solo han sido observados en la lámina propia de forma ocasional. Sin embargo, esta diferencia no fue estadísticamente significativa.
6. El edema fue significativamente mayor en el duodeno de los animales pertenecientes al grupo solución de Lactato Ringer, comparado con el grupo HES 130/0.4 y con el grupo control ($P<0.01$); cuando se considera la totalidad del intestino delgado, el edema estuvo incrementado de forma

significativa tanto en el grupo solución de Lactato Ringer como en el grupo HES 130/0.4 con respecto al grupo control ($P<0.05$).

7. La hiperemia fue significativamente mayor en el grupo HES 130/0.4 en comparación con el grupo solución de Lactato Ringer ($P<0.05$).
8. La congestion fue significativamente mayor en todo el intestino delgado en los grupos solución de Lactato Ringer y HES 130 / 0,4 en comparación con el grupo control ($P < 0,05$).
9. La intensidad de tinción de anticuerpo citocromo C no fue significativamente diferente entre grupos. Sin embargo, independientemente de la intensidad de tinción, en el duodeno de los animales pertenecientes a los grupos solución de Lactato Ringer y HES 130/0.4, hubo un patrón de tinción difuso del epitelio más pronunciado en el extremo de las vellosidades, mientras que en el grupo control se observó un patrón de tinción puntiforme en esta misma localización (epitelio del extremo de las vellosidades). No hubo diferencias evidentes en la tinción de yeyuno e íleon.
10. La intensidad de tinción (high score) cuando se usó M30 Cytodeath® Fluorescein para la detección de apoptosis fue significativamente mayor para la totalidad del intestino delgado en el grupo HES 130/0.4 en comparación con el grupo control ($P<0.05$).
11. El número total de células apoptóticas en el íleon positivas para M30 fue significativamente mayor en el grupo HES 130/0.4 en comparación con solución de Lactato Ringer ($P<0.05$). En relación al porcentaje de células apoptóticas en el íleon, este fue significativamente mayor en los grupos solución de Lactato Ringer y HES 130/0.4 cuándo comparados con el grupo control ($P<0.05$). El número de células apoptóticas por mm^2 en el íleon fue significativamente mayor en el grupo HES 130/0.4, en comparación con el grupo control ($P<0.05$). La detección de apoptosis usando M30 Cytodeath® Fluorescein fue realizada en el duodeno, yeyuno e íleon de seis cerdos de cada grupo.

12. El número y porcentaje de células apoptóticas con inmunoreactividad positiva a con la técnica de TUNEL no fue diferente entre grupos. Adicionalmente, las células desprendidas con marcación positiva por el método TUNEL no presentaron diferencias entre grupos.

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APPENDIX A

Appendix Table 1. Percentage of mucosal loss per animal and intestinal segment in Group 1.

Percentage of Mucosal Loss – Group 1 (RL)			
Animals	**Duodenum**	**Jejunum**	**Ileum**
Pig 1	1,47	2,35	1,34
Pig 2	4,65	1,85	0,00
Pig 3	2,48	0,00	0,00
Pig 4	2,41	2,15	3,32
Pig 5	1,65	0,00	0,95
Pig 6	1,48	1,42	0,25
Pig 7	9,34	1,18	1,19
Pig 8	8,23	2,56	1,19
Pig 9	3,44	2,44	1,23
Pig 10	5,74	0,00	0,56
Pig 11	2,73	3,27	3,19
Mean	**3,97**	**1,57**	**1,20**
Standard Deviation	**2,74**	**1,15**	**1,13**
Median	**2,73**	**1,85**	**1,19**

Appendix Table 2. Percentage of mucosal loss per animal and intestinal segment in Group 2.

