Departament de Medicina i Cirurgia Animals
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Intestinal anastomosis wound healing after platelet-rich plasma (PRP) application on pigs. Macroscopic, microscopic and breaking strength evaluations

PhD Thesis

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CERTIFICA:

Que la memoria titulada "INTESTINAL ANASTOMOSIS WOUND HEALING AFTER PLATELET-RICH PLASMA (PRP) APPLICATION ON PIGS. MACROSCOPIC, MICROSCOPIC AND BREAKING STRENGTH EVALUATIONS" presentada por **Otilia Rafael Bambo** para optar al grado de Doctor, ha sido realizada bajo su dirección y, considerándola finalizada, autoriza su presentación para que ésta sea juzgada por el tribunal correspondiente.

Y para que así conste, firmo el presente certificado.

Bellaterra, 4 de Mayo de 2009.

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Some Common Abbreviations

ADP - Adenosine diphosphate

ANOVA - analisys of variance

dpm - Decay per minute

DTS - Dense tubular system

EGF - Epidermal growth factor

ECM -Extracellular matrix

EDTA - Ethylenediamine tetra-acetic acid

FGF - Fibroblast growth factor

fl - Femtoliter

HGF- Hepatocyte growth factor

IGF - Insulin-like growth factor

IL-1- Interleukin-1

g - Gravitational force

GAGs - Glucosaminoglycans

GH - Growth hormone

GI - Gastro intestinal

GDP - Guanosine Diphosphate

GP - Glycoproteins

GTP - Guanosine Triphosphate

H&E - Hematoxylin-eosin

KGF - Keratinocyte growth factor

MPC - Mean platelet component

MCP-1 -Monocyte chemotactic protein-1

mRNA - messenger ribonucleic acid

MMP - Matrix metalloproteinase

MSC - Mesenchymal stem cells

MSSA -Methicillin-sensitive Staphylococcus aureus

OCS - Open canalicular system

PCG - Platelet concentrate gel

PDGF - Platelet-derived growth factor

PED - Persistent epithelial defects

(pg) - Picograms

PMNs - Polymorphonuclear leukocytes

PPP - Platelet-poor plasma

PRP - Platelet-rich plasma

TGF- β1 - Transforming growth factor- β1

TNF - Tumor necrosis factor

TLR - toll-like receptors

VEGF - Vascular endothelial growth factor

1. Introduction

Intestinal wound healing is a process for surgical reconstitution of the digestive tract (Ikeuchi *et al.*, 1999). Rapid and effective wound-healing is of vital importance to the surgeon and to the patient. Failure of wound-healing generally leads to potentially severe complications, additional surgical procedures, prolonged hospitalization, long-term disability, discomfort, diminished quality of life and mortality rates between 2 and 5 percent (Vignali *et al.*, 1997; Pickleman *et al.*, 1999). Despite many recent advances in surgical technologies, patients continue to suffer from anastomotic disruptions and strictures. Attempts to enhance anastomotic healing have included the use of various surgical techniques and materials (improved surgical technique, higher quality of suture threads, use of staples, control of sepsis by specific bowel preparation before surgery, use of parenteral nutrition, use of various sealants, fibrin-collagen patch, etc.), but none is sufficient to prevent the development of complications (Haukipuro *et al.*, 1988; Van der Hamm *et al.*, 1992; Golub *et al* 1997; Ikeuchi *et al.*, 1999; Ozel *et al.*, 2006).

One of the most significant advances in the field of modern molecular biology and biochemistry in the last three decades are certainly growth factors and cytokines. Growth factors are biologically active polypeptides influencing growth, differentiation and metabolism of target cells through specific receptors. Numerous growth factors have been identified so far, taking part in the complex wound healing process, with new ones being identified constantly. In the last several years a number of authors investigated the use of various recombinant growth factors (local or systemic) in order to enhance some of the phases of wound healing process (Ciacci *et al.*, 1993; Dignass *et al.*, 1993; Beck *et al.*, 2003). Some laboratory studies have suggested that there may be a hierarchy in the action of these growth factors and cytokines in stimulating intestinal restitution. *In vitro* studies using monolayers of intestinal epithelial cell lines indicate that transforming growth factor-β (TGF-β) plays a central role (Ciacci *et al.*, 1993; Dignass *et al.*, 1993; Beck *et al.*, 2003).

TGF-β regulates enterocyte proliferation and differentiation and helps to maintain intestinal integrity of the epithelial surface along the villi (Dignass and Sturm, 2001). During injury or disease, TGF-β stimulates epithelial cell migration (Ciacci *et al.*, 1993) and extracellular matrix production (O'Kane and Ferguson, 1997), thereby promoting wound healing. TGF-β is also known as a potent immunoregulator and plays a critical role in maintaining mucosal immune homeostasis (Letterio and Roberts, 1998), and local production of TGF-β in the intestinal mucosa increases in response to mucosal inflammation (Babyatsky *et al.* 1996).

One should also take into account that recombinant growth factors are pure human or animal growth factors, but they are not native growth factors. Human and animal cells such as platelets do not synthesize them. Another disadvantage of these recombinant growth factors is that they are expensive and concerns exist about their safety in human administration (Calabresi *et al.*, 1998). Alternatively, platelet-rich plasma (PRP), which is a volume of autogenous plasma that has a platelet concentration above baseline, is a proven source of growth factors (Marx *et al.*, 1998). The use of PRP is based on the premise that the large number of platelets in PRP release significant quantities of growth factors that promote chemotaxis of precursor cells, cell mitosis, collagen production, initiating vascular in-growth, and inducing cell differentiation (Spencer *et al.*, 1993; Anitua, 1999; Freymiller and Aghaloo, 2004).

PRP has been used for hard and soft tissue regeneration, particulary in maxillofacial and oral surgery with predictable clinical outcomes (Marx *et al*., 1998; Anitua, 1999; Margolis *et al*., 2001; Crovetti *et al*., 2004; Mazzuco *et al.,* 2004).
In recent times, strategies in clinical treatment plans encourage the production of autologous PRP containing high concentrations of platelet growth factors with the use of whole blood separation devices. PRP mixed with thrombin and calcium chloride will result in the production of platelet gel, which can be exogenously applied to surgical wounds, leading to the degranulation of the platelet α-granules and platelet growth factor release (Frechette *et al*., 2005). In several studies, investigators have appeared to take advantage of platelet growth factor delivery in support of hemostasis and wound healing (Englert *et al*., 2005; Everts *et al*., 2007). In oral and maxillofacial surgery, published results imply that earlier bone graft maturation can be expected when platelet gel is used in mandibular defects (Marx *et al*., 1998; Steigmann and Garg, 2005). In addition, platelet gel applications have also been reported to improve soft tissue healing in chronic non-healing wounds (Margolis *et al*., 2001; Crovetti *et al*., 2004; Mazzuco *et al.,* 2004).

PRP was been used in different tissues with promising results, but their application to gastrointestinal tract healing (end-to-end anastomosis) was poorly investigated (Yol *et al*., 2008). In this study we are investigating if the platelet concentrate gel could have a beneficial effect on healing of small bowel anastomosis such as early cicatritation and decreased incidence of leakage.

2. Literature revision

2.1. Platelets

Platelets are discoid anucleate cytoplasmic fragments generated from megakaryocytes in the bone marrow that travel through the bloodstream and play a critical role in hemostasis, as well as in inflammation and wound healing. They are the second most numerous corpuscles in the blood (Harrison, 2005).

2.1.1. Platelet structure and function

Platelet structure

Platelets are small, 5 to7 μm long and often less than 3 μm wide. Occasionally, in horse and guinea pig, larger and more lentiform platelets, as long as 20 μm, can be observed in circulation. They have a mean cell volume of around 5–6 fl and a life span of approximately 8–10 days before they are removed from the blood by macrophages (George, 2000; Kamath *et al.*, 2001). Mammalian platelets are not provided with a nucleus, in contrast to thrombocytes in birds, fish and reptiles (Weyrich *et al.*, 2003). Platelets circulate freely without adhesion to the vessel wall or aggregation with other platelets. If stimulated, platelets become spherical, extend pseudopods, and adhere to vessel walls and to each other. Platelets have a ring of contractile microtubules (cytoskeleton) around their periphery, containing actin and myosin. Inside the platelet, a number of intracellular structures are present containing glycogen, lysosomes and granules (George, 2000; Kamath *et al.*, 2001). A schematic view of the structural components of the platelet can be seen in (Fig. 2.1).

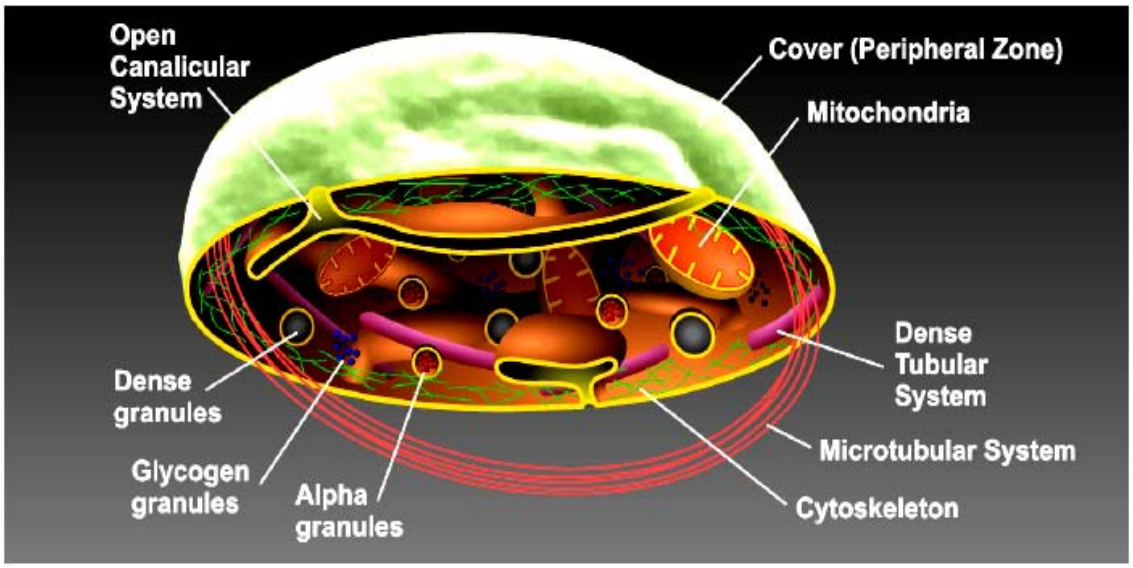

Figure 2.1- Platelet morphology. (From: http://www.platelet-research.org/1/intro.htm)

a) The Peripheral Zone – Platelet Surface

The peripheral zone mediates the adhesion and aggregation function and consists on the outer platelet membrane with downy glycocalyx coat, and the open canalicular system (OCS) (Weyrich *et al.*, 2003).
Platelet membrane is composed of a polarized phospholipid bilayer containing the membrane proteins. Asymmetric distribution of the phospholipids in resting platelets is an important factor for platelet function: the neutral phospholipids, phosphatidylcoline and sphingosine are mainly distributed in the outer layer; while the negatively charged aminophospholipids, phosphatidylserine and phosphatidylethanolamine are almost exclusively present in the inner layer. Such organization of phospholipids changes during activation of the platelets, and anionic phospholipids are able to accelerate several steps in plasma coagulation (Zwaal and Schroit, 1997; Tablin, 2000).
Glycocalyx is the outermost layer of the platelet surface and is reported to be 15-20 nm thick. Biochemically, much of the glycocalyx is composed of the extracellular domains of glycoproteins (GP), some of which are members of the integrin superfamily (such as GP I, GP II, GP III, GP IV, GP V), the leucine-rich glycoprotein family, the immunoglobulin superfamily , and the selectins (such as GMP 140, or P-selectin). These GP mediate platelet interactions with other cells and act as receptors for platelet agonists, such as those for ADP, collagen, epinephrine, thrombin, etc., which assist in platelet adhesion, aggregation and coagulation (Tablin, 2000; Kamath *et al.*, 2001).

The OCS, also termed the surface-connected canalicular system, represents canals that reach from their connections with plasma membrane far inside the platelet and are accessible from extracellular space by pores. It acts as an internal membrane reservoir to facilitate platelet spreading and filopodia formation after activation (Tablin, 2000). OCS is present in humans, pigs, rats, mice, guinea pigs, rats, rabbits, dogs, cats, primates, and horses, but (bovine and elephant are an exception) (Tablin, 2000).

b) Structural Zone – Cytoskeleton

Internal to the plasma membrane, there is the skeleton of the platelet which is responsible for contraction and for providing support to the microtubule system. The cytoskeleton is composed mainly of actin filaments, which are the essential contractile element of the platelet, that are linked to membrane receptors by actin-associated proteins (e.g. vinculin, spectrin, talin, α-actin). Together with these proteins, along with changes in the intracellular calcium concentration and in association with platelet myosin, actin filaments regulate shape change, extension of pseudopodia, platelet spreading and adhesion, platelet aggregation, secretion, and clot retraction (Tablin, 2000; Hartwig and Italiano, 2003).
Additional major cytoskeletal elements include microtubules and intermediate filaments (Yeaman, 1997; Tablin, 2000; Kamath *et al.*, 2001; Hartwig and Italiano, 2003). These components serve to maintain the discoid shape of resting platelets and actively participate in changing the shape of activated platelets (Tablin, 2000).

c) Organelle zone

Organelles in the cytoplasm include the mitochondria, glycogen stores, dense tubular system, and different storage granules: α-granules, dense granules, peroxisomes and lysosomes (Tablin, 2000; Kamath *et al.*, 2001).
Glycogen granules are found free in the cytoplasm of the platelet. Glycogen is the primary source of energy metabolism of resting platelets. The dense tubular system is derived from

the endoplasmic reticulum of the parent megakaryocyte and serves as the reservoir for calcium. The secretory granules possess molecules that affect platelet function, coagulation, fibrinolysis, vascular tone, inflammation, and wound healing. Some of the components are synthesized by megakaryocytes, other are taken up from plasma and incorporated into the granules. Upon activation, granules fuse with platelet surface membrane and extrude their content. It is a graded process depending on the number, nature, and concentration of the original stimuli (Reed and Fitzgerald, 2000; Kamath *et al.*, 2001; Hartwig and Italiano, 2003).

Platelet functions

Platelets are surprisingly multifunctional and are involved in many pathophysiological processes including haemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation, including promotion of atherosclerosis, host defence and even tumour growth and metastasis (table 2.1). Upon vessel wall damage, platelets undergo a highly regulated set of functional responses including adhesion, spreading, release reactions, aggregation, and exposure of a procoagulant surface, microparticle formation and clot retraction. All of these platelet responses function to rapidly form a haemostatic plug that occludes the site of damage to prevent blood loss (Harrison, 2005).

Platelet main physiological functions				
Haemostasis and thrombosis	**Maintenance/regulation of vascular tone**	**Inflammation**	**Host defence**	**Tumour biology**
Adhesion Activation Spreading Secretion Aggregation	Uptake of serotonin when resting	Atheroslerosis	Phagocytosis, internalisation of viruses and bacteria	Tumour growth
Procoagulant activity	Release of serotonin, thromboxane, prostaglandins upon activation	Allergic asthma; renal disease	Killing of bacteria	Tumour killing
Clot retraction		Chemotaxis	Release of platelet microbicidal proteins	Tumour metastasis
Tissue Repair		Platelet-leukocyte interactions	Superoxide production	

Table 2.1 - The multifunctional platelet. Platelets are involved in many patophysiological processes, in addition to hemostasis and thrombosis, namely maintenance of vascular tone, inflammation, host defence and tumour biology (adapted from Harrison, 2005).

2.1.2. Platelet granules

Platelets contain three distinct cytoplasmatic granules types:

1) Dense (σ) granules store mediators of vascular tone including serotonin, ADP, eicosanoids, thromboxane A2, calcium, and magnesium. Of all species studied, pig platelet dense granules release the most magnesium, (Yeaman, 1997; Tablin, 2000).

2) The α-granules are the largest granule population and contain proteins involved in hemostatic functions such as adhesion (e.g., fibrinogen, thrombospondin, vitronectin, laminin, and von Willebrand factor), modulation of coagulation (e.g. plasminogen, α2-plasmin inhibitor, and thrombasthenin), growth factors (such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insuline-like growth factor (IGF-I), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF), glycoproteins (GPIb, GPIIb-IIIa), thrombospondin, fibronectin, and α-granule specific protein P-selectin (Tablin, 2000; Anitua *et al.*, 2004).

3) Lysosomal (λ) granules contain acid hidrolases, guanine, phospholipases and kinases which act as proteolytic and hydrolytic enzymes, glycosidases, proteases and cationic proteins. Lysosomal granules are also believed to contain enzymes that principally mediate thrombus dissolution of residual megakaryocyte mRNA templates (Klinger, 1997; Yeaman, 1997; Werner, 2003).

These distinct platelet granules are subject to discrete or synchronous release, dependent on agonist specificity and potency. For example, low levels of thrombin or ADP induce dense (σ) and (α) degranulation, while lysosomal (λ) granules are not secreted until these agonists are present at much higher concentrations. From this perspective, platelets may be viewed as vehicles that respond to agonists and ligands expressed at sites of endovascular damage or microbial colonization and release a diverse array of preformed bioactive molecules (Klinger, 1997; Yeaman, 1997; Werner, 2003).

2.1.3. Pig Platelets

Porcine platelets are morphologically similar to those of other domestic species. They are variable in shape and are generally small, 1 to 3 μm in diameter, with a mean volume of 6,9 to 8,9 fl (Evans, 1994). Platelets are anucleate and have deeply staining purple cytoplasmic granules. Platelets are found in variable-sized clumbs discoid anucleate cytoplasmic fragments generated from megakaryocytes in the bone marrow that travel through the bloodstream and have a critical function in hemostasis (Fig. 2.2).

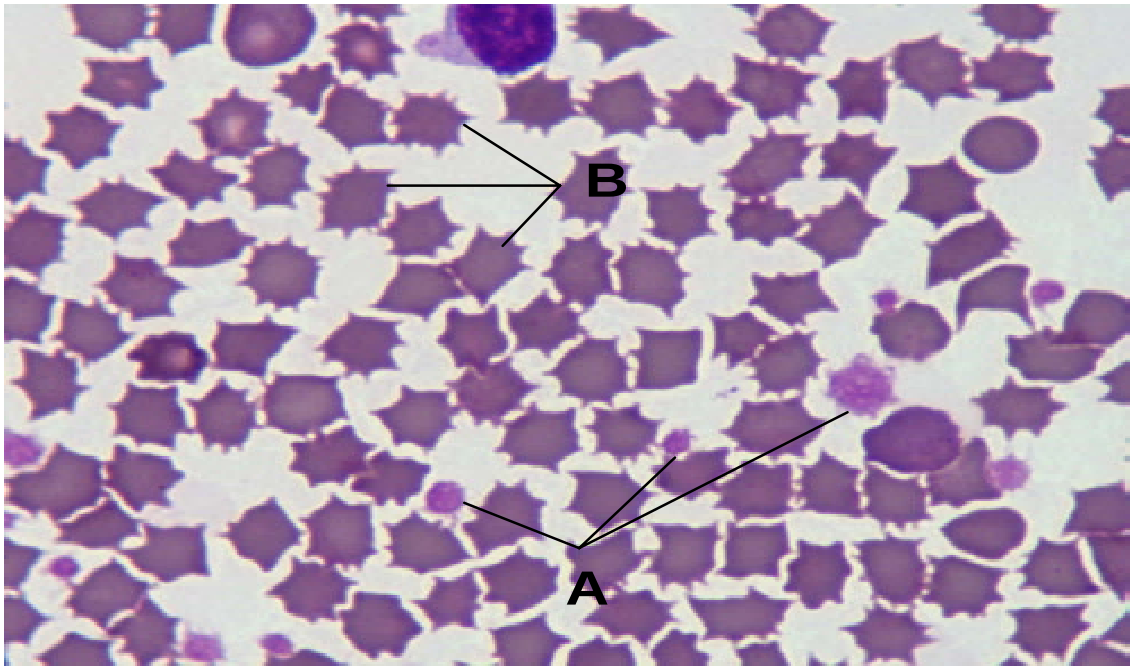

Figure 2.2 - Normal platelet density in peripheral blood smears (Giemsa stain, original magnification x l0). Scattered platelets correlating to a peripheral blood platelet count of 514,000 cells per μL. A - Platelets in different sizes. B - Red blood cells. The spiny crenation is common in pig.