Percentage of Mucosal Loss – Group 2 (RL)			
Animals	**Duodenum**	**Jejunum**	**Ileum**
Pig 1	1,27	0,00	0,00
Pig 2	1,03	1,05	0,00
Pig 3	1,48	0,91	0,00
Pig 4	0,00	0,00	0,00
Pig 5	0,00	0,00	0,00
Pig 6	2,26	0,00	0,00
Pig 7	1,80	0,00	0,83
Pig 8	4,38	0,58	1,46
Pig 9	0,11	0,13	0,14
Pig 10	0,00	0,00	0,84
Pig 11	0,00	0,00	0,00
Mean	**1,12**	**0,24**	**0,30**
Standard Deviation	**1,36**	**0,40**	**0,51**
Median	**1,03**	**0,00**	**0,00**

Appendix Table 3. Percentage of mucosal loss per animal and intestinal segment in Group 3.

Percentage of Mucosal Loss – Group 3 (Control)			
Animals	**Duodenum**	**Jejunum**	**Ileum**
Pig 1	2,61	1,07	0,00
Pig 2	1,45	2,94	0,47
Pig 3	0,00	0,00	0,48
Pig 4	0,00	0,00	0,14
Pig 5	0,92	0,67	0,36
Pig 6	1,17	0,15	0,65
Mean	**1,02**	**0,81**	**0,35**
Standard Deviation	**0,98**	**1,13**	**0,24**
Median	**1,05**	**0,41**	**0,41**

Appendix Table 4. Grade and cellular composition of the inflammatory infiltrate present in the duodenum, per group.

		Duodenum					
Group	Pig	**Infiltrate Grade**	**Neutrophils**	**Lymphocytes**	**Plasma Cells**	**Macrophages**	**Eosinophils**
Group 3 (Control)	Pig1	2		**	**	**	*
	Pig3	2		**	**	**	*
	Pig3	2	*	**	**	*	*
	Pig4	2	*	**	**	*	
	Pig5	2		**	**	*	
	Pig6	2	*	**	**	*	*
Group 1 (RL)	Pig1	3		*	***	*	
	Pig2	3	*	*	***	*	
	Pig3	3	*	***	**	**	*
	Pig4	2		**	**	**	*
	Pig5	2	*	**	**	**	*
	Pig6	2	***	**	**	*	
	Pig7	3	**	***	**	**	*
	Pig8	3		***	**	**	*
	Pig9	3	***	***	**	**	*
	Pig10	3		***	**	**	*
	Pig11	3		***	**	**	*
Group 2 (HES 130/0.4)	Pig1	3		*	***	*	
	Pig2	2	**	***	**	**	**
	Pig3	2	*	**	**	**	*
	Pig4	3	**	***	**	**	*
	Pig5	2		**	**	**	
	Pig6	2	**	**	**	**	
	Pig7	3		***	**	**	*
	Pig8	2	*	**	**	*	*
	Pig9	3		***	**	**	*
	Pig10	3		***	**	**	*
	Pig11	3		***	**	**	*

*Small numbers; **moderate numbers; ***large numbers.

Appendix Table 5. Grade and cellular composition of the inflammatory infiltrate present in the jejunum, per group

		Jejunum					
Group	Pig	**Infiltrate Grade**	**Neutrophils**	**Lymphocytes**	**Plasma Cells**	**Macrophages**	**Eosinophils**
Group 3 (Control)	Pig1	2		**	**	**	*
	Pig3	2		**	**	**	**
	Pig3	2		**	**	*	**
	Pig4	2		**	**	*	**
	Pig5	2		**	**	*	*
	Pig6	2		**	**	*	*
Group 1 (RL)	Pig1	3	*	***	***	***	***
	Pig2	2		**	**	**	**
	Pig3	2	**	**	*	*	***
	Pig4	2		**	**	**	**
	Pig5	1		*	*	*	
	Pig6	2		**	**	*	**
	Pig7	2		**	**	**	*
	Pig8	3		***	**	**	*
	Pig9	2		**	**	*	*
	Pig10	2		**	**	**	
	Pig11	2		**	**	*	*
Group 2 (HES 130/0.4)	Pig1	3	**	***	***	***	
	Pig2	3		**	**	**	**
	Pig3	2		**	**	**	**
	Pig4	2		**	**	**	**
	Pig5	3		***	***	**	**
	Pig6	2		**	**	*	*
	Pig7	3		***	**	**	*
	Pig8	3		***	**	**	*
	Pig9	3		***	**	**	*
	Pig10	3		***	**	**	*
	Pig11	2		**	**	*	*

*Small numbers; **moderate numbers; ***large numbers.