2.2. Pathobiology of wound healing and growth factors

2.2.1. Wound Healing Process

The wound healing process, or wound repair, is a complex series of events that begins at the moment of injury and can continue for months to years. These events overlap in time and are categorized into separate steps: the inflammatory phase, the proliferative phase, maturation and remodelling phase (table 2.2) (Falanga *et al.*, 1995; Martin, 1997).

The inflammatory phase
The inflammatory phase is characterized by hemostasis and inflammation.

Hemostasis

The initial response following tissue injury via an incision is usually bleeding. Collagen exposed during wound formation activates the clotting cascade (both the intrinsic and extrinsic pathways), initiating the inflammatory phase. Normal hemostasis is a coordinated sequence of cellular and biochemical interactions, starting with an injury and ending several minutes later with a stabilized platelet aggregate, a process that conserves blood and enables wound healing (White, 2000; Harrison, 2005). Blood platelets contribute to every aspect of hemostasis, from the initial adhesion of platelets to the vessel wall, spreading over the surface and forming a platelet aggregate, providing an activated cell surface that vastly accelerates coagulation and leads to stabilization of the platelet aggregate by fibrin. All of these processes are regulated, entirely or in part, by specific glycoprotein receptors on the platelet surface. The (GP) IbIX-V complex, a constitutively expressed receptor on the platelet plasma membrane, mediates the initial deposition of platelets on the subendothelium under high shear conditions (Falanga *et al.*, 1995; Martin, 1997).

Inflammation

Neutrophils are the first cells appear in the wound area; migration of those cells are stimulated by various chemotactic factors, and are important for controlling local infection of bacteria through endocytosis as well as the release of lysosomal enzymes. This causes additional tissue death at the wound site, initiating the debridement processes (Falanga *et al.*, 1995). The neutrophil attain their maximal numbers in 24-48 hours and commence their departure by hour 72. Neutrophil infiltration slows down as monocyte infiltration increases. Upon migration into the wound area, monocytes undergo phenotypic changes to become macrophages (Falanga *et al.*, 1995; Martin, 1997). The macrophages continue the cleansing process during days 3-4 as well as debride the wound, aided by the macrophage secretion of tumor necrosis factor-α and interleukin-1. In addition to cleaning the wound, the macrophages release numerous cytokines that are important for the attraction and proliferation of fibroblasts. These include PDGF, FGF, TGF-β, VEGF and plasma-activated complements (anaphylactic toxins). Other inflammatory cells that interact within the wound area include lymphocytes, plasma cells, and mast cells. These cells also contribute with cytokines, such as interleukin-4, to induce collagen production in fibroblasts (Falanga *et al.*, 1995; Martin, 1997).

Proliferative phase

The proliferative phase is characterized by angiogenesis, granulation tissue formation, collagen deposition and re-epithelization (Midwood *et al.*, 2004). In angiogenesis, new blood vessels grow from endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin. On collagen deposition, fibroblasts begin to commit apoptosis, converting granulation tissue from an environment rich in cells to one that consists mainly of collagen. The reepithelialization involves epithelial cells migration across the new tissue (granulation tissue) to form a barrier between the wound and the environment (Midwood *et al.*, 2004).

Angiogenesis

The process of angiogenesis occurs concurrently with fibroblast proliferation when endothelial cells migrate to the area of the wound (Kuwahara and Rasberry, 2007). Stem cells called endothelial cells originating from parts of uninjured blood vessels develop pseudopodia and push through the ECM into the wound site. Through this activity, they establish new blood vessels (Greenhalgh, 1998). The most important family of cytokines implicated in the neovascularization processes are VEGF family and FGF (Romo and Pearson, 2005). Endothelial growth and proliferation is also stimulated by hypoxia and presence of lactic acid in the wound (Falanga, 2005).

Fibroplasia and granulation tissue formation

Simultaneously with angiogenesis, fibroblasts begin entering the wound site two to five days after wounding as the inflammatory phase is ending and their numbers peak at one to two weeks post-wounding being the main cells in wound healing for that time (Stadelmann *et al.,* 1998). Fibroplasia ends two to four weeks after wounding. In the first two or three days after injury, fibroblasts mainly proliferate and migrate, while later, they are the main cells that lay down the collagen matrix in the wound site (Stadelmann *et al.*, 1998). Fibroblasts from normal tissue migrate into the wound area from its margins. Initially fibroblasts use the fibrin scab formed in the inflammatory phase to migrate across, adhering to fibronectin (Romo and Pearson, 2005). Fibroblasts then deposit ground substance into the wound bed, and later collagen, which they can adhere to for migration (de la Torre and Sholar, 2006). Growth factors (PDGF, TGF-β) and fibronectin encourage proliferation, migration to the wound bed, and production of ECM molecules by fibroblasts (Falanga *et al.,* 1995; Martin, 1997).

Granulation tissue begins to appear in the wound even during the inflammatory phase; two to five days post wounding, and continues growing until the wound bed is covered. It is needed to fill the void that has been left by a large, open wound that crosses the basement membrane. Basically consist of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and the components of a new, provisional ECM. The provisional ECM is different in composition from the ECM in normal tissue and includes fibronectin, collagen, glycosaminoglycans, and proteoglycans (Romo and Pearson, 2005).

Collagen deposition

In days 5-7, fibroblasts have migrated into the wound, laying down new collagen of the subtypes I and III. Early in normal wound healing, type III collagen predominates but is later replaced by type I collagen (Falanga *et al.,* 1995; Martin, 1997). One of fibroblasts most important duties is the production of collagen (Kuwahara and Rasberry, 2007). Fibroblasts begin secreting appreciable collagen by the second or third post-wounding day (Romo and Pearson, 2005), and its deposition peaks at one to three weeks (Mercandetti and Cohen 2005). Collagen deposition is important because it increases the strength of the wound; before it is laid down, the only thing holding the wound closed is the fibrin-fibronectin clot, which does not provide much resistance to traumatic injury (Greenhalgh, 1998). FGF-1 and FGF-2 are potent stimulators of fibroblasts, which are essential in extracellular matrix production (Podolsky, 1994).

Re-epithelization

Re-epithelization occurs with the migration of cells from the periphery of the wound and adnexal structures within 24 hours of injury. Division of peripheral cells occurs in hours 48-72, resulting in a thin epithelial cell layer, which bridges the wound. EGF is believed to play a key role in this aspect of wound healing. This succession of subphases can last up to 4 weeks in the clean and uncontaminated wound (Falanga *et al*., 1995; Martin, 1997; Werner *et al*., 2003).

Maturation and Remodeling phase

When the levels of collagen production and degradation equalize, the maturation phase of tissue repair has begun (Greenhalgh, 1998). The maturation phase can last for a year or longer, depending on the size of the wound and whether it was initially closed or left open (Mercandetti and Cohen, 2005). During maturation, type III collagen, which is prevalent during proliferation, is gradually degraded and the stronger type I collagen is laid down in its place. Originally disorganized collagen fibers are rearranged, cross-linked, and aligned along tension lines (Lorenz and Longaker, 2003).

I- Inflammatory phase	**a) Immediate to 2-5 days** **b) Hemostasis** - Instantly after endothelial disruption vascular contraction begins platelet adhesion and formation of a soft aggregate plug. Vasoconstriction (maintained by platelet secretion of serotonin, prostaglandin and thromboxane) slows blood flow, enhancing platelet adhesion and activation. The soft plug is solidified through a complex interaction between platelet membrane, enzymes, and coagulation factors. **c) Inflammation** - Neutrophils are the first cells appear in the wound area, stimulated by various chemotactic factors. Monocytes appear approximately 24 hours after injury and peak at 48 hours post-injury, and mature into macrophages. Macrophages secrete cytokines and growth factors (PDGF, FGF, TGF-β, VEGF), in matrix synthesis and degradation, and are able to mediate angiogenesis and fibroplasia.
II- Proliferative phase	**a) 2 days to 3 weeks** **b) Angiogenesis** - Stem cells called endothelial cells originating from parts of uninjured blood vessels develop pseudopodia and push through the ECM into the wound site. Through this activity, they establish new blood vessels. VEGF together with FGF may enhance neovascularization. **c) Fibroplasia and Granulation tissue** **Fibroplasia**, fibroblast proliferates in the deeper parts of the wound. These fibroblasts begin to synthesize small amounts of collagen which acts as a scaffold for migration and further fibroblast proliferation, stimulated by TGF- β, FGF and other related factors. **Granulation tissue -** involve the perfusion of fibrous connective tissue that replaces a fibrin clot in healing wounds. Consists of capillary loops supported in this developing collagen matrix, also appears in the deeper layers of the wound. **Collagen deposition** - 4 to 5 days after the injury occurs; fibroblasts begin producing large amounts of collagen and proteoglycans. Collagen fibers are laid down randomly and are cross-linked into large, closely packed bundles. FGF-1 and FGF-2 are potent stimulators of fibroblasts. **d) Reepitelization**, migration of cells from the periphery of the wound and adnexal structures within 24 hours of injury. Division of peripheral cells occurs in hours 48-72, resulting in a thin epithelial cell layer, which bridges the wound.
III-Remodelling Phase	**a) 3 weeks to 2 years** **b)** During **remodelling phase** loose provisional matrix is gradually replaced by collagen fibers, the provisional matrix collagen is almost exclusively of type III is main fibrillar collagen and it is degraded by collagenase activity and gradually replaced by collagen type I, the main collagen of the mature scar.

Table 2.2 - Phases of wound healing.

2.2.2. Regulation of wound repair

Platelets

Platelets are unique anucleate mammalian blood cells with specialized molecular repertoires that have evolved to accomplish crucial functions in host integrity, defense and repair (Klinger, 1997; Yeaman, 1997; Werner *et al.*, 2003). Platelets are the principal effectors of cellular hemostasis in humans and other mammals (Weyrich *et al.*, 2003). They adhere to the exposed subendothelial matrix and aggregate in response to prothrombotic signals, contributing to the formation of platelet - fibrin clot that is critical for sealing vascular disruptions and for ultimate vessel and wound repair (White, 2000). The formation of a platelet plug in response to prothrombotic stimuli involves several steps, namely adhesion, shape change, release of mediators, aggregation and development of a pro-coagulant surface, which will be covered more comprehensively later under platelet activation (Harrison, 2005). In addition, platelets have major roles in acute and chronic inflammation including: release of proinflammatory mediators, display of the surface molecules that have inflammatory functions and interactions with polymorphonuclear leukocytes and endothelial cells, regulating their adhesion and activation (McIntyre *et al.*, 2003; Weyrich *et al.,* 2003). Although they are highly differentiated for hemostasis and inflammation, they participate in the wound healing process through the release of cytokines, growth factors and other regulatory molecules together with the interaction with neutrophils and endothelial cells. Platelets also have antimicrobial systems and link clotting and immune cascades (Klinger, 1997; Yeaman, 1997; Werner *et al.*, 2003).

Growth factors

Growth factors are classified as cytokines, which are proteins that act as intercellular signals to allow cells to communicate with one another. Growth factors are involved in all stages of wound healing and also have the ability to regulate many other functions within cells including protein synthesis, cell proliferation and differentiation. They are specific for attracting useful cells and proteins to the wound, including immune cells to fight against infection and other cells to form connective tissue. Growth factors also stimulate an increased production of connective tissue; create a new supply of blood vessels to nourish the site, thus promoting maturation (Marx *et al.*, 1998; Kassolis *et al.*, 2000; Marx *et al.*, 2001; Appel *et al.*, 2002). These cytokines are released by a variety of activated cells (platelets, macrophages) at the wound site and act on the appropriate target cell or cells to carry out specific action (Giannobile, 1996; Nimni, 1997; Liebarman *et al.*, 2002).

Growth factors can exert their effect through three different ways:

- **Autocrine**, in which the growth factor influences the cell of its origin or other cells identical in phenotype to that cell (e.g. a growth factor produced by an osteoblast influences the activity of another osteoblast) (Liebarman *et al.*, 2002).

- **Paracrine**, in which the growth factor influences an adjacent or neighboring cell that is different in phenotype from its cell of origin (e.g. a growth factor produced by an osteoblast stimulates differentiation of an undifferentiated cell) (Liebarman *et al.*, 2002).

- **Endocrine**, in which the growth factor influences a cell that is different in phenotype from its cell of origin and located at a remote anatomical site (e.g. a growth factor produced by neural tissue in the central nervous system stimulates osteoblast activity). Thus, a growth

factor may have effects on multiple cell types and may induce an array of cellular functions in a variety of tissues (Giannobile, 1996; Nimni, 1997; Liebarman *et al.*, 2002).

Platelets contain several growth factors which are released after injury, including PDGF, TGF, IGF, HGF, VEGF, and FGF (Giannobile, 1996; Liberman *et al.*, 2002).The clinical importance of these growth factors is demonstrated by the finding that recombinant DNA-derived polypeptide growth factors significantly accelerate healing of soft tissue (Appel *et al.*, 2002).

Platelet Derived Growth Factor (PDGF)

PDGF is released from platelet alpha-granules, macrophages, monocytes, endothelial cells and smooth muscle cells immediately after injury.

PDGF is composed of two distinct polypeptide chains, A and B, that form homodimers (AA or BB) or heterodimers (AB). The AA and BB isoforms enhance proliferation of bone cells, increasing the production of PDGF-AA in osteoblast cultures (Martin, 1997; Liebarman *et al.*, 2002; Sánchez *et al.*, 2003).

PDGF plays a significant role in the formation of connective tissue during wound healing (Giannobile, 1996; Martin, 1997; Werner, 2003). PDGF is a very powerful regulatory growth factor and a sentinel growth factor that begins nearly all wound healing process. PDGF attracts neutrophils, macrophages, and fibroblasts to the wound and serves as a powerful mitogen. PDGF stimulates fibroblasts to synthesize new extracellular matrix, predominantly noncollagenous components such as glycoproteins, GAGs and adhesion proteins (Giannobile, 1996; Liebarman *et al.*, 2002). PDGF also increases the amount of fibroblast-secreted collagenase, indicating a role for this cytokine in tissue remodelling (Giannobile, 1996; Martin, 1997). PDGF's main function is to stimulate cell replication (mitogenesis) of healing capable stems and premitotic partially differentiated osteoprogenitor cells, which are part of the connective tissue-bone healing cellular make-up. PDGF also causes replication of endothelial cells, causing budding of new capillaries (angiogenesis) (Martin, 1997; Liebarman *et al.*, 2002). PDGF has also important functions during the embryogenesis, particulary in the development of the kidneys, blood vessels, lungs, and central nervous system (CNS). In these organs, connective tissue-like cell types are dependent on PDGF, including mesangial cells, pericytes, alveolar fibroblasts, and glial cells.

Transforming Growth Factor- β (TGF- β)

It is released from platelets, T-lymphocytes, macrophages/monocytes and neutrophils after injury. It acts stimulating and inhibiting endothelial, fibroblastic, and osteoblastic mitogenesis; regulating collagen synthesis and collagenase secretion; regulating mitogenic effects of other growth factors; stimulating endothelial chemotaxis and angiogenesis (Giannobile, 1996; Liebarman *et al.*, 2002).

TGF- β is a 2-chain polypeptide that is linked together by disulfide bonds. It exists as 3 different isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is found in high concentrations in bone and platelets (Giannobile, 1996; Liebarman *et al.*, 2002).

When released by platelets or secreted by macrophages, TGF-β exerts its effects on adjacent cells, including fibroblasts, marrow stem cells, endothelial cells, and preosteoblasts.

TGF-β stimulates angiogenesis and the production of fibronectin, GAGs, and collagen in connective tissue to aid in restitution and adequate wound closure (Giannobile, 1996; Martin 1997). One of the most important functions of TGF-β seems to be the chemotaxis and mitogenesis of osteoblast precursors. In addition, this polypeptide inhibits osteoclast formation and resorption, favoring bone formation. This local connective tissue response to TGF-β *in vivo* is strongly anabolic and leads to fibrosis and angiogenesis (Milani and Calabro, 2001). A fundamental mechanism of the antiproliferative (catabolic) action of TGF- β is its ability to antagonize the mitogenic effects of other peptide growth factors such as EGF and PDGF. Even in a single cell type, the nature of growth factor action may depend on the context set by other substances present. For example, TGF-β stimulates growth of certain fibroblasts *in vitro* in the presence of PDGF but inhibit their growth if EGF is present (Milani and Calabro, 2001). Indeed, TGF-β may play a central role in the regulation of migration of intestinal epithelial cells. TGF-β has several potentially important functions in intestinal bowel disease (Ciacci *et al.*, 1993; Beck *et al.*, 2003).

Fibroblast Growth Factor (FGF)

It is released from platelets, macrophages, neural tissue, adrenal, corpus luteum, placenta and fibroblasts (Kandel *et al.*, 1991; Blotnick *et al.*, 1994) and can be released from mechanically wounded endothelial cells. FGF acts increasing angiogenesis and vessel permeability and stimulates mitogenesis for endothelial cells.

There are at least 19 distinct members of the FGF family of growth factors. The two originally characterized FGFs were identified by biological assay and are termed FGF-1 (acidic-FGF, aFGF) and FGF-2 (basic-FGF, bFGF) (Beck and Podolsky, 1999).

Both FGF-1 and FGF-2 stimulate proliferation of all cells of mesodermal origin as well as numerous cell populations of endodermal and ectodermal origin including fibroblasts, keratinocytes, smooth muscle cells, endothelia1 cells, and various cell types within the central and peripheral nervous system (Galzie *et al.*, 1997; Hu *et al.*, 1998). Furthermore, FGF-1 and FGF-2 are both mitogenic and chemotactic for endothelial cell, which contribute to angiogenesis following injury. These factors can induce the production of collagenase and plasminogen activator, which in turn leads to extracellular matrix breakdown. FGF-1 and FGF-2 are also potent stimulators of fibroblasts, which are essential in extracellular matrix production and wound healing (Podolsky, 1994).

Vascular Endothelial Growth Factor (VEGF)

This growth factor is released from platelets and endothelial, macrophages, fibroblasts, and keratinocytes cells after injury. The VEGF family currently includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E.

The biological functions of VEGF-A have been characterized in much detail. Based on a series of *in vitro* and *in vivo* studies, VEGF-A has been identified as a major regulator of vasculogenesis and angiogenesis during development (Yeaman, 1997), indicating that it might also be involved in the regulation of angiogenesis during wound healing (Giannobile, 1996; Nimni, 1997).

Its expression is also increased in hypoxic conditions, such as those found at the wound site (Lieberman *et al.*, 2002). In wound healing, VEGF promotes the vascularization of injured tissues and thus facilitates the arrival of inflammatory and reparative cells

(Giannobile, 1996; Martin, 1997; Yeaman, 1997). VEGF-A is vital for endochondral bone formation and also promotes the proliferation of retinal pigment epithelial, pancreatic duct and Shawn cells *in vitro* (Martin, 1997).

Insulin- like Growth Factor (IGF)

IGF is a peptide hormone synthesized and secreted into the circulation primarily by the liver via a growth hormone (GH)-dependent process. It is also released from platelets, osteoblasts, macrophages, monocytes, and chondrocytes. IGF has 2 forms, IGF-I and IGF-II each of which has 2 single chain peptides with structural homology to insulin.

IGF was shown to contribute to the regulation of tissue growth and cellular differentiation *in vivo* and *in vitro*. IGF-I serves as a systemic anabolic function, it is notable that intestine is also a site of IGF-I synthesis and one of the most sensitive IGF-I target tissues (Read *et al*., 1992). This growth factor also acts in an autocrine way and as insulin factor. In combination with PDGF can enhance the rate and quality of wound healing. Other functions of this growth factor include cartilage growth stimulating, bone matrix formation, and replication of preosteoblasts and osteoblasts.

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IGF-I is important for both the regulation of normal physiology, as well as a number of pathological states, including cancer. The IGF has been shown to play roles in the promotion of cell proliferation and the inhibition of cell death (apoptosis). IGF-II is thought to be a primary growth factor required for early development while IGF-I expression is required for achieving maximal growth (Giannobile, 1996; Lieberman *et al*., 2002; Sánchez *et al*., 2003). While IGF-II may be primarily fetal in action it is also essential for development and function of organs such as the brain, liver, and kidney. Beyond general growth, insulin-like growth factors are involved in both pre and postnatal development and function of a large number of organs such as the prostate gland, mammary glands, spleen, pancreatic beta cells, lung, inner ear, sympathetic neurons, thymus and teeth. They are also important factors for muscle development and skeletal growth as well as for skeletal mass maintenance (Sánchez *et al*., 2003).

Epidermal Growth Factor (EGF)

It is released from platelets, macrophages and monocytes. EGF stimulates endothelial chemotaxis and angiogenesis; regulates collagenase secretion and stimulates epithelial and mesenchymal mitogenesis.

This growth factor is present in various body fluids and tissues, and is continuously secreted into the gastrointestinal lumen in humans by submandibular glands, mucous neck cells of the stomach, Brunner's glands of the duodenum, Paneth cells of the small intestine, and ulcer-associated cell lineage (a recently identified glandular structure induced at the sites of injury) (Marti *et al*., 1989; Wright *et al*., 1990).

EGF and EGF family of related peptides are involved as key constituents in the maintenance and repair of gastrointestinal mucosa (Jones *et al*., 1999). Both EGF and TGF-α has numerous functions within the gastrointestinal tract in addition to stimulation of proliferation. It inhibits gastric acid secretion and increases the release of mucus, also up-regulates intestinal electrolyte and nutrient transport, and induces expression of brush border enzymes such as disaccharidases (Goodlad *et al*., 1991; Uribe and Barrett, 1997). EGF is also responsible for cell differentiation and stimulates re-epitheliation, angiogenesis

and collagenase activity. In wound healing, EGF can induce cell proliferation, differentiation and motility. EGF is highly expressed in the margin of wounds promoting re-epithelization (Martin, 1997; Lieberman *et al.*, 2002) and up-regulates matrix metalloproteinase-1 (MMP-1) expression and regulates type I collagen turnover. MMP-1 facilitates epithelial cell migration across the collagen type I matrix in several tissues, including the dermis (Martin, 1997).

Hepatocyte Growth Factor (HGF)

It is released from platelets, fibroblasts or other mesenchymal cells. HGF is a multifunctional polypeptide secreted by mesenchymal cells, functions as a mitogen, morphogen, and/or motogen for multiple subsets of epithelial cells, including gastrointestinal epithelial cells (Jones *et al.*, 1999; Tahara *et al.*, 2003).

HGF activator, HGF activator inhibitor type-1 and HGF-associated molecules involved in the activation of HGF in injured tissues, are associated with colonic mucosal repair (Tahara *et al.*, 2003). Additionally, HGF expression was reported to be up-regulated in inflamed colonic mucosal tissue in patients with ulcerative colitis (Kitamura *et al.*, 2000), and plasma HGF levels are increased in animal models of acute colitis (Nishimura *et al.*, 1998; Ortega-Cava *et al.*, 2002).

This growth factor is also a hepatotrophic factor that promotes liver regeneration. It has also been shown to stimulate the growth of various epithelial cells, such as renal tubular cells, epidermal melanocytes and keratocytes (Nishimura *et al.*, 1998). HGF is a heparin-binding protein that is mitogenic for endothelial and epithelial cells (Nishimura *et al.*, 1998; Ortega-Cava *et al.*, 2002). HGF and VEGF show a synergistic action on endothelial cells and tubulogenesis, a response that does not happen with any of the growth factors alone. HGF is also implicated in cutaneous wound pathologic neovascularization and exuberant granular tissue formation (Martin, 1997).

Extracellular Matrix (ECM)

The ECM is defined as any material produced by cells and excreted to the extracellular space within the tissues. It takes the form of both ground substance and fibres and is mostly made up of fibrous elements, proteins involved in cell adhesion, GAGs and other space-filling molecules (Agren and Werthen, 2007).
The ECM serves as a scaffolding to hold tissues together, its form and its composition help to determine tissue characteristics. In epithelia, it includes the basement membrane. The ECM undergoes dynamic interactions with cells that are pivotal for cell adhesion, motility, growth, differentiation, ECM synthesis, in addition to offering passive support to the cells. The ECM is also a depository of growth factors in latent forms under normal, unwounded conditions. With injury and matrix destruction, the previously bound and inactive growth factors are released in active form and thereby assist in initiating and regulating the repair process (Agren and Werthen, 2007).

2.2.3. Healing of gastrointestinal anastomosis

Anastomotic dehiscence remains a major problem in gastrointestinal surgery. Even if small bowel anastomoses carry the least risk of complications among the other gastrointestinal anastomoses, the healing sequence of anastomoses are necessary to define the underlying mechanisms and find ways to improve surgical outcome in high risk patients (Hendriks and Mastboom, 1990). For a better understanding of the intestinal healing, knowledge of intestinal histology is essential. The gastrointestinal tract has a uniform general histology with some differences which reflect the specialization in functional anatomy. The intestine is divided in the following layers: 1) the mucosa, 2) the submucosa, 3) the muscularis propria, and 4) the serosa.

Mucosa

The mucosa is the innermost layer of the gastrointestinal tract that is surrounding the lumen, within the tube. This layer comes in direct contact with bolus, and is responsible for absorption and secretion, important processes in digestion (Hedlund *et al.,* 2002). The mucosa of the small intestine is highly modified. The luminal surface is completely covered by a number of finger or leaf-like projections called villi. The core of a villus is an extension of the lamina propria, and its surface is covered by a simple columnar epithelium (Hedlund *et al*., 2002).

The mucosa has three parts: (1) an **epithelium** (and of course the associated basement membrane), (2) a layer of loose connective tissue called the **lamina propria**, and (3) a band of smooth muscle, the **muscularis mucosa**, denoting the limit of the mucosa (Hedlund *et al*., 2002).

1) The epithelium acts as a barrier separating the organism's body from the environment, which is continuous with the lumen of the alimentary canal. In different areas, the epithelium may simply be a route (esophagus, anal canal), may have a secretory function (stomach, goblet cells in intestines) or may have an absorptive function (intestines). The type of epithelium present reflects the function of the particular segment of the alimentary canal.

2) The lamina propria consists of loose connective tissue, with numerous blood and lymphatic vessels, and an abundance of diffuse lymphoid tissue. In addition to lymphocytes, plasma cells, eosinophils and macrophages are present. Not infrequently, lymphocytes are seen to be aggregated into lymph nodules. The lymphatic tissue of the lamina propria is referred to as galt, or gut-associated lymphatic tissue, and acts as an immunological barrier for pathogens (Hedlund *et al*., 2002).

3) The muscularis mucosa allows for movement of the mucosa independent of the wall of the digestive tract, thereby increasing its contact with food. In most of the alimentary canal, the muscularis mucosa consists of an inner circular and outer longitudinal layer of smooth muscle (Hedlund *et al*., 2002).

Submucosa

The submucosa consists of a dense irregular layer of connective tissue with large blood vessels, lymphatics and nerves branching into the mucosa and muscularis (Hedlund *et al*., 2002). It contains Meisser`s plexus, an enteric nervous plexus, situated on the inner surface of the muscularis externa. The submucosa provides the gastrointestinal tract with most of the tensile strength and is responsible for anchoring the sutures that hold the anastomosed bowel ends together (Halsted, 1887; Greatorex, 1970).

Muscularis externa

The muscularis externa consists of an inner circular layer and an outer longitudinal layer. The circular muscle layer prevents the food from going backwards and the longitudinal layer shortens the tract. Contractions of these layers are coordinated and called peristalsis, propels the bolus, or balled-up food, through the gastrointestinal tract (Hedlund *et al.*, 2002).

Serosa

The serosa is the outermost layer which is made up of loose connective tissue and coated in mucus so as to prevent friction damage from the intestine rubbing against other tissue. Serosa is important for forming a quick seal at site of injury or incision (Furst *et al.,* 1994; de Waard *et al.,* 1995; Hedlund *et al.,* 2002).

Intestinal wound healing

The three overlapping phases of healing in intestine are named: lag phase, proliferative phase and the maturation phase.

The lag phase occurs during days 0 to 4 and is associated with inflammation and edema of the healing intestine. A fibrin seal forms during the first few hours. Although the fibrin clot contributes to wound strength, during this phase most of the wound strength is attributed to sutures. During the lag phase macrophages are important in wound debridement and production of growth factors that modulate fibroplasia and angiogenesis (TGF- β, EGF, PDGF, and cytokines) (Jones *et al.*, 1999; Hedlund *et al.*, 2002). Healing is functionally weakest at the end of lag phase, because of fibrinolysis and collagen deposition, therefore dehiscence most commonly occurs 3 to 5 days after intestinal surgery (Hedlund *et al.*, 2002).

The proliferative phase begins with the arrival of fibroblasts at the wound site. Fibroblasts become the major cell type by day 4, and their arrival is regulated by various growth factors. Fibroblasts replace the provisional matrix (established through the inflammation phase) with collagen-rich granulation tissue. The strength of the repair site approximates that of normal intestine 10 to 17 days after surgery (Hedlund *et al.*, 2002).

The maturation phase occurs between 10 and 180 days. With time, newly formed granulation tissue undergoes remodeling, and the density of macrophages and fibroblasts is reduced. Collagen is reorganized and remodelled during this phase (Hedlund *et al.*, 2002). Thin collagen fibers transform into thick bundles, and the percentage of type III collagen is reduced to 20%. Wound contraction occurs as fibroblasts pack thick collagen bundles into contractile units.

Factors that contribute to intestinal anastomosis healing

In small animals the reported incidence of small intestinal dehiscence rates 7-16%, with 74 -80% of those patients dying (Hedlund *et al.*, 2002). As for any wound, a compromised anastomosis represents the convergence of a number of factors-systemic, local, and operative that disrupts the normal sequence of wound healing (Gordillo and Sen, 2003). Many factors have been implicated in the success or failure of the anastomotic healing process. The anastomosis oxygenation is of paramount importance and depends primarily on the intrinsic vasculature (Thornton and Barbul, 1997). However, several factors have been described to affect intestinal healing.

Operative factors: It is well known that technical factors relating to the construction of an anastomosis (gentle handling of tissue; aseptic technique; sharp anatomic dissection of tissue; careful hemostasis; obliteration of dead space; avoidance of tension; use of fine instruments for sharp dissection; accurate suture placement and blood supply, these are known as Halsted's principles of surgery) are of paramount importance to ensure optimal anastomotic healing, most likely resulting from effects on tissue perfusion and oxygen delivery (Thornton and Barbul, 1997). Optimal intestinal healing depends on a good blood supply, accurate mucosal apposition, and minimal surgical trauma. Approximating suture patterns facilitate a rapid healing. Everting and inverting suture patterns were found to retard intestinal healing and may result in greater stricture formation. Healing is also facilitated by adjacent serosal surfaces and omentum, which help seal wounds and contribute to the blood supply (Hedlund *et al.*, 2002). Good serosal apposition is necessary to minimize the risk of leakage (Furst *et al.*, 1994; de Waard *et al.*, 1995; Hedlund *et al.*, 2002). Unfortunately, even the most experienced and technically proficient surgeons have a certain leakage rate which suggests that there are other factors at play.

Systemic factors: Wound healing requires energy and adequate nutritional intake by the patient. Malnourished patients are predisposed to wound healing failure as they lack of the necessary proteins, vitamins and minerals for repair. These include vitamins A, C, and B6, all required for collagen synthesis and cross-linking, as well as zinc, iron and copper. Zinc and iron acts as cofactors to many reactions involved in DNA synthesis, protein synthesis, and cellular proliferation. Zinc or copper deficiencies result in fewer fibroblasts, and impacts collagen synthesis (Thornton and Barbul, 1997). Other systemic factors such as shock, hipoproteinemia, debilitation and concurrent infections may delay healing and increase the risk of incisional breakdown. Tension on the repair caused by accumulation of ingesta, fluid, gas or mobilization of the bowel increases intestinal suture breakdown (Hedlund *et al.*, 2002).

Local factors: Such as peritonitis impairs wound healing by prolonging the inflammatory phase, and inducing the increased expression of tissue proteases. Elevated tissue proteases digest growth factors, and thus delay epithelization and collagen deposition. Localized preoperative or perioperative bowel irradiation was not shown to compromise anastomotic healing in animal model (Thornton and Barbul, 1997).

The effect of ischemia on intestinal healing

It is clear that the availability of oxygen is critical for wound healing. Oxygen is essential for normal cell metabolism and energy production in the absence of tissue injury (Gordillo and Sen, 2003). Oxygen availability becomes especially important during wound healing as a result of the increased metabolic demands for processes such as cellular proliferation and collagen synthesis (Gordillo and Sen, 2003; Tandara and Mustoe, 2004). Adequate tissue oxygenation is required for normal oxidative function of neutrophils, leukocyte activation, fibroblast production, angiogenesis, and reepithelialization, which all are essential in wound healing (Grim *et al.*, 1990; Hirn, 1993). Fibroblasts require oxygen for their energy production, and collagen can not be synthesised in the absence of molecular oxygen (Roberts and Harding, 1994).

Oxygen is necessary in the hydroxylation of lysine and proline during collagen synthesis, which is ultimately responsible for conferring tensile strength to the wound (anastomosis) (Gordillo and Sen, 2003; Tandara and Mustoe, 2004).

Hypoxemia, caused by disrupted vasculature, is a key factor that limits wound healing. The central area of the wound is the most hypoxic, with a progressive increase in the oxygen gradient toward the uninjured tissue at the periphery (Khanna and Wallace, 2002). Disruption of microcirculatory blood flow at the level of the anastomosis is one of the major factors leading to anastomotic dehiscence and leakage. Decreased tissue perfusion begins intraoperatively, and can lead to necrosis and tissue breakdown in the early postoperative period. Studies showed that the anastomosis was at highest risk during the first 24h, and that microvascular recovery has improved anastomotic perfusion by 96h (Schroder *et al.*, 2004).

In addition to oxygen, many growth factors and cytokines are required for wound healing to proceed normally. Considering the numerous different substances produced by the wound, nitrous oxide has a particularly important role. The effect of nitrous oxide on surgical wound infections has been investigated previously. *In-vitro* evidence indicates that exposure to nitrous oxide inactivates vitamin B12 and thus methionine synthase (Perry *et al.*, 1983). Methionine synthase is the enzyme responsible for conversion of homocysteine to methionine and methyltetrahydrofolate to tetrahydrofolate, which are critical pathways for thymidine formation, which, in turn, is essential for DNA formation. Without functioning methionine synthase, protein cannot be produced. Protein expression is a critical aspect of scar formation and tissue repair (Hassanain *et al.*, 2005). Thus, nitrous oxide toxicity could impair healing. Nitrous oxide also has been shown to depress chemotactic migration by monocytes, apparently by interfering with microtubules. Chemotaxis is a key part of the bacterial killing process, which needs chemotaxis, phagocytosis, and killing (Benhaim and Hunt, 1992; Fleischmann *et al.*, 2005).

2.2.4. Parameters for anastomotic repair evaluation

Substantial investigative effort has been dedicated to develop strategies to improve anastomotic healing, including improved surgical technique, bowel preparation, nutritional and pharmacological interventions. Investigating wound healing and attempting to improve its outcome necessitates process quantification. Parameters to evaluate the anastomotic repair may be:

a) Mechanical parameters.
b) Biochemical parameters.
c) Histological parameters.

a) Mechanical parameters: two mechanical parameters have been used to evaluate the healing of intestinal anastomosis i.e., bursting pressure and breaking strength (Hendriks and Mastboom, 1990; Ikeuchi *et al.*, 1999, Månsson *et al.*, 2002).

Bursting pressure: is a measure of the resistance of the intestinal wall to increasing intraluminal pressure and is considered a good mechanical parameter to monitor the anastomotic repair as long as the rupture takes place within the anastomosis. Experimentally, the bursting pressure is the pressure at which disruption occurs when the segment of bowel (containing the anastomosis) is progressively distended with gas or liquid (Hendriks and Mastboom, 1990; Ikeuchi *et al.*, 1999; Månsson *et al.*, 2002). The bursting pressure measurement apparatus consisted of an air-filled 60-ml syringe placed in an infusion pump and connected via plastic tubing to an in-line sphygomanometer. Generally, bursting pressure is recorded in millimetres of mercury (mmHg). This method of measurement is found to be cheap and easy to perform. The bursting pressure is a more

reliable measure to evaluate early postoperative anastomotic mechanical strength, especially within a week of operation (Hendriks and Mastboom, 1990; Ikeuchi *et al.*, 1999; Mansson *et al.*, 2002). In addition, the bursting pressure is generally considered to reflect the physiological strain in the intestine more accurately than the breaking strength (Hendriks and Mastboom, 1990; Månsson *et al.*, 2002).

However there are differences in results in diverse studies. The existence of a certain degree of variation between data from these types of experiments is not unexpected, since tissue repair can be affected by a multitude of factors. At first sight, non-uniformity in suturing techniques could be envisaged to contribute to the numerical divergence apparent from the various publications. It is regrettable that the techniques employed and the suture materials used are not always described fully. Evidently, these have differed widely and consequently variations on bursting pressure results (Hendriks and Mastboom, 1990).

According to Hendriks and Mastboom, (1990), bursting pressure measurements also depends on the treatment of the bowel after sacrifice. In most cases, the anastomotic segment is isolated, one end is tied off, and the other end is connected to a nipple through which gas or liquid is infused. This procedure requires careful handling. Also, adhesions involved in sealing the closures might be disturbed. It often remains unclear if adhesions are removed or left in place. Most authors only refer to excision of the anastomosis without mentioning adhesions at all. The fact that this factor is indeed of considerable influence, certainly during the first post-operative days, may be illustrated by the bursting pressures measured in anastomoses which have been left in place (Hendriks and Mastboom, 1990).

Hendriks and Mastboom (1990) and Månsson *et al.* (2002) have shown that the rate of inflation of the bowel is an important determinant in the outcome of bursting pressure measurements, and this confounding factor has been suggested to help explain the difficulty in comparing bursting pressure data from different studies. Furthermore, the bursting pressure analysis depends critically on whether the anastomosis is left *in situ* or is excised (Hendriks and Mastboom 1990), some authors determinate a bursting pressure *in situ* on the anaesthetised animal to preserve vascular supply of the colon and to minimise the mechanical manipulation and possible *post-mortem* changes of an anastomosis before testing (Christensen *et al.*, 1991; Petersen *et al.*, 1996; Colak *et al.*, 2003) and other determinate bursting pressure before sacrifice the animal (Ikeuchi *et al.*, 1999; Siemonsma *et al.*, 2003; Posma *et al.*, 2007). This fact can lead some differences in the results of various studies.

Few investigators have compared breaking strength and bursting pressure measurements in the same study. Smith *et al.* (1982) evaluated the impact of drainage materials on anastomosis repair and demonstrated that breaking strength values were similar while data on bursting pressure differed significantly between groups, indicating that bursting pressure measurements are more sensitive than those of breaking strength. In addition, it has been reported that there is no correlation between breaking strength and bursting pressure in colonic anastomosis (Ikeuchi *at al.*, 1999).

Breaking strength: reflects the resistance of the intestinal wall to forces exerted in a longitudinal direction. Breaking strength is measured by applying an increasing force in a longitudinal direction to anastomotic segments. The peak force necessary to induce disruption is taken as the breaking strength (Hendriks and Mastboom, 1990; Ikeuchi *et al.*, 1999; Månsson *et al.*, 2002).

Generally, for breaking strength determination the intestinal segment with the anastomosis in the middle is placed between the two clamps of the tensiometer with sutures left in place or removed according to the rules of the investigator. Testing force is applied perpendicular to the direction of the anastomosis line. The anastomosis is stretched until rupture at a constant force.