Appendix Table 6. Grade and cellular composition of the inflammatory infiltrate present in the ileum, per group.

		Ileum					
Group	Pig	**Infiltrate Grade**	**Neutrophils**	**Lymphocytes**	**Plasma Cells**	**Macrophages**	**Eosinophils**
Group 3 (Control)	Pig1	2		**	**	**	*
	Pig3	2		**	**	**	*
	Pig3	1	*	*	*	*	*
	Pig4	2		**	**	*	*
	Pig5	2		**	**	*	*
	Pig6	1		*	*	*	*
Group 1 (RL)	Pig1	3	*	***	***	***	***
	Pig2	2		**	**	**	**
	Pig3	1	*	**	*		
	Pig4	2		**	**	**	*
	Pig5	1		*	*	*	
	Pig6	2		**	**	*	*
	Pig7	2		**	**	**	*
	Pig8	2		**	**	*	*
	Pig9	2		**	**	*	*
	Pig10	3		***	***	**	*
	Pig11	3		***	**	**	*
Group 2 (HES 130/0.4)	Pig1	3	**	**	**	**	
	Pig2	1	*	**	**	*	
	Pig3	2		**	**	**	*
	Pig4	1		*	*	*	*
	Pig5	2		**	**	**	*
	Pig6	2		**	**	*	*
	Pig7	3		***	**	**	*
	Pig8	3		***	**	**	*
	Pig9	2		**	**	*	*
	Pig10	3		***	**	**	*
	Pig11	2		**	**	*	*

*Small numbers; **moderate numbers; ***large numbers.

Appendix Table 7. Mucosal loss grading for the duodenum, jejunum and ileum in groups 1, 2 and 3.

Groups	Pigs	Mucosal loss grading		
		Duodenum	**Jejunum**	**Ileum**
Group 1 (RL)	Pig 1	2	2	0
	Pig 2	4	1	1
	Pig 3	3	1	2
	Pig 4	2	1	1
	Pig 5	2	0	0
	Pig 6	2	3	2
	Pig 7	4	1	0
	Pig 8	1	0	0
	Pig 9	3	1	1
	Pig 10	3	0	0
	Pig 11	1	2	1
Group 2 (HES 130/0.4)	Pig 1	3	1	1
	Pig 2	2	1	0
	Pig 3	2	2	2
	Pig 4	2	0	1
	Pig 5	1	0	0
	Pig 6	2	1	0
	Pig 7	3	2	1
	Pig 8	0	0	0
	Pig 9	2	0	0
	Pig 10	3	0	0
	Pig 11	1	1	0
Group 3 (Control)	Pig 1	4	1	0
	Pig 2	2	3	0
	Pig 3	0	0	0
	Pig 4	1	0	0
	Pig 5	0	1	0
	Pig 6	2	0	0

Appendix Table 8. Grading data for each semi quantitative parameter for all the small intestine segments in groups 1, 2 and 3.

		Group 1				Group 2				Group 3			
Grade		**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Oedema													
n	**Duodenum**	1	5	5	0	2	9	0	0	2	4	0	0
	Jejunum	2	8	1	0	2	3	5	1	3	3	0	0
	Ileum	2	8	1	0	1	8	2	0	3	2	1	0
Hyperaemia													
n	**Duodenum**	11	0	0	0	7	3	1	0	5	1	0	0
	Jejunum	10	1	0	0	9	1	1	0	5	1	0	0
	Ileum	11	0	0	0	8	3	0	0	4	2	0	0
Haemorrhage													
n	**Duodenum**	11	0	0	0	11	0	0	0	6	0	0	0
	Jejunum	11	0	0	0	11	0	0	0	6	0	0	0
	Ileum	10	0	1	0	10	1	0	0	6	0	0	0
Congestion													
n	**Duodenum**	7	3	0	1	9	2		0	2	1	3	0
	Jejunum	7	4	0	0	9	2		0	2	2	2	0
	Ileum	7	2	2	0	4	6	1	0	0	5	1	0
Inflammatory Infiltrate													
n	**Duodenum**	0	0	4	7	0	0	3	8	0	0	6	0
	Jejunum	0	1	8	2	0	0	4	7	0	0	6	0
	Ileum	0	2	7	2	0	5	1	5	0	1	5	0
Cellular Degeneration													
n	**Duodenum**	0	4	4	3	1	2	6	2	1	4	1	0
	Jejunum	4	3	4	0	5	3	3	0	0	4	2	0
	Ileum	7	2	1	1	7	2	2	0	6	0	0	0

Appendix Table 9. Intensity of the cytoplasmic immunoreactivity for cytochrome c antibody and predominant cytoplasmic staining pattern in the epithelium of the villi, crypts and glands, in the duodenum.

Cytochrome c oxidase	Pig	Duodenum						
		Villi epithelium	Crypts		Glands within lamina propria		Predominant cytoplasmic staining pattern	
		Intensity of reaction	Intensity of reaction	% of crypts	Intensity of reaction	% of glands	Epithelium of the villi	Crypts and glands
Group1 (RL)	Pig1	3	2	100%	2	100%	Punctate	Punctate
	Pig2	3	0	0	0	0	Diffuse	Staining absent
	Pig3	3	2	100%	3	50%	Diffuse	Punctate
	Pig4	3	2	100%	2	100%	Diffuse	Punctate
	Pig5	3	2	100%	3	50%	Diffuse	Punctate
	Pig6	3	2	100%	2	100%	Diffuse	Punctate
Group2 (HES130/0.4)	Pig1	3	2	100%	1	100%	Punctate	Punctate
	Pig2	3	2	100%	2	100%	Diffuse	Punctate
	Pig3	2	2	100%	2	100%	Diffuse	Punctate
	Pig4	3	2	100%	1	100%	Punctate	Punctate
	Pig5	3	2	100%	2	100%	Diffuse	Punctate
	Pig6	2	2	100%	2	100%	Diffuse	Punctate
Group3 (Control)	Pig1	2	2	100%	2	100%	Punctate	Punctate
	Pig2	2	2	100%	2	100%	Punctate	Punctate
	Pig3	2	2	100%	2	100%	Punctate	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	3	2	100%	2	100%	Punctate	Punctate
	Pig6	3	2	100%	2	100%	Punctate	Punctate

Staining intensity was evaluated as 0, undetectable; 1, weak; 2, moderate; 3, intense; and 4, very intense.

Appendix Table 10. Intensity of the cytoplasmic immunoreactivity for cytochrome c antibody and predominant cytoplasmic staining pattern in the epithelium of the villi, crypts and glands, in the jejunum.

Cytochrome c oxidase	Pig	Jejunum						
		Villi epithelium	Crypts		Glands within lamina propria		Predominant cytoplasmic staining pattern	
		Intensity of reaction	Intensity of reaction	% of crypts	Intensity of reaction	% of glands	Epithelium of the villi	Crypts and glands
Group1 (RL)	Pig1	2	2	100%	2	100%	Punctate	Punctate
	Pig2	2	2	100%	2	100%e	Punctate	Punctate
	Pig3	2	2	100%	2	100%	Punctate	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	2	2	100%	2	100%	Punctate	Punctate
	Pig6	2	2	100%	2	100%	Punctate	Punctate
Group2 (HES130/0.4)	Pig1	3	2	100%	3	50%	Punctate	Punctate
	Pig2	3	2	100%	2	100%	Diffuse	Punctate
	Pig3	2	2	100%	2	100%	Punctate	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	3	2	100%	3	50%	Punctate	Punctate
	Pig6	3	2	100%	2	100%	Diffuse	Punctate
Group3 (Control)	Pig1	3	2	100%	3	100%	Punctate	Punctate
	Pig2	2	1	100%	3	75%	Punctate	Punctate
	Pig3	2	2	100%	2	100%	Punctate	Punctate
	Pig4	2	1	100%	2	100%	Punctate	Punctate
	Pig5	3	2	100%	3	100%	Punctate	Punctate
	Pig6	2	1	100%	3	75%	Punctate	Punctate