The peak force necessary to induce disruption is taken as the breaking strength in kilograms force (Kgf). The minimal tensile strength (MITS) necessary to break part of anastomosis and maximal tensile strength (MATS) needed to disrupt the whole anastomosis are registered for each sample. Unfortunately, many of the tensiometers described in the literature are not well suited to evaluate the tensile strength of intestinal anastomoses because they are not sensitive enough to reproducibly quantify such small forces. In addition, some of these have proven to be sensitive and reproducible enough for this application but frequently prohibitively expensive.

However, data may vary between studies. Weiber *et al.* (1994) found that breaking strength is constant up to 4 days after formation of colonic anastomosis while others have reported that breaking strength may decrease markedly 2 days postoperatively (Hogstrom and Haglund, 1985). The reason for this inconsistency in breaking strength data is not completely known yet, but it may be because breaking strength is very sensitive to the surgical technique, being a critical aspect the distance between the wound edges and the site at which the sutures are placed (Hogstrom and Haglund, 1985). Another possible explanation is related to the fact that some studies use constantly increasing forces (Jiborn *et al.*, 1978), while others use increasing forces by intervals (Hillan *et al.*, 1988).

It stands to reason that the outcome of breaking strength measurements is equally dependent on the procedure followed for the isolation of the segment and the actual testing method applied (Hendriks and Mastboom, 1990; Månsson *et al.*, 2002). Anastomotic breaking strength has been reported to increase progressively from day 7 to day 28 after operation, suggesting that breaking strength may be used to study to late healing of colonic anastomoses, although outside the clinically relevant period (Weiber *et al.,* 1994). Interestingly, this late increase in breaking strength is correlated in time with the accumulation of fibrillar collagens (types I and III) (Brasken, 1991), indicating that breaking strength may be of value in later phases of anastomotic healing.

b) Biochemical parameters: The biochemical description of anastomotic repair is limited to the behavior of collagen, as represented by its rather unique constituent amino acid hydroxyproline. Usually early anastomotic strength depends on the ability of the existing collagenous network to retain the sutures while newly formed collagen fibrils should restore the original strength to the healing bowel. Thus, postoperative collagen degradation and synthesis are expected to affect anastomotic strength, and the course of various parameters for collagen metabolism has been taken as a measure for anastomotic healing. As yet, the majority of the data reported concern to quantitative aspects of collagen-concentration, content, and synthesis, while its quality, in terms of solubility, crosslinking, or type, has received little attention (Hendriks and Mastboom, 1990).

Collagen concentration and content: Quantification of collagen in enteric anastomoses has been synonymous with quantification of hydroxyproline, an amino acid unique to collagenous proteins in most tissues. The hydroxyproline level is always taken as a measure for the amount of collagen present. Anastomotic hydroxyproline concentrations, usually expressed on the basis of dry weight, change massively during the first postoperative

period. For collagen determination a sample containing part of the anastomosis is usually stored in liquid nitrogen for subsequent hydroxyproline assay. After weighing, the sample is frozen, lyophilized, and pulverized. The hydroxyproline content, as a measure of the collagen content, is measured by high-performance liquid chromatography after hydrolysis with 6N hydrochloric acid and derivatization with dabsylchloride. The hydroxyproline content is expressed as μg/mg of wet tissue weight (Hendriks and Mastboom, 1990; Posma *et al.*, 2007).

Generally, a lower anastomotic hydroxyproline concentration reflects the presence of less collagen and thus poorer healing. However this is not necessarily the case: a changed concentration may be caused by changes in noncollagenous substances and an unchanged concentration may be the result of, for instance, a loss of collagen attended by an increased amount of noncollagenous material. (As part of the healing process, noncollagenous fibrous tissue is always present in the region of an anastomosis site. During removal of the anastomosis, this adhered tissue cannot be separated completely from the anastomotic segment, and this noncollagenous tissue comprises part of the tissue weight). Without this type of precaution, it seems advisable to show some restraint in concluding that a certain treatment impairs or improves anastomotic repair, or anastomotic collagen metabolism (Hendriks and Mastboom, 1990).

One way to determine that differences in the postoperative course of hydroxyproline concentrations between various experimental groups are attributable to changes on collagen metabolism is to ensure that changes in total dry weight of the anastomotic segments in the groups are similar. Another precaution must take during postoperative course of collagen content description, a control value should be available that represents the collagen content at the time of surgery. If the anastomotic hydroxyproline content is to be measured in an intestinal segment containing the entire anastomosis, the control cannot simply be a segment of uninjured intestine of similar length (obtained at operation from the same animal or from an unoperated control animal) since the anastomotic segment will always contain more tissue per centimeter than a biopsy from normal intestine (Hendriks and Mastboom, 1990).

Collagen synthesis and degradation: The occurrence of collagen synthesis around experimental intestinal anastomoses has been shown *in vivo*, in both ileum (Jönsson *et al.*, 1987) and colon (Jiborn *et al.*, 1980) from two days after operation onwards. The methodology used for such experiments requires administration of large amounts of radioactive-labeled proline to animals and precludes the measurement of synthesis in the immediate postoperative period as the precursor needs to be injected at least 24 hours before sacrifice.

Subsequently, the incorporation of label into hydroxyproline is determined and it's specific (dpm labeled hydroxyproline per mole hydroxyproline present) or total (dpm labeled hydroxyproline per biopsy) activity is a measure for the amount of newly synthesized collagen. The course of anastomotic collagen synthesis may thus be followed and compared under various conditions thought to affect anastomotic repair. So far, application of this technique in the research on intestinal healing has been limited, possibly because it requires administration of large amounts of (expensive) labeled proline to living animals (Hendriks and Mastboom, 1990).

Quality of collagen: Ultimately, collagen mass have been found to be responsible of anastomotic strength. It is important to point out that the strength of collagenous tissue may also depend on the types of collagen present. In this respect, the collagen types present and the stability of the constituting crosslinks are of importance for the strength of the collagenous network (Hendriks and Mastboom, 1990).

In this point of view not only the collagen mass determines the anastomotic strength but also the quality of the collagen fibrils. For instance, the major collagen types in soft tissues, type I and type III, form fibrils with large and small diameters, respectively. In addition to the fact that fibril diameter plays a major part in determining its mechanical properties, it has also been suggested that collagen in small fibrils shows a higher turnover rate than collagen in large fibrils (Hendriks and Mastboom, 1990).

Thus, a changing type distribution will certainly affect tensile strength. Healing tissue shows changing patterns of collagen types: type III transiently becomes more abundant than in uninjured tissue and an important role has also been suggested for type V (Hendriks and Mastboom, 1990). While it is known that the intestinal wall contains the collagen types I, III, and V, nothing has been reported about its composition after resection and anastomosis. This knowledge might be valuable in the search for disorders on the molecular level, which may explain impaired anastomotic healing (Hendriks and Mastboom 1990).

c) Histological parameters: is based on the observation of the sample by means of a conventional binocular light microscopy, after the tissue has been fixated, dehydrated, embedded, cut and stained. Common stained used are haematoxylin-eosin (HE). Histological evaluation certainly it is very useful to describe the course and eventual result of the healing sequence at tissue level, and the successive infiltration of various cell types into the wound area (Hendriks and Mastboom 1990; Verhofstad *et al.*, 2001).Various histological parameters of wound repair have been studied in intestinal anastomosis to compare the healing processes on treated and untreated groups in an experimental model. Those parameters include: healing of the mucosa based on restored glandular epithelium; edema expressed as the ratio of maximum thickness of the wall at the anastomosis to the thickness of the normal intestinal wall; degree of necrosis; creation of fibrin network; sequential appearance and migration of polymorphonuclears (PMNs) and macrophages; proliferation and orientation of fibroblasts. Creation of new extracellular matrix especially, collagen fibres, neoangiogenesis and granulation tissue (Verhofstad *et al.*, 2001).

Restoration of tissue strength is an important aspect of wound healing. This process requires the deposition of adequate amounts of extracellular matrix components, particularly collagen fibers, in the wound area. Generally classic (HE) stain is used to determine the number of inflammatory cells and the overall architectural completion of the anastomosis (presence of gaps and healing by layers). However the organization of collagen fibers is essential in understanding the process of anastomotic healing. Special collagen stain (e.g. Masson's trichrome), rather than routine histopathological stain (e.g. HE), can visualize collagen fiber organization. Masson's trichrome stain is used for the detection of collagen fibers and their increases in tissues, on formalin-fixed, paraffin-embedded sections, and may be used for frozen sections as well. The collagen fibers will be stained blue, the nuclei will be stained black and the background is stained red (Rabau *et al.*, 1995).

2.2.5. Studies performed in the intestine to improve healing

Gastrointestinal anastomosis is among the most frequently performed procedures in general surgery clinics. Numerous experimental and clinical studies in the literature have investigated the factors that have an effect on healing of gastrointestinal anastomosis. Although many local and systemic factors affecting anastomotic healing have been determined, anastomotic leaks still cause considerable morbidity and mortality in daily surgical practice. In human medicine and large collective review, general incidence of leakage after gastrointestinal anastomosis was found to be 1.8% with a mortality rate of 24% (Pickleman *et al.*, 1999). On the other hand, overall incidence of leakage for colonic anastomosis is between 5.5% and 9% (Chandramouli *et al.*, 2004).

Attempts to enhance anastomotic healing have included the use of various surgical techniques and materials: quality of suture threads, use of staples, control of sepsis by specific bowel preparation before surgery, use of parenteral nutrition; mechanical protection (endoluminal latex prosthesis, biofragmental ring) and local application of fibrin bioadhesive, fibrin and collagen patches, omental flaps with the most promising results. Nowadays a number of authors investigated the (local or systemic) use of various growth factors, hormones (GH) and pharmacological agents such as the antibiotic doxycycline (orally or subcutaneously) in order to enhance some of the phases of the wound healing process (Christensen *et al.*, 1990; Adams *et al.*, 1992; Christensen *et al.*, 1991; Christensen and Oxlund, 1994; Christensen *et al.*,1995; Harrison *et al.,* 1995; Petersen *et al.*, 1996; Egger *et al.,* 1998; Merad *et al.*, 1998; Egger *et al.*, 1999; Zilling *et al.*, 1999; Pierie *et al.*, 2000; Egger *et al.*, 2001; Kanellos *et al.*, 2002; Kanellos *et al.*, 2003; Siemonsma *et al.*, 2003; Kanellos *et al.,* 2004; Papalambros *et al.,* 2004; Ozel *et al.*, 2006; Nordentoft *et al.*, 2007).

Clinical and experimental gastrointestinal anastomoses have been sealed with omental flaps. The omentum has been found to be effective in preventing leakage after the reestablishment of gastrointestinal continuity. Omentum can reabsorb fluid collections as an ideal biologic drain because it is rich in lymphatics and vascularity (Morison *et al.,* 1906; Merad *et al.*, 1998; Pierie *et al.*, 2000). Indeed, Adams *et al.* (1992), reported an animal model of a vascular anastomosis that developed between the omental blood supply and bowel vessels as early as the third postoperative day to aid in anastomotic healing, and they concluded that the omental wrap can be used to protect a compromised anastomosis by providing both a biologically viable plug to prevent early leakage and neovasculature for later wound repair.

Fibrin glue (FG) has many different uses across a broad range of surgeries, where they have proved to be successful in controlling bleeding, supporting angiogenesis and stimulating the macrophages, that secrete the necessary factors for fibroblast proliferation and collagen production, to go to the related region, providing suture support and tissue sealing (Zilling *et al.*, 1999; Kanellos *et al.*, 2002; Kanellos *et al.*, 2003). In gastrointestinal surgery, FG has been used as a protective seal in normal and high risk anastomoses and also has been claimed to strengthen the anastomoses and one study demonstrated a reduction of anastomotic leakage (Kanellos *et al.*, 2004). The combination of suture and FG has been proposed by Hjortrup *et al.* (1989) in their experiment using dog rectums, anastomoses was made combinating both fibrin adhesive internally and sutures externally. In other experimental studies, sutureless anastomoses have been made successfully with liquid FG on small bowel (Detweiler *et al.*, 1999; Zelling *et al.*, 1999; Bonanomi *et al.*, 2004). Kanellos *et al.* (2002) also applied FG on the anastomotic colon edges by an applicator tip before suture pattern; and in another study they performed first suture and FG after around the

anastomosis line (Kanellos *et al.*, 2003). Ozel *et al.* (2006) used fibrin-collagen patch to seal intestinal anastomosis in rats and Nordentoft *et al.* (2007) sealed small bowel anastomosis in pigs with collagen patch coated with FG components with hopeful results.
In attempt to improve intestinal wound healing, various pharmacological agents have been used in experimental studies to define their role in the healing of bowel anastomoses. Doxycycline has been demostrated to improve wound strength after intestinal anastomosis in the rat but only at day 3, when it was at its lowest (Siemonsma *et al.*, 2003).

Growth hormone (GH) is a very potent anabolic agent released in the physiological situation in a pulsative manner from the pituitary gland. As a result of its anabolic actions, exogenous GH has beneficial effects on protein conservation and has a pronounced stimulatory effect on the wound healing. The gastrointestinal tract is highly responsive to the trophic effect of GH, which stimulates the healing of intestinal anastomoses (Christensen and Oxlund, 1994; Petersen *et al.*, 1996). The effects of GH on healing of bowel anastomosis are currently being evaluated in an attempt to identify agents that may promote and improve the anastomotic healing process, especially in seriously ill patients with altered metabolism and impaired tissue healing (Christensen *et al.*, 1991; Christensen and Oxlund, 1994; Christensen *et al.,* 1995; Gomez de-Segura *et al.,* 1995; Harrison *et al.,* 1995). Zaizen *et al.* (1990) had demonstrated that GH administration after celiotomy for 5 post-operative days improves wound healing in malnourished rats.

The investigators suggest that GH may be clinically applicable in preventing wound complications in malnourished patients requiring urgent or emergency laparotomy. The colon is highly responsive to exogenous GH as demonstrated by increases in weight and collagen content in intact left colon of adult male and female rats after GH treatment for 30 days (Christensen *et al.*, 1990). Wheeless *et al.* (1998) demonstrated that post-operative recombinant human growth hormone (rhGH) treatment increased the bursting pressure of the ileal anastomotic segment in an irradiated rat model. GH also has a stimulating effect on left colonic anastomoses by increasing the strength and collagen deposition rate in non-irradiated bowel in rats (Christensen *et al.*, 1991; Christensen and Oxlund, 1994; Harrison *et al.*, 1995).

For the last decade, it has been acknowledged that growth factors are essential for regulating the cellular events involved in the formation of granulation tissue and in wound healing. Recently, clinical trials were initiated to study the wound healing effect of applying growth factors in wounds. Several growth factors such as FGF, IGF-I, EGF, keratinocyte growth factor (KGF), PDGF have been demonstrated to stimulate intestinal wound healing (Mustoe *et al.*, 1990; Nanney *et al.,* 1990; Mustoe *et al.*, 1991; Slavin *et al.*, 1992). These factors are dependent on being applied directly in the wound or systemically. Intraluminal administration of EGF has been shown to increase tensile strength in intestinal wounds in pigs (Kingsnorth *et al.,* 1990) and has minimised the increase in intestinal permeability and reduced histological changes following ischaemia and reperfusion in the rat (Villa *et al.*, 2002).

Parenteral administration of IGF-I reduced catabolism post intestinal ischaemia (Haglind *et al.*, 1998) and restored serum total protein, reduced weight loss, and enhanced intestinal regeneration following small intestine resection (Yamaguchi *et al.*, 2000). Exogenous administration of this growth factor has also been shown to induce intestinal wound healing in dexamethasone-treated rats (Read *et al.*, 1992) and enhance mucosal adaptation after jejuno-ileal resection (Vanderhood *et al.*, 1992). Intra-ileal infusion of IGF-I, has been shown to have trophic effects on the intestine (Olanrewajutt *et al.*, 1992). IGF-I also

partially prevents experimental colitis in rats (Hawarth *et al.*, 1998), and has demonstrated to reverse diabetes-induced impairment of wound healing in rats (Bitar, 1997). Treatment of colon-operated rats with IGF-I increased the postoperative body weight and stimulated the collagen deposition of left colonic anastomoses, whereas the anastomotic strength remained unaffected by this treatment (Petersen *et al.*, 1996).

In inflammatory bowel disease, increased expression of KGF has been described in mucosa suggesting a potencial role of KGF in mediating mucosal repair mechanisms (Brauchle *et al.*, 1996; Finch *et al.*, 1996). Systemic administration of KGF ameliorated colonic mucosal injury in different models of colitis in rats and mice (Zeeh *et al.*, 1996; Egger *et al.*, 1999). In other study systemic administration of KGF promoted anastomic healing in the left colon in rats (Egger *et al.*, 1998) and increased anastomic bursting pressure compared with values in control animals.

As a source of growth factors PRP has been clinically applied for accelerating healing either on hard and soft tissues (Anitua, 1999; Sanchez *et al.*, 2003; Anitua *et al.*, 2004; Marx, 2004), but limited studies have been performed on gastrointestinal healing. Brady *et al.* (2006) suggested that the use of platelet concentrate gel via an endoscopic delivery system, after a laparoscopic Roux-en Y gastric bypass procedure, contributed to an enhanced hemostatic response, accelerated tissue healing, and improved collagen synthesis, thus preventing anastomotic leaks and thereby improving outcome. Yol *et al.* (2008) also used PRP in colonic anastomosis on rats and they observed the positive effects of PRP on inflammation, increasing the strength and collagen deposition rate after colonic anastomosis, concluding that PRP may be used in colon anastomosis, especially in patients with impaired wound healing, to obtain a better anastomosis.

2.3. Platelet-Rich Plasma (PRP)

Before PRP was first introduced by Whitman *et al.* (1997) and Marx *et al.* (1998) for its use in oral and maxillofacial surgery, fibrin sealants and biomaterials were being employed to improve tissue healing.

Fibrin sealants are any substance with characteristics that allow for polymerization, holding tissues together or serving as a barrier to leakage (Reece *et al.,* 2001). There are three basic types of fibrin sealants: 1) autologous, 2) homologous (both obtained from cryoprecipitate), and 3) synthetic/commercial. The major advantage of a single donor for fibrin sealant is the fact that the donor's cryoprecipitate is readily available, can be stored and it allows a sufficient time for adequate screening for transmissible diseases. Cryopreserving is a laborious, intensive and costly process that requires several steps to add and to remove the cryoprotective. These reasons seem to contribute to the limitations in its widespread use. The synthetic/commercial fibrin sealants advantage includes their availability and ease of use. However, the disadvantage is related with thrombin component which usually is a commercial bovine one, and is expensive, not readily available, with storage problems and cause adverse reaction (Landsberg *et al.,* 2000). Synthetic/commercial products are slightly more expensive than autologous and homologous fibrin sealants (Matras, 1982; Matras, 1985; Whitman *et al.,* 1997).

A **biomaterial** is defined as any substance synthetic or natural in origin, which can be used for any period of time, as whole or part of a system which treats, augments or replaces any tissue, organ or function of the body and is continuously or intermittently in contact with body fluids. There are currently five types of biomaterials in use: polymers, composites, metals and alloys, ceramics (often used in combination with metals) and biological materials (Lloyd and Cross, 2002). Especially, biodegradable biomaterials play an important role in medical applications (Anitua *et al.,* 2007). The advantages of biomaterial include their degradability and can disappear in the body, before accomplishment its action; it is not necessary to retrieve the material from the body. Disadvantage, is that some are not reasonable cost-effective.

PRP is an autologous product that concentrates a high number of platelets in a small volume of plasma, produced from anticoagulated freshly drawn autologous whole blood. In general, this blood is obtained by point-of-care devices and sequestered into different blood components, namely, PRP, platelet-poor plasma (PPP), and erythrocyte concentrate (Whitman *et al.,* 1997; Marx *et al.,* 1998; Anitua, 1999; Zimmerman *et al.* 2001; Appel *et al.,* 2002; Everst *et al.,* 2006).