Staining intensity was evaluated as 0, undetectable; 1, weak; 2, moderate; 3, intense; and 4, very intense.

Appendix Table 11. Intensity of the cytoplasmic immunoreactivity for cytochrome c antibody and predominant cytoplasmic staining pattern in the epithelium of the villi, crypts and glands, in the ileum.

Cytochrome c oxidase	Pig	Ileum						
		Villi epithelium	Crypts		Glands within lamina propria		Predominant cytoplasmic staining pattern	
		Intensity of reaction	Intensity of reaction	% of crypts	Intensity of reaction	% of glands	Epithelium of the villi	Crypts and glands
Group1 (RL)	Pig1	3	0	100%	0	0	Punctate	Staining absent
	Pig2	3	2	100%	2	100%	Punctate	Punctate
	Pig3	3	2	100%	2	100%	Diffuse	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	2	3	100%	2	100%	Punctate	Punctate
	Pig6	3	0	100%	0	0	Punctate	Staining absent
Group2 (HES130/0.4)	Pig1	3	3	100%	3	100%	Diffuse	Punctate
	Pig3	3	3	100%	2	100%	Punctate	Punctate
	Pig3	3	2	100%	2	100%	Punctate	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	2	2	100%	2	100%	Punctate	Punctate
	Pig6	3	3	100%	3	100%	Diffuse	Punctate
Group3 (Control)	Pig1	4	3	100%	3	75%	Punctate	Punctate
	Pig2	3	3	100%	3	100%	Diffuse	Punctate
	Pig3	2	2	100%	2	100%	Punctate	Punctate
	Pig4	2	2	100%	2	100%	Punctate	Punctate
	Pig5	3	3	100%	3	100%	Punctate	Punctate
	Pig6	3	3	100%	3	100%	Diffuse	Punctate

Staining intensity was evaluated as 0, undetectable; 1, weak; 2, moderate; 3, intense; and 4, very intense.

Appendix Table 12. High-score, total number of apoptotic cells, percentage of apoptotic cells and apoptotic cells per mm^2 obtained with the TUNEL method for each pig in all groups.

		H-score			Total number of apoptotic cells			% apoptotic cells			Apoptotic cells / mm^2		
	Pig	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Group 3 (Control)	1	4,53	16,13	0,67	61	262	12	3,09	12,41	0,63	129,78	557,41	25,52
	2	112,2	6,27	4,13	528	130	35	42,86	5,66	2,14	1,12	276,58	74,46
Group 1 (RL)	1	1,25	19,29	0,11	15	179	2	0,86	12,02	0,07	31,91	380,83	4,26
	2	10,6	3,83	4,66	127	43	52	8,47	2,3	2,48	270,2	91,48	110,63
Group 2 (HES 130/0.4)	1	19,21	17,95	7,21	139	111	155	9,29	7,88	6,64	295,73	236,16	329,77
	2	2,21	7,49	10,97	11	104	152	0,53	5,44	9,74	23,4	221,26	323,38

Appendix Table 13. Detached cells, negative detached cells, positive detached cells and percentage of positive detached cells obtained with the TUNEL method, for each pig, in all groups.

		Detached cells			Negative detached cells			Positive detached cells			% of positive detached cells		
	Pig	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Group 3 (Control)	1	0	13	21	0	12	17	0	1	4	0	7,69	19,05
	2	35	131	2	8	114	2	27	17	0	77,14	12,98	0
Group 1 (RL)	1	77	44	34	73	32	29	4	12	5	5,19	27,27	14,71
	2	1	37	169	0	35	151	1	2	18	100	5,41	10,65
Group 2 (HES 130/0.4)	1	152	44	0	68	20	0	84	24	0	55,26	54,55	0
	2	56	201	19	48	188	19	8	13	0	14,29	6,47	0

Appendix Table 14. High-score, total number of apoptotic cells, percentage of apoptotic cells and apoptotic cells per mm^2 obtained with M30 antibody for each pig in all groups.

		H-score			Total number of apoptotic cells			% apoptotic cells			Apoptotic cells / mm^2		
	Pig	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Group 3 (Control)	1	62,22	5,13	5,02	399	64	40	31,47	4,63	2,05	419,56	67,3	42,06
	2	9,81	4,65	0.91	93	40	13	8,55	5,08	0,88	97,79	42,06	13,67
	3	56,41	3,22	9,77	313	29	60	18,9	1,65	3,4	329,13	30,49	63,09
	4	0,46	8,39	2,16	7	49	27	0,38	3,27	1,55	7,36	51,52	28,39
	5	2,43	15,57	6,23	38	70	37	2,24	5,84	2,45	39,96	73,61	38,91
	6	66,96	5,56	5,73	292	41	37	17,53	2,9	2,24	307,05	43,11	38,91
Group 1 (RL)	1	47,33	3,73	23,12	333	26	120	22,73	1,67	6,98	350,16	27,34	126,18
	2	52,8	62,22	3,99	381	399	66	27,973	31,47	4,03	400,63	419,56	69,4
	3	10,95	14,88	12,05	92	103	88	6,44	8,58	5,43	96,74	108,31	92,53
	4	4,89	1,33	8,82	65	17	61	4,21	1,46	3,73	68,35	17,88	64,14
	5	21,56	5,5	12,66	139	31	87	7,76	2,08	5,23	146,16	32,6	91,48
	6	32,61	69,7	7,6	164	451	56	10,16	29,61	3,14	172,45	474,24	58,89
Group 2 (HES 130/0.4)	1	59,77	112,31	117,88	375	566	846	28,28	47,48	45	394,32	595,16	889,59
	2	74,97	6,23	5,84	414	61	33	33,15	4,31	2,22	435,33	64,14	34,7
	3	7,05	19,65	12,13	71	88	132	4,99	8,67	8,13	74,66	90,43	138,8
	4	24,56	14,97	10,61	96	63	82	7,28	5,99	4,45	100,95	66,25	86,23
	5	66,08	17,09	17,15	266	89	103	19,66	6,58	6,18	279,71	93,59	108,31
	6	9,09	0,63	6,61	40	7	57	3,18	0,36	2,84	42,06	7,36	59,94

Appendix Table 15. Detached cells, negative detached cells, positive detached cells and percentage of positive detached cells obtained with the M30 antibody for each pig, in all groups.

		Detached cells			Negative detached cells			Positive detached cells			% of positive detached cells		
	Pig	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**	**Duodenum**	**Jejunum**	**Ileum**
Group 3 (Control)	1	2	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	30	0	0	30	0	0	0	0	0	0	0
	5	36	234	0	36	196	0	0	38	0	0	16,24	0
	6	0	38	0	0	31	0	0	7	0	0	18,42	0
Group 1 (RL)	1	16	0	0	11	0	0	5	0	0	31,25	0	0
	2	83	2	0	37	2	0	46	0	0	55,42	0	0
	3	6	0	0	6	0	0	0	0	0	0	0	0
	4	64	34	0	35	31	0	29	3	0	45,31	8,82	0
	5	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	72	0	0	72	0	0	0	0	0	0
Group 2 (HES 130/0.4)	1	31	10	0	22	4	0	9	6	0	29,03	60	0
	2	31	0	0	26	0	0	5	0	0	16,13	0	0
	3	49	49	0	35	28	0	14	21	0	28,57	42,86	0
	4	57	0	0	30	0	0	27	0	0	47,37	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0

Publications related to this thesis

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