Platelet rich plasma (PRP), although other terms such as, platelet concentrate (PC), autologous platelet gel (APG), plasma very rich in platelets (PVRP), platelet rich in growth factors (PRGF), are used for the same preparations (Whitman *et al.,* 1997; Marx *et al.,* 1998; Anitua *et al.,* 1999; Marx *et al.,* 2001; Appel *et al.,* 2002; Ervest *et al.,* 2007). In the present investigation we used the term PRP.

The advantage of the PRP is to be autologous, disease transmission is not an issue; can be generated in the clinical setting and with small volumes of blood, the patient does not need to incur the expense of the harvesting procedure in hospital or at the blood bank; is easy to handle and actually improves the ease of application of bone substitute materials, bone grafting products and other tissues by making gel-like. Obviously, patients with bleeding disorders or hematologic diseases do not qualify for this in-office procedure (Marx *et al.,*

1998; Anitua *et al.,* 1999; Zimmerman *et al.,* 2001; Appel *et al.,* 2002; Weibich and Kleis, 2002; Weibrich *et al.,* 2003).

The crucial step was begun after some investigations (Whitman *et al.,* 1997; Marx *et al.,* 1998), which introduce the use of PRP in oral and maxillofacial surgery. Marx *et al.* (1998) presented the use of PRP in the reconstruction of mandibular continuity defects with cancellous marrow grafts and described a higher maturation rate and greater bone density radiographically compared with grafts without PRP. Autologous platelet as a source of healing factors have been shown to enhance bone regeneration and soft tissue maturation in the fields of orthopaedics, periodontics, maxillofacial surgery, urology, and cosmetic surgical treatments (Man *et al.,* 2001; Anitua *et al.,* 2004; Anila and Nandakumar, 2006; Murray *et al.,* 2007).

PRP contain a number of important growth factors such as PDGF, TGF- β, IGF, EGF, HGF and VEGF. Additionally, PRP also contains proteins (i.e. fibrin, fibronectin, vitronectin) known to act as cell adhesion molecules for osteoconduction and as a matrix for bone, connective tissue and epithelial cell migration (Slater *et al.,* 1995; Marx *et al.,* 1998; Marx *et al.,* 2004; Everts *et al.,* 2006). Thus, it is logical to use platelets as a wound healing stimulator.

The most significant benefit of using an autologous growth factors (AGF) concentrate is its being autologous, endogenously derived, and easily available. There are no issues about immunogenicity or transmission of infection. There are no known local or systemic side effects or adverse effects. The process is also considerably cost effective as compared with the use of purified or recombinant growth factors, and also it may be a more physiologically source to provide a combination of all factors in the platelets rather than individual factors (Lowery *et al.,* 1999).

Usually, PRP is applied after its activation by platelet agonist to form a gel. The most used PRP activators are calcium chloride (a citrate inhibitor that allows the plasma to coagulate) and thrombin (an activator that allows polymerization of the fibrin into a viscous gel) (Marx *et al.,* 1998; Anitua *et al.,* 1999; Landesberg *et al.,* 2000; Appel *et al.,* 2002). Platelets become immediately activated because of interaction with thrombin and calcium, the most potent platelet activators, and a sticky platelet aggregate is formed. Afterwards, platelet α-granules release growth factors into the extracellular environment, where they bind to specific platelet growth factor receptors. After this, through intracellular tissue signaling, a number of pathways are triggered that initiate the healing process (Harrison, 1993; Kamath *et al.,* 2001).

Thereafter, the PRP gel can be exogenously applied in various forms: as biologic membranes, sprayed on soft tissue, with a syringe or as a solid clotted jelly mass applied to soft tissues or applied on the surface of the graft immersion (embedded titanium implant and tendon) (Anitua *et al.,* 2007) or successive local application (diabetis and ulceral wound healing) (Margolis *et al.,* 2004; Saldalamacchia *et al.,* 2004) to surgical wound sites during closure and to soft tissue structures to stimulate tissue regeneration, or it is mixed with bone or bone substitutes to accelerate bone healing (Aghaloo *et al.,* 2004; Schlegel *et al.,* 2004; Suba *et al.,* 2004).

In fact, the interest in PRP is increasing as novel potential effects are being discovered. For example, recent reports suggest that PRP exerts potent angiogenic effects (Anitua *et al.,* 2006) and also antibacterial effects against *Staphylococcus aureus* and *Escherichia coli* (Bielecki *et*

al., 2007). A reduction in the development of severe postoperative wound infections with the application of PRP during incision wound closure after a cardiac surgical procedure has been reported (Trowbridge *et al.,* 2005). Platelet-rich products act also as anti-inflammatory agents by blocking monocyte chemotactic protein-1 (MCP-1) released from the monocytes and lipoxin A4 generation (El-Sharkawy *et al.,* 2007). In addition, serotonin, a neurotransmitter and hormone present in platelets, has been reported to directly mediate liver regeneration (Lesurtel *et al.,* 2006).

The outcome of multiple studies on the efficacy of PRP treatment has been published. Proponents of PRP application refer to improved wound healing and increased bone growth to warrant its use (Marx *et al.,* 1998; Anitua, 1999; Man *et al.,* 2001; Sanchez *et al.,* 2003; Anitua *et al.,* 2004; Anitua *et al.,* 2007).

There is a large variety of animal studies on PRP gel research in the literature (Marx *et al* 1998; Aghaloo *et al.,* 2002; Kim *et al.,* 2002; Li *et al.,* 2004; Weibrich *et al.,* 2004; Intini *et al.,* 2007), however, the results tend to be confusing and the reader might conclude that the animal data on PRP studies is conflicting. One concern is that a variety of different animal species has been used, and often no information of platelet counts or growth factor numbers in the PRP is provided. Furthermore, methods showing how PRP was produced are sometimes lacking. Some investigators even used damaged platelets (activated platelet) whereas others did not activate the PRP at all, as most clinicians would do in a clinical setting to release PRP growth factors (Nikolidakis *et al.,* 2006). Also, "true" autologous PRP is not always achieved in small animals.

Conversely, there are publications that have concluded that there was little or no benefit from PRP. For example, in an animal study, Aghaloo *et al.* (2002) grafted rabbit calvarial defects with autogenous bone, PRP alone, or autogenous bone and PRP; the control had no treatment. The histomorphometric evaluation showed a tendency for slightly more bone when PRP was combined with autogenous bone than for autogenous bone alone, but this difference was not significant. Also, Plachokova *et al.* (2006) and Plachokova *et al.* (2007) showed that PRP had no effect on early bone healing in addition to an osteoconductive material in a rat model. On other study, Choi *et al.* (2004) did not find any effect of PRP on bone formation in the mandible reconstruction of a canine model. Li *et al.* (2004) also concluded that the combination of beta tricalcium phosphate biomaterial and PRP did not improve the bone-forming capacity in anterior spine fusion on pigs. Therefore, the analysis of the animal studies outlines the controversial nature of this issue and generates confusion regarding the beneficial effect of PRP on other studies.

The positive observations of PRP indicate that its application is a promising technique to deliver autologous platelet growth factors to the wound/tissue. However, more scientific evidence and data to support the use of PRP in clinical settings are mandatory for progress in the use of these autologous biotechnology procedures to be achieved. Recently, novel applications have emerged in the field of PRP applications and in a variety of surgical disciplines.

2.3.1. Applications on soft and hard tissue, properties, contraindications and risks.

- Clinical use in humans and animal models

Effects of PRP on chronic nonhealing diabetic foot ulcer wounds

Diabetic foot ulcerations are multifactorial in origin and are known for their slow healing rate and difficult to treat because of poor wound healing, persistent sepsis and underlying neuropathy. Complications, such as infection and gangrene, are frequent and amputation is often necessary (Crovetti *et al.,* 2004; Driver *et al.,* 2006). Recently, the topical application of autologous PRP gel provides an ulcer management option that may avoid or prevent loss of a limb by major amputation. Saldalamacchia *et al.*(2004), in a controlled study of the use of autologous platelet gel for the treatment of diabetic foot ulcers have reported promising results obtained for patients with chronic nonhealing (diabetic) wounds after topical PRP application. Some authors demonstrated that the application of PRP was more effective than standard care methods for wound healing, and that this treatment was even more effective for patients with deeper wounds and chronic non-healing wounds when treated with PRP dressings (Margolis *et al.,* 2004; Mazzucco *et al.,* 2004). Pain reduction following PRP application was observed in a study by Crovetti *et al.* (2004), an effect that is still not understood.

Effects of PRP on tendons and ligaments

Soft tissue trauma such as tendon and ligament ruptures and joint capsular injuries, which frequently occur, often require a surgical intervention (Kannus *et al.,* 1997; Sharma *et al.,* 2005). It is supposed that surgical repair combined with the application of biologically active PRP should accelerate healing with an improved outcome. The mechanism of action by which tendon repair is improved with the application of PRP is based on release of VEGF. This growth factor stimulates angiogenesis, leading to an improved blood supply, which is mandatory for the tendon repair process (Sanchez *et al.*, 2003; Anitua *et al.,* 2004). Furthermore, released platelet growth factors induce a proliferation of tendon cells and stimulate production of VEGF and HGF, a potent antifibrotic agent. The latter may be of importance in reducing scar formation and fibrosis in newly reconstructed tendon tissue, which may lead to poor outcomes (Sanchez *et al.*, 2003; Anitua *et al.,* 2004; Everts *et al.,* 2007).

Aspenberg and Virchenko (2004) showed, in an *in vivo* rat model, that PRP applied to traumatize Achilles tendons increased tensile strength and stiffness by about 30% after the first week. The effect persisted for as long as 3 weeks after the injection, suggesting that the use of PRP in tendon repair improved the physiological healing process. Sanchez *et al.* (2003) used PRP to anterior cruciate reconstructive ligament surgery with an autologous graft and improved fixation of the graft within the bone tunnels. Recently, Mishra and Pavelko (2006) used platelet gel in the treatment of chronic elbow tendinitis. Treated patients had less pain and better function when compared to other non operative or conservative standardized physical therapy protocols (Anitua *et al.,* 2004).

Effects of PRP on ophthalmic surgery

In vivo ophthalmic clinical studies were performed using autologous platelet-rich plasma to treat diverse ocular surface disorders such as idiopathic macular holes, ocular surface disorders, superior limbal keratoconjunctivitis, persistent epithelial defect, recurrent erosion syndrome and symptomatic dry eye (Gehring *et al.,* 1999; Paques *et al.,* 1999; Hartwig *et al.,* 2004; Liu *et al.,* 2006; Alio *et al.,* 2007a.). Alio *et al.* (2007b.) also produced autologous PRP to use as topical eyedrops for the treatment of recalcitrant neurotrophic, herpetic and immunologically induced corneal ulceration, and also observed that pain was particularly reduced in all patients. Eye drops made from autologous serum are thought to be superior to artificial tears in several aspects (Tsubota *et al.,* 1999; Geerling *et al.,* 2001). First, their pH, osmolality and biomechanical properties are similar to natural tears. Second, they contain essential ocular surface nutrients, such as growth factors, vitamins, and bacteriostatic components such as IgG, lysozyme, and complement. Third, they are free of preservatives, stabilizers, and other additives that potentially induce toxic or allergic reactions (Liu *et al.,* 2006).

Effects of PRP on bone growth and bone surgery

Impaired bone healing after fractures, with the development of pseudarthrosis, or fusion operations in the case of nonunions cause pain and disability. Attempts are being made to create bone substitutes and technologies to improve bone healing by adding biological materials, such as PRP which were shown to stimulate osteogenesis and osteoconduction. In bone healing (i.e., callus formation), platelets act as an exogenous source of growth factors stimulating the activity of bone cells on the basis of a unique role in bone growth (Everts *et al.,* 2007). Marx *et al.* (1998) used platelet concentrates as a source of autologous growth factors in bone and have shown, radiographically and histologically, an increase in bone formation and density after autologous bone grafting in 44 patients. Kim *et al.,* (2002) suggested that platelet concentrate may be useful in accelerating the osseous integration of titanium implants after its use as a dental implant.

Another therapeutic application of PRP involves the combination of PRP with different bone substitutes. Aghaloo *et al.* (2004) used natural deproteinized bovine matrix and measured improved bone growth when it was used with PRP gel. On another study, Suba *et al.* (2004) used β-tricalcium phosphate in combination with PRP gel and observed more intense bone regeneration. Furthermore, due to the sticky structure of PRP gel, caused by the fibrin strands present in the gel, bone substitutes are kept together, avoiding unwanted migration of bone particles. Recently, the percutaneous application of PRP gel in a diabetic femur fracture model was described. Normalized cellular proliferation and chondrogenesis, with improved mechanical strength, were observed when PRP gel was injected in this model (Gandhi *et al.,* 2006).

Further application of PRP includes combining of PRP and biomaterials. Anitua *et al.* (2007) applied a PRP gel to an embedded titanium implant and created a new dynamic surface that could potentially show biological activity. One potential field of interest is the incorporation of platelet-rich products in bone grafting technology. This development is extremely relevant in different surgical fields including orthopaedic and maxillofacial surgery and oral implantology. In this context a wide array of composite biomaterials can be created by mixing PRP gel with either artificial or natural biomaterials (Fig. 2.3).

Figure 2.3 - The liquid PRP used to bio-activate dental implant surfaces. (Adapted from: Anitua *et al.,* 2007)

Effects of PRP on cardiovascular surgery

Recent work shows that it is now possible to apply activated-PRP (by either calcium or thrombin) to surgical wound sites of patients undergoing cardiac surgery (Vang *et al.,* 2007). PRP enriched growth factor has been directed applied at the sternum before and after re-approximation and before skin closure, to enhance osteointegration and facilitate sternal (bone) healing, thereby reducing the incidence of post-operative sternal disruption, which is a documented complication of thoracic surgery. During cardiopulmonary bypass surgery, withdrawal of PRP by plateletpheresis immediately before start of surgery appears to be promising because it avoids cardiopulmonary bypass-related platelet damage and also limits post surgery blood loss (Boldt, 1995; Misumi *et al.,* 1995; Crowther *et al.,* 2000; Hiramatsu *et al.,* 2002). PRP was also shown to confer beneficial effects on pain, blood loss and bruising in the postoperative phase following cardiac surgery. Apart from the wound healing properties of PRP, the use of PPP is also valuable as a sealant, and haemostatic agent (via fibrin deposition), thereby reducing haematoma formation and fluid collections in dead spaces (Vang *et al.,* 2007).

- Clinical use of PRP in the pig model

In general pigs are, because of their size and short cycles of reproduction, suitable experimental animals for many surgery techniques requiring the dimensions of a human (e.g., liver, kidney, heart, blood circulation, and intestine) (Swindle *et al.,* 1988; Clark *et al.,* 1999). Furthermore there is a strong morphological and functional resemblance of the aforementioned organic systems between human and pigs. New surgical techniques can first be evaluated and investigated in pigs for feasibility, safety, and efficiency.

For many of these reasons, the pig becomes the animal of choice in our study. Also, pigs can become very adaptable, and willing to cooperate if they are maintained in low-stress conditions and they are especially suitable for the obtention of platelets concentrate. Pig platelets were found to be similar in biochemical properties as well in terms of platelet count and clotting parameters to human platelets than platelets from other non-primate species (Hönig and Merten, 1993).

Frequently, platelet concentrate requires a relatively high whole blood volume. However, this volume can vary according to the lesion/tissue in which PRP will be applied and its application technique (gel, liquid, or with autologous bone graft, etc). Generally, pigs dispose of an overall blood volume of 65 mL/kg (6.5% of the body weight). So with this volume of blood per body weight, sampling to obtain PRP can be made without disturbing of the pig hematologically (Smith *et al.,* 1991; Brundage *et al.,* 2003). Pigs have been used as an animal model in platelet concentrate application in different clinical areas see table 2.3.

Tissue/lesion, studied	**PLT-derived product**	**Recovery or regeneration**
Maxillary bone (Schlegel *et al.,* 2004)	PRP and autogenous bone graft	Not significant
Spinal bone (Li *et al.,* 2004)	PRP and tricalcium phosphate biomaterial	Poor
Maxillary bone(Thorwarth *et al.,*2005)	PRP	Significant (early phase)
Anterior cruciate ligament (Murray *et al.,* 2007)	Collagen-PRP Hydrogel	Significant (early phase)
Superficial skin burns (Henderson *et al.,* 2003)	PRP held in place by a standard OpSite dressing	Poor
To stimulate articular chondrocyte proliferation and matrix biosynthesis (Akeda *et al.,* 2006)	PRP	Significant

Table 2.3 - Some studies in which pigs were used as a model in platelet concentrate application.
PLT - Platelet

- Application of PRP on tissue engineering

A diversity of methods have been used for the restoration of bone or soft tissue defects in different surgical settings, including orthopaedic surgery, maxillofacial surgery, and reconstructive surgery (Langer and Vacanti, 1993; Yamada *et al.,* 2004). However, the manipulation and reinforcement of biocompatible materials in surgery is not always easy to achieve.

Mixtures of autologous tissues have been used to accomplish restoration of defects. Tissue engineering, a technology involving the morphogenesis of new tissues using isolated cells with biocompatible matrices, is often combined with growth factors. Mesenchymal stem cells are multipotent cells that can replicate as undifferentiated cells with the possibility of differentiating into mesenchymal tissues (i.e., bone, cartilage, tendon, muscle). This ability has made mesenchymal stem cells a potential component of tissue engineering concepts. Recently, several research groups have been studying activated PRP as a matrix for tissue-engineering models because the activated platelet gel releases numerous platelet-derived growth factors (Lucarelli et al., 2003; Zhu *et al.,* 2006).

The recent interest in obtaining higher regeneration rates for injured mesenchymal tissues has lead to the development of therapeutic protocols based on the non transfusional use of hemocomponents, including platelet lysate. Platelet lysate is a proteic extract obtained from the lysis of concentrated purified platelets. Platelets lysate contains very high levels of growth and chemotactic factors and, when applied to injured mesenchymal tissues, can stimulate proliferation and differentiation of stem cells which are locally present (Anitua *et al.,* 2004).

- Other PRP properties: Infection prevention

Clinical studies have shown that PRP may have advantageous effects on wound healing due to the growth factors platelets release when activated. Since PRP also contains leukocytes, or white blood cells, it is hypothesized that application of the gel to surgical wounds will reduce or possibly eliminate bacterial growth. However, the mechanism of antibacterial effect of PRP gel is not yet fully discovered (Bielecki *et al.,* 2007). Dohan *et al.* (2006) suggested that PRP gel is also an immune node able to stimulate defense mechanisms. Bielecki *et al.* (2007) demonstrated *in vitro* a strong activity comparable to gentamicin and oxacillin for PRP gel against methicillin-sensitive *Staphylococcus aureus* and *Escherichia coli*. In a study evaluating the effect of PRP on the postoperative wound healing process in patients receiving total knee prosthesis, 5% of patients not treated with PRP developed a superficial wound infection compared to the patients in the PRP group (Everts *et al.* 2006). Finally, PRP was demonstrated to avoid superficial and deep wound infections in a study on the use of PRP in cardiac surgery (Trowbridge *et al.,* 2005).

- Risks and contraindications of using PRP gel

We are not currently aware of any serious adverse effects that have occurred when PRP was used for wound healing and bone grafting. Still, a possible risk arises from bovine thrombin that is used to activate PRP. Landsberg *et al.* (1998) and Marx, (2004) described that the use of bovine thrombin may be associated with the development of antibodies to factors V, XI, and thrombin, resulting in the risk of life-threatening coagulopathies.

- Contraindications of PRP

In the literature there are reported contraindications for the use of autologous platelet gel in patients with: critical thrombocytopenia; hypofibrinogenemia; platelet dysfunction syndrome; sensitivity to bovine thrombin; unstable patients and pregnancy. The use of PRP are also contraindicated in patients with acute and chronic infections; chronic liver pathology; anti coagulation therapy and sepsis that cause significant bleeding disorders, and, therefore, inhibit platelet viability, may not benefit from such therapy. Some pharmacologic agents may inhibit platelet viability and also may hinder the quality of PRP (Landesberg *et al.,* 1998; Floryan and Berghoff, 2004).

2.3.2. Methods for preparation of PRP

PRP was first introduced to the oral surgery community by Whitman *et al.,* (1997). The authors thought that through activation of the platelets within the gel and the resultant release of growth factors, enhanced wound healing should be expected. Platelet gel is formed by activating PRP, derived from differential centrifugation of autologous whole blood, with thrombin and calcium chloride. Platelets, once activated in the presence of thrombin, release a myriad of factors and begin to form the scaffold for the developing fibrin clot (Whitman *et al.,* 1997).

Various protocols have been used to obtain PRP, but many of these techniques have been criticized for their inability to obtain an adequate concentration of platelets. These variations in the procedures used to obtain PRP, undoubtedly modified both the amount of PRP obtained and the corresponding GF concentrations. All the devices use the general principle of centrifuge technology. Among the existing procedures, we can distinguish between single and two-step centrifugation techniques (Whitman *et al.,* 1997; Anitua, 1999; Marx, 2001; Landsberg *et al.,* 2000; Appel *et al.,* 2002; Weibrich *et al.,* 2004).

Preparation of PRP in human

The preparation of PRP in humans is well known and different methods for the preparation of locally usable platelet-rich plasma (PRP) components have been described (Marx *et al.,* 1998; Anitua, 1999; Landsberg *et al.,* 2000; Zimmerman *et al.,* 2001; Appel *et al.,* 2002; Weibich and Kleis, 2002; Weibrich *et al.,* 2003). These methods include: apheresis automated devices; buffy coat devices and tube methods.

a) Apheresis automated devices

Apheresis, from the Greek "to take away", means that platelets are collected using a device which draws blood from the donor and centrifuges the collected blood to separate out the platelets and other components to be collected (Westphal, 1984; Weibrich and Kleis, 2002; Anila and Nandakumar, 2006). The advantage of this method is that the remaining blood is returned to the donor and single donation provides at least one therapeutic dose, as opposed to the multiple donations for whole blood. This means that a recipient is not exposed to as many different donors and has less risk of transfusion transmitted disease and other complications. PRP obtained by the apheresis technique have been extensively assessed for their platelet collection efficiency and quantity of some growth factors (Weibrich and Kleis, 2002). With this technique, PRP can be produced at a concentration of 300% of normal blood levels (Marx *et al.,* 1998; Sonnleitner *et al.,* 2000). The most advantage of this technique is its low risk of bacterial contamination during PRP

preparation (Zimmerman *et al.,* 2001). It seems reasonable that this method is used when both red cell salvage and PRP application are both indicated.

However the disadvantage of the apheresis system is the high technology and experienced personnel required. This technique is not applicable for small clinical settings and larger predonation volumes (250 to >500 mL) of whole blood are needed, resulting in a PRP volume ranging from (20 to >50 mL) compared to the buffy coat and tube methods (Zimmerman *et al.,* 2001; Appel *et al.,* 2002; Weibrich and Kleis, 2002). Limitations of this method are the high cardiovascular stress caused to the recipient, known health risks and high production costs (Westphal, 1984; Weibrich and Kleis, 2002; Anila and Nandakumar, 2006).

b) Buffy coat devices

Several commercial systems are available for preparing PRP, using buffy coat procedure have been developed and evaluated for use in human medicine. Generally double spin centrifugation is required. Some of those specialized custumized divices include: (i) Platelet Concentrate Collection System (PCCS®, Biomet-Merck, Bridgend) (fig.2.4). (ii) SmartPrep Platelet Concentrate System (Harvest Technologies, Plymouth, MA) (fig.2.5). (iii) Gravitational Platelet Separation System (GPS System, Biomet Merck Biomaterials, Darmstadt, Germany) (Fig.2.6). (iv) Secquire PRP System® (PPAI Medical, FL, USA). (v) Magellan®Autologous Platelet Separator System, and amongst others.

The buffy coat systems have important clinical advantages in comparison with the apheresis such as permitting the procurement of PRP using smaller volumes of blood, increasing the platelet concentrations and avoiding the need for red blood cells (RBC) and platelet poor plasma (PPP) reinfusion (Zimmerman *et al.,* 2001; Weibrich and Kleis, 2002; Anila and Nandakumar, 2006). A higher number of platelets and growth factors are collected when compared with apheresis and tube methods. The risk of contamination is lower than with the manual method and can be used in small clinical settings (Zimmerman *et al.,* 2001; Appel *et al.,* 2002). Buffy coat devices platelet concentrating systems also provide higher platelet levels (>1000x10^3 PLTs/μl) (Zimmermann *et al.,* 2001; Weibrich and Kleis, 2002) than both the apheresis procedure (Whitman *et al.,* 1997; Marx, 1998) and the tube method, when single spin is performed (Weibrich *et al.,* 2005). However, the disadvantage of those devices is that concentrate a high number of leukocytes, and they require expensive and cumbersome centrifuges and collection/preparation kits. It is important to point out that the exact role of leukocytes in PRP intended for regenerative objectives has not been established, but it is thought that a high number of inflammatory cells could be detrimental for some treated tissues (Zimmerman *et al.,* 2001).

Among of these buffy coat methods mentionated above, the most studied ones regarding centrifugation steps (rpm, g/min), amount of blood collected preoperatively (immediately before surgery), baseline number of platelets, amount of PRP obtained, number of platelets increase above baseline and number of growth factors on PRP are: SmartPrep Platelet Concentrate System (Harvest Technologies, Plymouth, MA), Platelet Concentrate Collection System (PCCS®, Biomet-Merck, Bridgend, UK) and Gravitational Platelet Separation System (GPS System, Biomet Merck Biomaterials, Darmstadt, Germany) (Sonnleitner *et al.,* 2000; Weibrich and Kleis, 2002; Floryan and Berghoff, 2004; Marlovits *et al.,* 2004; Weibrich *et al.,* 2005; Everts, 2006).

SmartPrep and PCCS

SmartPrep and PCCS use tailored centrifuge containers to manipulate the blood cells to achieve the separation and sequestration of platelets. Similar quantities of fresh whole blood are required in both units. Both systems consist of a desktop centrifuge and individual disposable kits that are designated to come into contact with blood (see Fig. 2.4 and Fig. 2.5). Each system uses centrifugal forces to separate blood cells through centrifuge cycles with short and long-duration spins. After the first spin (short), SmartPrep automatically decants the plasma into the plasma chamber, where during the second spin (long), the PPP is separated from a platelet concentrate. After the first spin in the PCCS system, the operator manipulates a valve and inserts air into an air bladder, forcing the plasma into the second chamber. After the second spin, the operator forces air into a bladder that separates the PPP from the PRP (Weibrich and Kleis, 2002; Weibrich *et al.,* 2002).

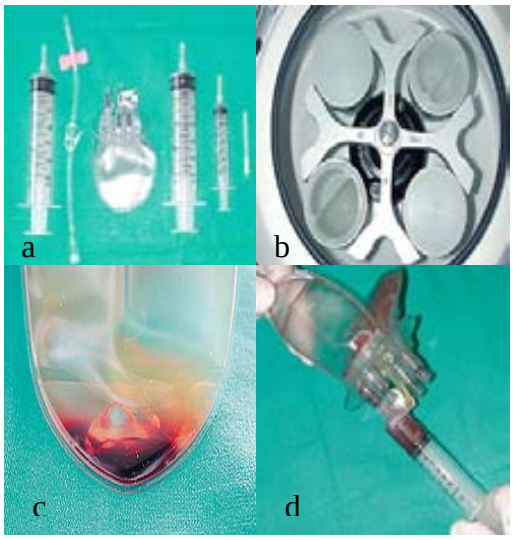


Figure 2.4 - Platelet Concentrate Collection System (PCCS®, Biomet-Merck, Bridgend, UK). a) Kit for blood collection. b) Centrifuge. c) Platelet concentrate obtained by centrifugation. d) Platelet concentrate is packed in a plastic syringe.

Smart PReP's process has been shown to capture exceptional platelet yields regardless of the patient's hematocrit or the level of the operator's experience. The SmartPReP method assures platelet yields 4-fold greater than the patient's baseline platelet count. It provides simplicity in operation and may provide a good platelet count before plasma resuspension. Smart PReP's second technology platform consist of two devices designed to produce point-of-care, autologous hemobiologic products. Specifically, Smart PReP separates autologous whole blood into RBC, platelet poor plasma and pure PRP with the full compliment of associated growth factors. Since the PRP and growth factor levels are easily controlled by volume, the clinician can achieve a predetermined level of growth factors tailored to a specific clinical application (Weibrich and Kleis, 2002; Weibrich *et al.,* 2002). (see Fig. 2.5)

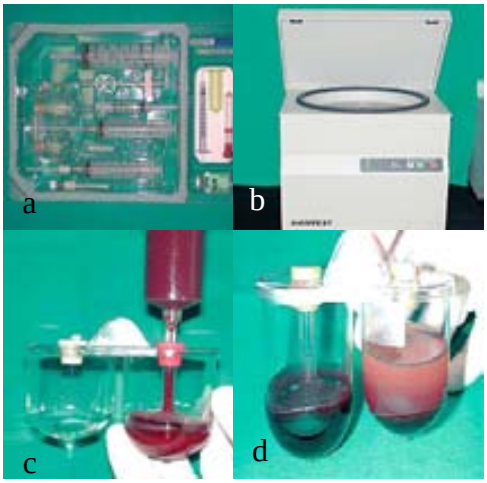


Figure 2.5 - Smart PReP's system (Harvest Technologies, Munich, Germany). a) Kit for blood collection. b) Harvest Centrifuge. c- d) Smart PReP's disposable system before and after centrifugation.

Gravitational Platelet Separation System (GPS)

This system consists of a disposable GPS tube that is used together with a bench-top centrifuge. Anticoagulated blood is inserted into the GPS disposable. The disposable is then centrifuged. During the centrifugation step, tuned-density buoy floats in between the RBC and the buffy coat, which contains the white blood cells and platelets. A second buoy moves from the top of the disposable, separating the buffy coat and plasma from the rest of the blood plasma (Fig. 2.6). After centrifugation, the excess plasma is removed, and the platelets are resuspended in the PRP by vigorously shaking the disposable for 30 seconds (Floryan and Berghoff, 2004; Marlovits *et al.,* 2004; Everst *et al.,* 2006).

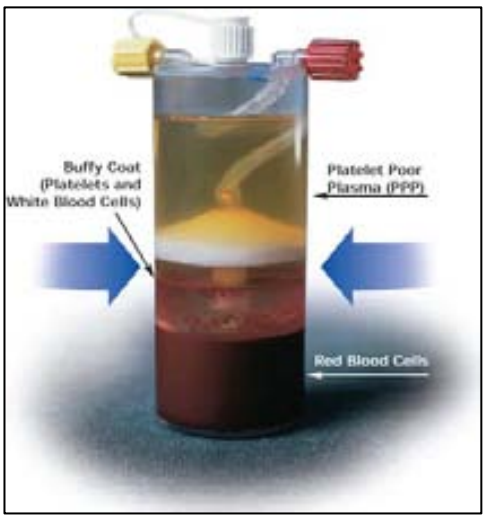


Figure 2.6 - Gravitational Platelet System (GPSTM, Biomet-Merck, Bridgend, UK)

c) Tube method. This method is also termed chair side technique, platelet rich in growth factors (PRGF) procedure. Since 2001 the PRGF system (G.A.C. Medicale San Antonio, 15-5°, 01005 Vitoria-España) is available (Fig. 2.7) (Anitua *et al.,* 2006).

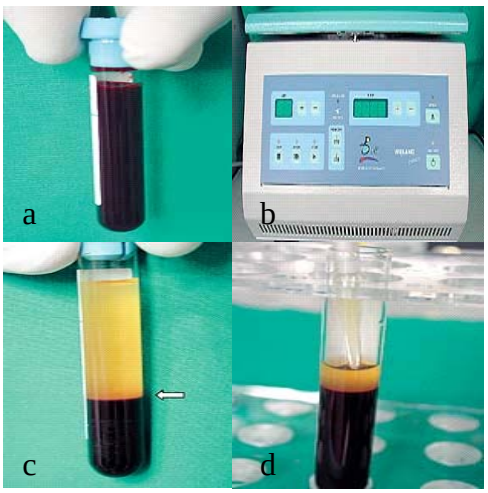


Figure 2.7- Platelet rich growth factors (PRGF kit®). a) 4.5mL of whole blood (combined with 0.5 mL of ACD-A, anticoagulant). b) PRGF centrifuge. c- d) Platelet concentrate is packed in a tube.

The advantages of this technique are the low cost and the minimal technical requirements in comparison to the apheresis and buffy coat methods. Only a simple centrifuge machine, 5 mL citrated tubes and 1 butterfly catheter are all that is needed to prepare PRGF (Marx *et al.,* 1998; Zimmermann *et al.,* 2001; Appel *et al.,* 2002; Weibrich and Kleis, 2002; Weibrich *et al.,* 2005). It is important to point out that with this technique the lowest number of leukocytes is collected when compared with other techniques such as buffy coat and apheresis devices (Zimmernan *et al.,* 2001). The clinical use of autologous growth factors using this method has obtained good results regarding the number of platelets and leucocytes content in PRP (Anitua *et al.,* 1999; Sanchez *et al.,* 2003; Anitua *et al.,* 2004).

The main disadvantage of this system for perioperative platelet preparation is to be open-system devices that force the maintenance of a maximal 8 hours interval between platelet preparation and use on surgical setting needs sterile conditions (Marx *et al.,* 2001, Zimmerman *et al.,* 2001). However, the PRP must be separated from the PPP soon after centrifugation because the concentrated platelets will slowly diffuse into the PPP over time and would reduce the platelet count of the PRP preparation. When single spin centrifugation is performed, this technique concentrates a mean lower number of platelets (400-500x10^3 PLTs/µl) (Weibrich *et al.,* 2005) than the apheresis and buffy coat techniques and tube method double spin centrifugation (Whitman *et al.,* 1997; Marx *et al.,* 1998, Zimmerman *et al.,* 2001; Appel *et al.,* 2002).

The question of the actual quantity of platelets required is often put forward by clinicians who need to know the minimal therapeutic level of the platelets in the PRP that would result in a significant improvement, using PRP compared with standard treatments (Everts, *et al.,* 2007). At present, not many data are available to answer this question directly, and only indirect information exists. Marx *et al.* (1998) performed the first study showing a significant improvement in mandibular continuity defects when PRP was mixed with autogenous bone grafts. Their PRP contained three to four times higher platelet count

compared with baseline values, although the average PRP platelet count found in their patients was just below $8 \times 10^5/\mu L$, a number that is lower than in most other studies. Nevertheless, they observed a significantly faster radiographic maturation and histomorphometrically denser bone regeneration.

Nowadays, the latest separation devices produce PRP platelet counts in excess of 6–10 times the baseline platelet count values. Manufacturers tend to interpret a high platelet concentration as a quality performance indicator of their separation devices, regardless of the fact that these high concentrations may not be necessary or might even contribute to a negative outcome (Everts *et al.,* 2007). Weibrich *et al.* (2004) observed an advantageous effect with platelet concentrations of approximately $10^6/\mu L$. Furthermore, they state that higher concentrations might have a paradoxically inhibitory effect.

Comparison between different methods

In humans some studies have been published comparing these methods with regard to PRP volume, platelet count on PRP and platelet yields (see table 2.4) (Zimmerman *et al.,* 2001; Appel *et al.,* 2002; Weibrich and Kleis, 2002; Weibrich *et al.,* 2002; Everts *et al.,* 2006).

Device	Mean PRP Volume (mL)	Mean platelet Concentrate (x 10^3)	Platelet Yield (%)	Increase above baseline (%)
Anitua protocol	9.5±4.1	433±129	35±16.8	190
Landsberg protocol	10.6±2.4	336±141	30±10.3	150
Clinaseal Sealed	7.6±1.5	401±267	39±16.3	164
ACE Surgical	7.8±0.6	493±245	33±10.2	180
AG Curasan	7.6±1.5	344±192	29±14.1	139
3i PCCS	7.0±1.5	939±284	61±8.9	324
Smart PReP	7.4±0.5	1.086±227	62±4.4	404

Table 2.4 - Platelet yields per device (adapted from Marx, 2004).

The clinical effectiveness of each type of PRP preparation method can vary within the technique and efficiency, and current methods to evaluate the platelet concentration efficiency of PRP systems have several limitations. Different techniques of preparation have been known to lead to substantially different amounts of cells, i.e. platelets and leucocytes, as well as different levels of growth factors (Zimmermann *et al.,* 2001; Appel *et al.,* 2002; Weibrich and Kleis, 2002; Weibrich *et al.,* 2002; Zimmermann *et al.,* 2003; Schlegel *et al.,* 2004; Wiltfang *et al.,* 2004).

Some studies have questioned the effect of differential centrifugation on the integrity of the remaining platelets. Ledent *et al.* (1995) showed that whole blood centrifuged at 1,500 g for 90 seconds resulted in a minimal release of growth factors from the platelets. However, PRP subjected to centrifugation forces of 2,500 g resulted in a significant release of growth factors from the platelet alpha granules. Therefore in other study Dugrillon *et al.* (2002) demonstrated that spins greater than 800 g may decrease the amount of TGF- β in the PRP released.

There are also several choices of anticoagulants that can be used during PRP preparation. Anticoagulant citrate dextrose-A is a preferable agent (Marx, 2001). The citrate binds calcium and prevents coagulation, whereas the dextrose and other ingredients support platelet metabolism and viability. Citrate phosphate dextrose is also useful for PRP preparation. It is similar to anticoagulant citrate dextrose-A, but it has fewer supportive ingredients and therefore may be less effective at maintaining platelet viability (Marx, 2001). The use of ethylenediaminetetraacetic acid (EDTA) is potentially more harmful than citrate in the preparation of PRP, and a large number of damaged platelets have been observed (Landesberg *et al.,* 2000). Trisodium citrate solution is suggested as an anticoagulant with no negative effects on PRP preparation (Anitua, 1999).

Preparation of PRP in Swine

To date, various devices for obtaining porcine PRP have been described, such as apheresis or discontinous platelet concentrate system which include reinfusion of red cells and remainder plasma (Henderson *et al.,* 2003). On pigs three commercial devices for obtaining PRP have been experimentally assessed. And also have been analyzed for platelet and leukocyte counts as well as for TGF-β, PDGF-AB, and IGF-I levels (Li *at al.,* 2004; Schlegel *et al.,* 2004; Thorwarth *et al.,* 2005, Akeda *et al.,* 2006) (table 2.5).

Device	PCCS (Thorwarth *et al.,* 2005)	AG Curasan (Thorwarth *et al.,* 2005)	Symphony system (Li *et al.,* 2004)
Platelets in WB ($x10^3$)	118±12	118±12	398,4
Platelets in PRP ($x10^3$)	767±108,7	483,8±97,2	1786,6
WBC in WB ($x10^3$)	4,3±1,9	4,3±1,9	18,7
WBC in PC ($x\ 10^3$)	14,7±68	24,8±8,9	29,5
TGF- β (ng/mL)	467,1	79,7	ND
PDGF-AB (ng/mL)	251,8	314.1	ND
IGF-I (ng/mL)	91	69	ND

Table 2.5 - Devices used to prepare platelet concentrate in pigs. ND- Non determined

The buffy coat and apheresis techniques are acceptable, but they present some economical restrictions according to the kind of study and size of the wound for PRP application. Platelet concentrate methods, like the apheresis method, can only be performed in a specialized laboratory (Whitman *et al.,* 1997; Marx *et al.,* 1998; Lowery *et al.,* 1999).

2.3.3. Methods used to trigger GF release from PRP

To release the growth factors and cytokines, platelets need to be activated. *In vivo* this happens through platelet agonists like thrombin, collagen, ADP, serotonin, and thromboxane A2. PRP can be used in clinical practice as a liquid state or forming a gel. PRP forms a gel when platelets are activated by any activator substance. The *ex vivo* activation of platelets or PRP can be done chemically or physically:

a) Chemical activation includes the addition of substances such as: bovine thrombin, autologous thrombin, calcium chloride, and ionophore and protease enzyme batroxobin from *bothrops atrax snake* venom which have a thrombin-like effect on platelet activation.

PRP is usually applied as a gel, by mixing it immediately before use with calcium chloride and bovine thrombin (Marx 1998; Zimmerman *et al.,* 2001; Appel *et al.,* 2002; Carter *et al.,* 2003; Martineau *et al.,* 2004; Weibrich *et al.,* 2004). However, the use of chemicals like bovine thrombin or ionophore is questionable. Particularly the use of bovine thrombin has been reported to be associated with the development of antibodies to factors V and XI, which can result in the risk of life threatening coagulopathies and diseases transmition and is also an expensive activator (Landesberg *et al.,* 1998). Everts *et al.* (2006) used autologous thrombin which was obtained during platelet concentrate procedure. Because of autologous nature of the thrombin, no imunogenicity and risk of diseases transmition is to be occurred. It is also a low cost activator. Other authors (Anitua, 1999; Dugrillon *et al.,* 2002) have proposed the formation of a PRP gel by adding calcium chloride which is an inert substance and either immunogenicity or risk of disease transmition is expected, and has a relatively lower cost than bovine thrombin.

b) Physical activation includes: repeated freeze-thawing cycles and sonification.

Repeated freeze-thawing cycles procedure are common methods for releasing intracellular growth factors for determination of growth factors content in platelet concentrate. According to Sekido *et al.* (1987) and Zimmerman *et al.* (2001), freezing and thawing seems to be the superior process to release growth factors. However, repeated freeze-thaw cycles are time consuming, and difficult to ensure platelet rich plasma ready to use for clinical propose. Advantages of this method include its low cost and that it does not uses potentially dangerous chemicals like bovine thrombin. Another available physically method is sonification which is used to lysed PRP. This method proved to be a simple, yet quick and powerful method to achieve PRP activation. Activation efficiency is comparable to the known methods, but sonification is less time-consuming than freeze-thaw cycles and also does not use potentially dangerous chemicals like bovine thrombin (Guillén *et al.,* 2005).

2.3.4. Evaluation of platelet activation and determination of transforming growth factor-β1 (TGF-β1)

Platelet activation during PRP preparation

Two main requirements must be met to avoid platelet activation *ex vivo* during platelet collection (a) a venipuncture procedure must be used that minimizes spontaneous platelet activation and (b) blood must be collected into a medium that will not only prevent coagulation, but will also preserve the activation status of platelets until the samples can be analyzed (Wu, 1994; Anitua, 1999; Landesberg et al., 2000; Marx, 2001).

Platelets can also be activated by excessive pipetting, the use of metallic or glass instruments and the use of a tourniquet over the vein to be punctured for blood extraction, or when not eliminating the first 2 mm of the extracted blood (Michelson, 1996). This premature platelet activation leads to an early release of growth factors. So when the blood sample is centrifuged, the growth factors will move to the most superior part. This way, the obtained PRP will be poor in growth factors (Tamimi *et al.,* 2007).
Another critical step in PRP preparation is the high spin centrifugation to concentrate the platelets. The mechanical forces may activate platelets, with the consequence of loosing the granular load that contains the growth factors into the plasma supernatant (Ledent *et al* 1995; Dugrillon *et al.,* 2002).

Determination of platelet activation (platelet activation markers)

Direct and indirect measures of platelet activation have been described. As a direct measure of platelet activation, detection and quantification of antigens exposed during platelet degranulation can be determined. The cell adhesion molecule P-selectin (CD62P) is one of such activation antigens. P-selectin is restricted to the inner surface of the alpha granule membranes of resting platelets. Platelet activation and granule release results in an increase of P-selectin (CD62P) expression on the external surface of the platelet. The increase in P-selectin may be rapidly and conveniently detected by immunofluorescent flow cytometry and used as a reliable marker of platelet activation (Macey *et al.,* 1999; Zimmerman *et al.,* 2001; Lalko *et al.,* 2003).

Recently new platelet activation markers are available and are named toll-like receptors (TLR). TLR family recognizes pathogen-associated molecular patterns not found on host cells. TLR1 and TLR6 have been observed on human platelets (Shiraki *et al.,* 2004); and TLR2 and TLR4 have been observed on murine platelets (Semple *et al.,* 2004; Andonegui *et al.,* 2005). TLR4 expression on resting human and rodent platelets has been previously demonstrated (Andonegui *et al.,* 2005; Aslam *et al.,* 2006; Ward *et al.,* 2005). Despite these findings, the functional significance of platelet TLR expression is unknown. However, Andonegui *et al.* (2005) demonstrated that megariocytes express TL4 and Coppinger *et al.* (2004) showed that TLR is released from human platelets on thrombin activation, suggesting that at least some of platelet TLR expression may originate from megakaryocytopoiesis and from alpha granules. That suppose that during platelet activation, α-granule content release may results in an increase of TLR expression on the external surface of the platelet, conveniently detected by flow cytometry and used as a reliable marker of platelet activation (Coppinger *et al.,* 2004; Andonegui *et al.,* 2005; Ward *et al.*, 2005; Aslam *et al.,* 2006).

As an indirect measure, the mean platelet component (MPC) parameter has been proposed as a potentially useful screening test for platelet activation (Macey *et al.,* 1999; Ifran *et al.,* 2005). MPC is a new platelet parameter detected by current complete blood counter (CBC) ADVIA 120 ™, which represents the mean refractive index of the platelets. MPC is linearly related to platelet density and is reduced when platelets degranulate, thus indicating that platelets have undergone activation. Several studies in human medicine have recently demonstrated that MPC values are inversely correlated with platelet membrane P-selectin expression, (Macey *et al.,* 1999; Ahnadi *et al.,* 2004) and has been used as an indicator of platelet activation in different disease and physiological states in people (Ahnadi *et al.,* 2004). In animals, MPC has been evaluated in dogs (Moritz *et al.,* 2003; Moritz *et al.,* 2005) and cats (Zelmanovic and Hetherington, 1998) and has been shown to decrease with platelet activation in both species and to be inversely related to platelet P-selectin expression (Moritz *et al.,* 2003; Moritz *et al.,* 2005). Thus, determination of the MPC by the ADVIA 120™ is becoming a quick method for the easy detection of platelet activation. Although no studies are available demonstrating the relationship between MPC and platelet activation in pigs, we hypothesized that MPC may also be an indicator of platelet activation in this specie and that it may be reduced in PRP obtained by tube method and double spin centrifugation in pigs if platelets become activated during procedure.

Determination of transforming growth factor-β1 (TGF-β1)

Wound healing, whether in bone or soft tissue, involves a complex chain of events. During this wound-healing cascade, platelets are trapped and activated, resulting in the release of growth factors present in the α-granules of the platelets and a fibrin clot generation in which a matrix for migration of tissue-forming cells will be formed (Slater *et al.,* 1995; Everts *et al.,* 2006; Everts *et al.,* 2007). The platelet released growth factors have mitogenic and chemotactic properties that may contribute to improve wound healing. Of particular importance is the TGF-β1 which most likely promotes the early influx of cells and stimulation of proliferation (Landesberg *et al.,* 2000).

Usually, the TGF-β1 concentrations are determined by enzyme-linked immunosorbent sandwich assay (ELISA) developed with commercial antibodies for TGF-β1.

3. Objectives

The healing of intestinal anastomoses remains a topic of ongoing research interest because clinical practice shows it to be disturbed rather frequently. Compromised healing may lead to dehiscence and leakage, which is attended by high morbidity and mortality. The occurrence of anastomotic leakage appears to be unavoidable in large bowel surgery in contrast with surgery of the small bowel (Hesp *et al.,* 1984). In small animals the reported incidence of small intestinal dehiscence rates 7-16%, with 74-80% of those patients dying (Hedlund *et al.,* 2002). Such a situation has led many surgeons throughout the world to devise and investigate new and more effective methods, techniques and materials to prevent dehiscence. Local protective methods to "protect" intestinal anastomoses during their complex biologic healing process have been the interest of many surgeons. Those methods included the use of various surgical techniques and materials, as well the use of various sealants, but none is sufficient to prevent the development of complications (Haukipuro *et al.,* 1988; Van der Hamm *et al.,* 1991).

Using these facts, in the last several years a number of authors have investigated the use of various growth factors (local or systemic) in order to enhance the intestinal wound healing with some encouraging results (Mustoe *et al.,* 1990; Slavin *et al.,* 1992). The disadvantage of these growth factors is that they are expensive and concerns exist about their safety in human administration (Calabresi *et al.,* 1998). Alternatively, PRP, which is a volume of autogenous plasma that has a platelet concentration above baseline, is a proven source of growth factors (Marx *et al.,* 1998). However, the most significant benefit of using the autologous growth factors (AGF) concentrate is its being autologous, endogenously derived, and easily available. There are no issues about immunogenicity or transmission of infection. There are no known local or systemic side effects or adverse effects. The process is also considerably cost effective as compared with use of purified or recombinant growth factors, and also it may be a more physiologically source to provide a combination of all factors in the platelets rather than individual factors (Lowery *et al.,* 1999).

Recently, PRP has been exogenously applied in many different clinical applications, demonstrating the effectiveness and importance of the product for a variety of medical procedures. Even with those related promising results the use of platelet concentrate on gastrointestinal tract healing, especially for end-to-end anastomosis, was poorly investigated. In our knowledge only one study is available in colonic rats and with promising results by Yol *et al.* (2008). Therefore this study was performed in rat model and only 7 days after anastomosis construction. On other hand any study are available on the lag phase (3 days after anastomosis construction) when clinically the anastomotic dehiscence occurs.

Based on the considerations above, the aims of the present study are:

1. To validate the tube method technique double spin centrifugation for obtaining autologous PRP derived from pig blood.

2. To evaluate platelet activation during double spin method procedure to obtain platelet concentrates in pigs, by monitored MPC values on whole blood and PRP obtained by flow cytometry haematology system.

3. To validate the TGF-β1 level determination technique with an enzyme-linked immunosorbent sandwich assay (ELISA) in pig PRP obtained by double centrifugation tube method.

4. To evaluate the quality of PRP obtained by tube method technique double spin centrifugation, determination TGF-β1 level with an ELISA technique.

5. To comprehensively characterize the effects of PRP gel at 24h, 48h, 72h, 96h, and 7 days after anastomotic construction on the development of wound histology.

6. To quantify the anastomotic resistance using the breaking strength at days 3 and 7 post anastomosis construction.

4. Material and methods

Animals

Large White breed pigs, males and females, weighting 55±5 Kg, were recruited in the present study. All animals were used in accordance with the appropriate standard for the use of experimental animals in research studies and in accordance with the Spanish Code of Practice for the Care and Use of Animals for Scientific Purposes, published by the Spanish Government Publishing Service, (Directiva CE 86/609- BOE RD 1201/05- 21/11/05). All procedures were approved by the Ethical Commission of Animal and Human Experimentation (CEEAH 644) of the Autonomous University of Barcelona.

Upon arrival, the animals were adapted for 14 days before surgery. The pigs were housed in groups of 2 or 3 in pens ranging from 3,6 - 5,4 m^2. During the acclimatization period, the environment was controlled to remain within a temperature range of 20 - 24 ºC and 65% of humidity. The light/dark cycle was 13 and 11 h, respectively, with the photoperiod between 6.30 a.m. and 7.30 p.m. with 30 min of dusk and dawn. After surgery each pig was kept in a separate box (1,83 - 3,92 m^2). They were fed with standard swine diet according to their weight, before and after the surgery, and received an uninterrupted supply of clean, fresh water.

The pigs were monitored 24 h/day throughout the observation period. This monitoring included observation of general behaviour, food intake, fecal production, and temperature. The pre and post-operative care and observation were performed by trained animal technicians, supervised by veterinarians, who attended the animals daily.

Experimental design

The present study was divided into 3 main stages:

- Stage I: PRP obtention and quality evaluation
- Stage II: Surgical procedure
- Stage III: Evaluation of the intestinal healing

4.1. Stage I – PRP obtention and quality evaluation

4.1.1. PRP obtention

In order to design an appropriate technique for PRP obtention in pigs, a pilot study was carried out.

a) Pilot study

On this preliminary study, pigs were used only to prepare PRP in order to obtain the best method to concentrate platelets in pigs.
Thirty pigs were used to collect 80 mL of whole blood for PRP preparation. For all these procedures, digital centrifuge (Ortoalresa unicent 20) was used. At the beginning, Anitua's (1999) protocol was used (460g for 8 minutes and single spin centrifugation), but attempts to produce PRP using the same parameters failed to yield satisfactory enrichment percentages. For this reason, a large group of animals was used. Blood obtained was spun

at different cycles of centrifugations forces, different times, and pellet obtained ressuspended in different volumes of plasma, in order to obtain an optimal concentration. The PRP preparation technique was considered to be a success when concentration of the platelets within the PRP was around 1.000.000 platelets/μl, "pellet" volume of 400-500 μL of viable undamaged platelets, and an increase in concentrate of platelets just about 3-fold above the physiologic levels.
The preparation of PRP, including the amount of blood collected, the centrifugal forces, and the final volume of the obtained PRP was optimized for each bioassay in order to obtain that concentration. The successful on PRP preparation technique was repeated twice to test reproducibility, and when the results appeared to be reproducible, the experiments for definitive purpose were begun (application of PRP gel on intestinal anastomosis wound).

To evaluate the efficacy of PRP tube method and double spins centrifugation, several quality parameters were taken into consideration. The essential parameter was the total number of platelets obtained in the PRP, from the whole blood volume drew from the animal. The total number of platelets in the PRP preparation was the most meaningful parameter because it is a measure of the ability of the system to obtain as many of the platelets within the PRP as possible.
The total number of the platelets within the PRP (number of platelets per μL) was related to the concentration within the original whole blood. The volume of the PRP were compared to the volume of blood drew from the animal. These last two parameters are proportional and both give an indication of the volume of the plasma fraction, in which the platelets are yielded.

Thus, the quality parameters used to evaluate the tube method and double spin centrifugation on PRP preparation in pig were: increase in platelet concentrate, platelet increase over baseline, and collection efficiency. These parameters where calculated using the following equations (Marx *et al.*, 1998; Carter *et al.,* 2003; Efeoglu *et al.*, 2004; Weibrich *et al.*, 2005; Akeda *et al.*, 2006):

$$\text{Increase in platelet concentrate (\%)} = \frac{Platelet\ Count\ of\ PRP}{Platelet\ Count\ of\ WB} \times 100$$

$$\text{PLT increase over baseline (fold)} = \frac{Platelet\ Count\ of\ PRP - Platelet\ Count\ of\ WB}{Platelet\ Count\ of\ WB}$$

$$\text{Collection efficiency (\%)} = \frac{Platelet\ Count\ of\ PRP \times Volume\ of\ PRP}{Platelet\ Count\ of\ WB \times Volume\ of\ WB} \times 100$$

PLT- platelet; PRP- platelet rich-plasma; WB- whole blood, PPP- platelet poor- plasma

After this preliminar study, a double spin centrifugation was chosen using the following spin method: for the initial spin 786g for 10 minutes, and for the second spin, 526 g for 10 minutes. Final pellet was ressuspended in 500μL of plasma. This combination yielded the best results in large white breed pigs.

b) Definitive study

Based on the pilot study, PRP obtention in pigs which were going to be submitted to the surgical procedure was performed as follows:

Collection of blood samples

Whole blood collection was done immediately prior to anaesthetic induction and surgery. Sixty-five cubic centimetres of arterial blood were withdrawn from the femoral artery of each pig into 13 (65mL) 5 mL-glass tubes containing 0.5 mL buffered sodium citrate (Venoject®, Terumo Europe N.V.) (Fig. 4.1) using a butterfly needle. One of the tubes was used for baseline measurements.

An additional blood sample was taken in heparin tubes for biochemistry evaluation. The heparinised whole blood was centrifuged at 1000g for 15 minutes and no less than 1 ml of plasma was placed into Eppendorf tubes that were held in a conventional freezer at -20ºC for further biochemistry analysis.

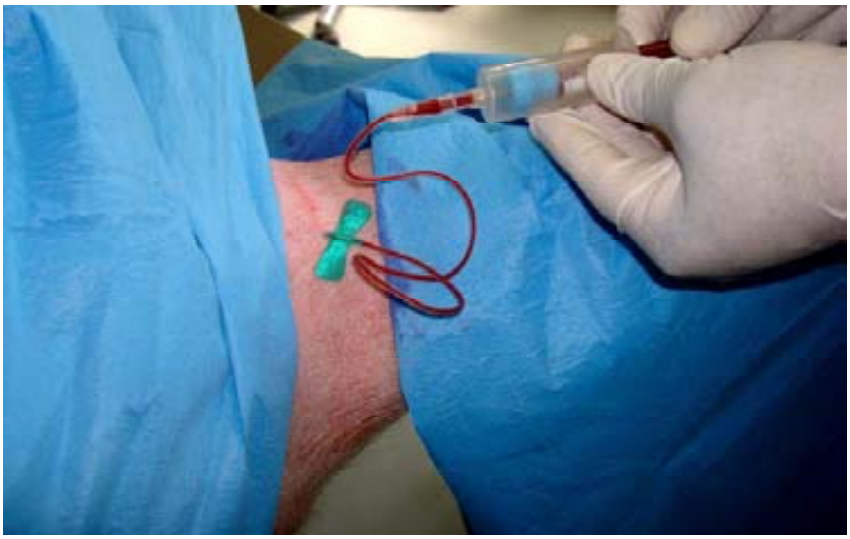


Figure 4.1-Withdrawal of whole blood on citrated tubes using a butterfly needle, from the femoral artery in the pig.

Double spin centrifugation

Platelet-rich plasma was prepared using the 12 tubes (60mL) via a 2-step centrifugation procedure. The citrated whole blood samples were first centrifuged at 786g during 10 minutes at room temperature. The blood was then separated into its three basic components; red blood cells, buffy coat, and platelet-poor plasma (PPP). The first supernatant plasma fraction, adjacent to the buffy coat, composed of platelets and plasma was collected under aseptic conditions, and transferred into silicone-coated glass tubes (Venoject®, Terumo Europe N.V.) and centrifuged again in order to pellet the platelets. This second centrifugation was 526 g for 10 minutes. The resulting supernatant was removed and platelet pellet was resuspended in about 500 µL of plasma per tube. The "pellet" was resuspended in the residual plasma by carefully 'hand shaking' the siliconed

tube for a few seconds (Fig. 4.2). All the PRP samples obtained were introduced into 10-mL silicone-coated glass tubes ready for use. Normally, 60 mL of citrated whole blood was used to prepare 6 ml of the PRP evaluated in this study. A fraction of PRP (1 mL) of each pig was analyzed for platelet and leukocyte count (ADVIA 120^{TM}). And the remained 5 mL of PRP was used to forming gel for anastomosis application.

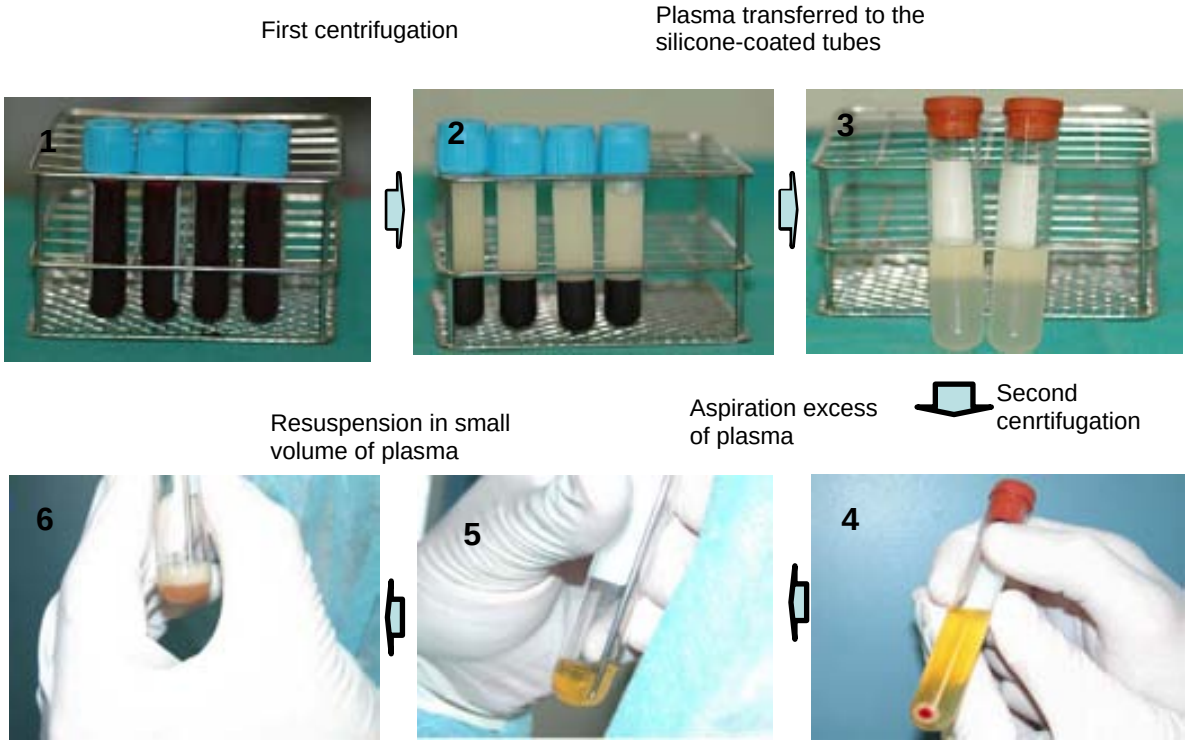


Figure 4.2 - Schedule of PRP preparation steps. Step 1: whole blood was collected in tubes containing citrate as an anti-coagulant. Step 2: immediately after being drawn, blood was centrifuged to separate RBCs from platelets and plasma. Step 3 and 4: The supernatant composed of platelets and plasma was collected on silicone-coated tubes and centrifuged again in order to pellet the platelets. Step 5 and 6: After this second centrifugation, the excess of plasma was aspired and platelets were resuspended in an appropriate volume (500 μL) of PRP to achieve a platelet concentration mean of 3,8-fold above the physiologic levels.

PRP gel obtention and gelation time

The PRP gel was obtained by mixing the PRP fraction with 10% calcium chloride (Calcium chloride 10%, B.Braun Medical SA) to antagonize the anticoagulative effect of citrate. For this purpose, Anitua's (1999) formulation was used (50 μL of calcium chloride per 1mL of PRP). All procedures were performed at room temperature under sterile conditions.

The gelation time was measured considering two macroscopic end-points: the appearance of visible fibrin polymerization and the occurrence of the gel solidification (the gel remaining attached to the surface of the Petri dish after turning it upside down).

4.1.2. Evaluation of PRP quality

Once the PRP obtention technique was designed, quality evaluation of all the PRPs performed was assessed by means of the following determinations:

a) Erythrocyte, platelet and leukocyte count, and quality parameters
b) Determination of platelet activation by flow cytometry
c) Determination of TGF- β1

a) Erythrocyte, platelet and leukocyte count, and quality parameters

Erythrocyte, platelet and leukocyte count was determined in samples of all animals submitted to surgical procedure (35 pigs) and in animals used for TGF-β1 determination (12 pigs), representing a total of 47 pigs. Quality parameters were calculated in all animals using the formulas described above.

Hematological and platelet parameters analysis in all studied pigs were performed few minutes after blood collection and before PRP obtention. Erythrocytic parameters, leukocytic series count, and platelet parameters, on whole blood and PRP, were automatically determined using a flow cytometry hematology system (ADVIA 120 Analyzer, Bayer Lab, NY, USA). All samples were measured using the same automatic cell counter, to ensure that the evaluations of the PRP preparations were comparable and not the result of measurement error; as a control, double measurements were made in all specimens, which showed repeatable results.

Evaluated hematological parameters included: red blood cell count (RBC, $10^6/\mu L$), hemoglobin concentration (HGB, g/dL), haematocrit (Ht %), mean corpuscular volume (MCV, fl), mean corpuscular haemoglobin (MCH, pg), red cell distribution width (RDW %), mean corpuscular hemoglobin concentration (MCHC, g/L). Total leukocyte count (WBC, 10^3 cell/μL), as well as the relative and absolute numbers of neutrophils, lymphocytes, monocytes, eosinophils, basophils (10^3 cell/μL ;%), and large unstained cell (LUC, 10^3 cell/μL; %) counts were determined. Platelet parameters: absolute platelets count (PLT, $10^3/\mu L$), number of large platelets ($10^3/\mu L$), mean platelet component (MPC, g/dL), mean corpuscular volume (MCV, fl); mean platelet volume(MPV, fl), mean platelet mass (MPM, pg), platelet distribution width (PDW, %), platelet mass distribution width (PMDW, pg), platelet component distribution width (PCDW, g/dL), plateletcrit (PCT, %), large platelets (LP, 10^3 cell/μL), amongst others were determined.

For biochemical analysis plasma specimens obtained from heparinised whole blood were analyzed for glucose, urea, alanine aminotransferase, lactate dehydrogenase, and total protein, to further confirm their health status.
Biochemical analysis were undertaken on serum using Olympus® AU400 Analyzer (serial number 3112676 Germany) and reagents from OSR (Olympus System Reagent®, Olympus®, Ireland). For serum proteins determination Hydrasys and Hyrys Analyzer (serial number 517; Sebia, French) and Hydragel protein (E) gels, (Sebia) were used.

b) Determination of platelet activation by flow cytometry

The MPC values were determined in the 47 animals mentioned above. The MPC values on citrated whole blood and PRP were obtained during routinely determination of the fundamental platelets parameters on whole blood and PRP using the ADVIA 120^{TM} Analyser.

c) Determination of TGF-β1 by ELISA

Twelve animals not submitted to the surgical procedure were included in this group. In this animals, sampling and PRP obtention was performed as described before, and TGF- β1 levels were determined on different PRP fractions (PPP, PRP, and PRP gel) using an enzyme-linked immunosorbent assay (ELISA).
All PRP fractions samples were obtained by centrifugation of citrated whole blood at 756g for 10 min first spin and the supernatant were centrifuged at 526g for 10 min second spin. The upper half of the PRP preparation after the second centrifugation was designated as platelet-poor plasma (PPP) and, after collecting a volume for analysis, the rest was subsequently discarded. The platelet pellet was resuspended in 500μL of plasma resulting in the PRP fraction. PRP gel was obtained by adding calcium chloride on fresh PRP. All PRP fractions (PPP, PRP and PRP gel) were frozen at -80ºC before growth factor analysis.

The PRP fractions were thawed and mixed well prior to growth factor analysis. TGF-β1 concentrations were determined by enzyme-linked immunosorbent sandwich assay (ELISA) developed with commercial antibodies for TGF-β1 (Human TGFbeta DuoSet®, R&D Systems) according to manufacturer instructions. The ELISA was performed in triplicate for each sample. Briefly, a capture antibody specific to TGF-β1 was added to a polystyrene 96-well plate (MaxiSorp, Nunc, Roskilde, Denmark) and incubated overnight at 4° C. After incubating the plate with blocking solution (phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.05% Tween 20), for 45 minutes at 37ºC, test samples and the standard recombinant cytokines (R&D Systems) were added, and the plate was incubated at 37ºC for 2 hours. For total TGF-β1 levels, the samples were diluted 1:4 in Dulbecco's (PBS) and treated with 1N HCl for 15 min (to obtaining a sample pH of approximately 2.6); afterwards samples were neutralized with 1N NaOH to a pH of approximately 7.6.
After washing four times with PBS containing Tween 20, a detection antibody specific for TGF-β1 was added, and the reactions were allowed to proceed for 1 hour at 37ºC. After washing three times, OPD (Sigma, USA) was added to induce the colour reaction for 30 minutes. A microplate reader (Victor3, Perkin Elmer) was used to measure the optical density at 450nm. A standard curve four parameter logistic (4 -PL) and logaritmic curve was drawn by plotting optical density versus the log of the concentration.

The increase in TGF-β1 concentrate was calculated using the following equation:

$$\text{Increase in TGF-β1 levels (pg/mL)} = \frac{TGF\ \beta 1\ levels\ of\ PRP}{TGF\ \beta 1\ levels\ of\ PPP}$$

4.2. Stage II- Surgical procedure

A preliminar study was carried out in order to decide the PRP gel application technique on intestinal anastomoses.

a) Pilot study

Six pigs divided into 2 groups of three animals each were used for macroscopic and histological evaluation of intestinal wound healing and evaluation of PRP gel application technique. The animals were euthanized at 24 and 48 hours after surgery. These animals underwent a laparatomy and a double enterectomy and anastomosis of the jejunum. One of the anastomosis was closed with suture repair alone (control), while the other was treated with PRP gel using a "sandwich technique".
Another six pigs, divided into the same groups as describe above, were treated in a similar fashion, but PRP gel application technique was modified, as an "immersion technique" was performed.

Pre-operative protocol and anesthesia

The pre-operative protocol included bowel preparation which consisted of a 24 - hour period of fasting before operation. Free access of water was allowed.

The pigs were premedicated with a combination of ketamine 10 mg/kg IM (Imalgene 1000®, Merial Laboratories), azaperone 2 mg/kg IM (Stressnil®, Janssen Pharmaceutical) and buprenorphine 20 μg/kg IM (Buprex®, Schering-Plough Laboratories). Immediately prior to induction, 65 mL of whole blood were drawn for PRP obtention. Then, a single dose of 4 mg/kg of propofol (Propofol Lipuro 1%, B.Braun Medical SA) was given IV for anaesthetic induction through an 18-gauge polyurethane catheter (Vasocan, B.Braun Medical SA) placed in the peripheral auricular vein.

The trachea was intubated and anesthesia was maintained with 1.5-2.5% isofluorane (IsoFlo, Abbott Laboratories) in 100% oxygen through a semi-closed circular anaesthetic system. To compensate fluid loss, all animals received an infusion of Ringer Lactate, at a rate of 10 mL/kg/h, during the perioperative period, and until completely recovered from anaesthesia, perioperative IV antibiotic therapy (cephalexin, 20 mg/kg) were administered via the auricular vein. Heart rate, pulse oximetry, respiratory rate and capnography, and temperature, were monitored during anaesthesia, using a Datex Ohmeda Cardiocap II Monitor.

Surgical procedure

Each pig was positioned in dorsal recumbency on the operating room table and the ventral abdominal region was prepared aseptically. After the surgical field was draped, a ventral midline abdominal incision was made and the *linea alba* was incised from 5 cm cranial to the umbilicus and extending 7-8 cm caudally. The jejunum was exteriorized from the abdomen, the lumen was occluded using digital pressure, and a complete transversal section was made with Metzenbaum scissors (Fig 4.3.a).

"Sandwich technique"

Autologous PRP gel was activated with calcium chloride in a glass Petri dish, and in few minutes PRP gel was obtained (Fig. 4.3.b). Thus the intestinal edges were opposed and a film of PRP gel was applied between the two intestinal edges. Intestinal anastomosis was performed suturing the gel in between ("sandwich technique"). Intestinal end-to-end anastomosis was performed using synthetic monofilament glyconate 3/0 USP suture material (Monosyn®, B.Braun Medical SA) and a simple interrupted pattern (Fig. 4.3.c).

A second enterectomy (control) was performed in the mid-jejunum at about 50 cm from the first enterectomy and was sutured using the same pattern as describe above without any treatment. When the intestinal suture was completed, tightness was checked for leakage and the anastomotic site was omentalized. Both anastomosis (treated and untreated) were performed on the same pig. The laparatomy was closed in a routine manner. During surgery, the body temperature was kept at 38 °C by using a heating pad and warm Lactated Ringer's solution given intravenously during the operation.

The animals were euthanized at 24 and 48 hours after surgery for histological evaluation.

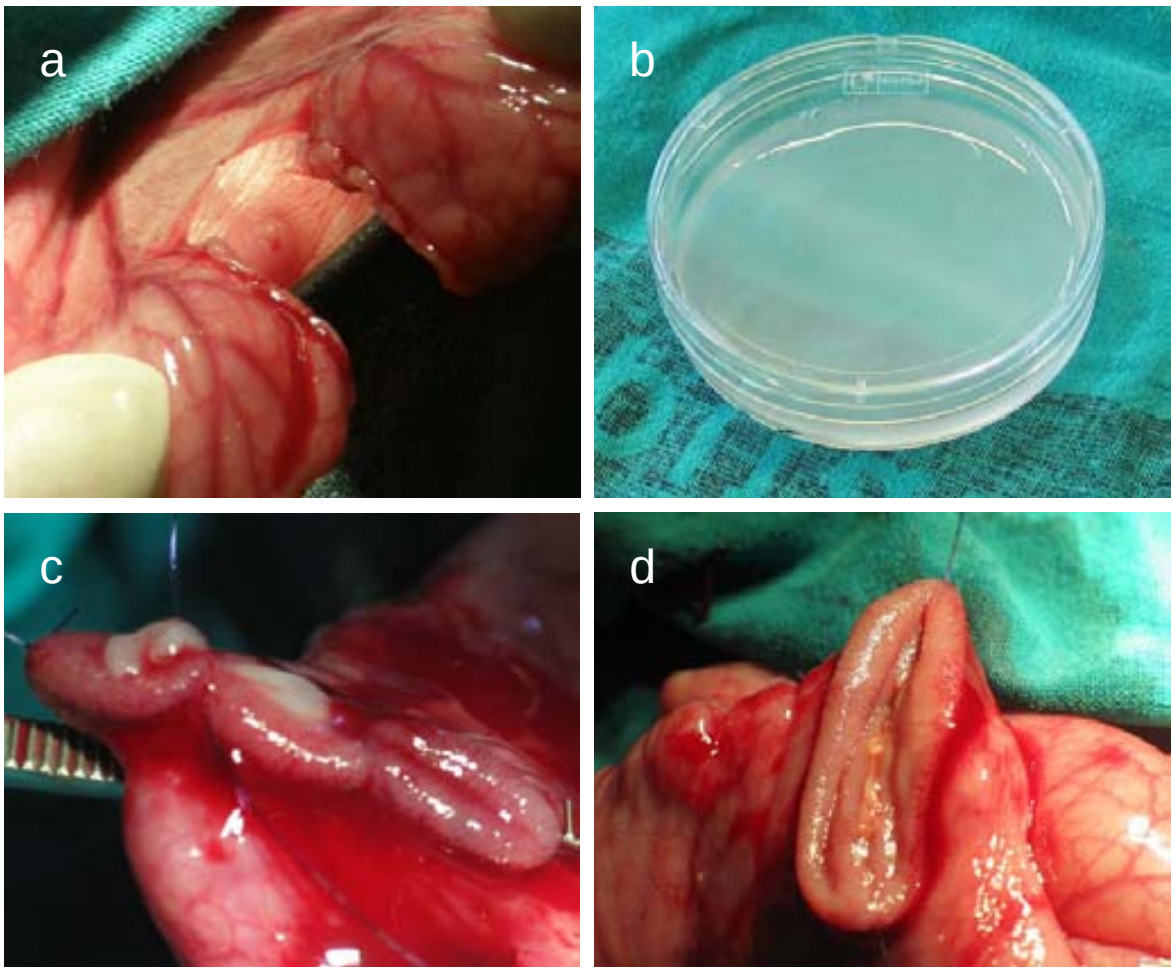


Figure 4.3- Application of calcium activated platelet concentrate "sandwich technique". (a) end-to-end intestinal anastomosis; (b) Petri dish with calcium chloride activated PRP; (c) application of PRP gel between the intestinal edges ("sandwich technique"); (d) intestinal lumen after performing the suture of the opposite side.

"Immersion technique"

Immediately before the first enterectomy was performed, platelet-rich plasma prepared and located in the glass Petri dish was activated. Afterwards, a complete transversal section of the small intestine was performed, in the mid jejunum, and intestinal edges were immediately submerged in the activated PRP, for 10 minutes, until the PRP was completely gelified (Fig. 4.4). After this procedure, anastomosis of the intestinal edges was performed using a simple interrupted suture pattern with synthetic monofilament glyconate 3/0 USP suture material (Monosyn®, B.Braun Medical SA). Careful manipulation of the intestine was done to avoid removal of the PRP-gel. When the intestinal suture was completed, tightness was checked for leakage (Fig. 4.5) and the anastomotic site was omentalized (Fig. 4.6).

A second enterectomy was performed in the mid-jejunum at about 50 cm from the first enterectomy. After complete transversal section of the small intestine, the edges were immediately immerged in physiological saline solution (NaCl 0.9%) for 10 minutes. Anastomosis and omentalization was performed as described before for the first enterectomy. The laparotomy was closed in a routine manner.

The animals were euthanized at 24 hours and 48 hours after surgery for histological evaluation.

All operative procedures including the jejunal anastomosis were performed by the same experienced surgeon.

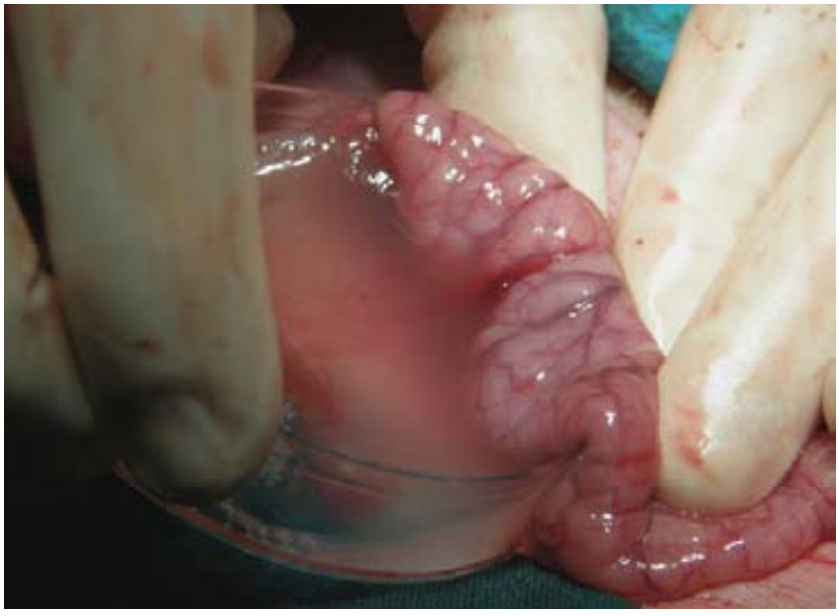


Figure 4.4 - Immersion of the intestinal edges in PRP after activation with calcium chloride during 10 minutes.

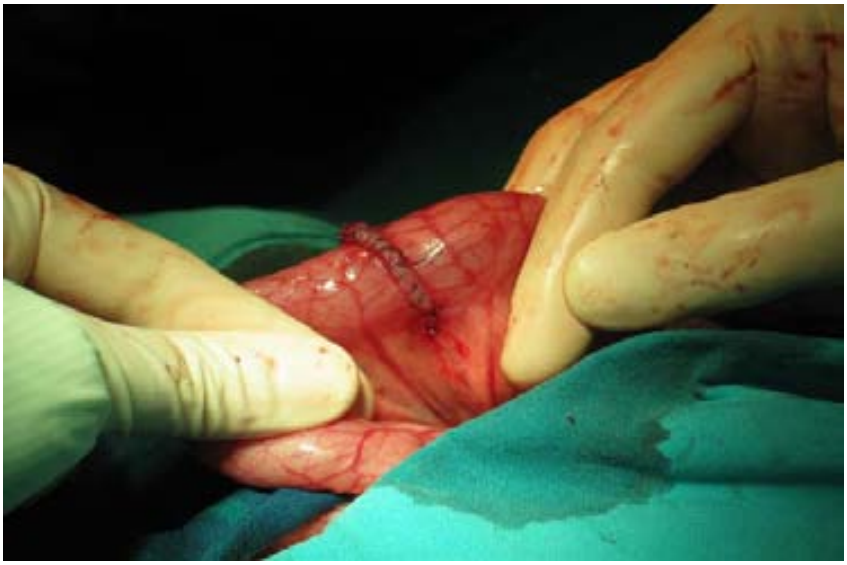


Figure 4.5 - Checking leakage before omentoplasty.

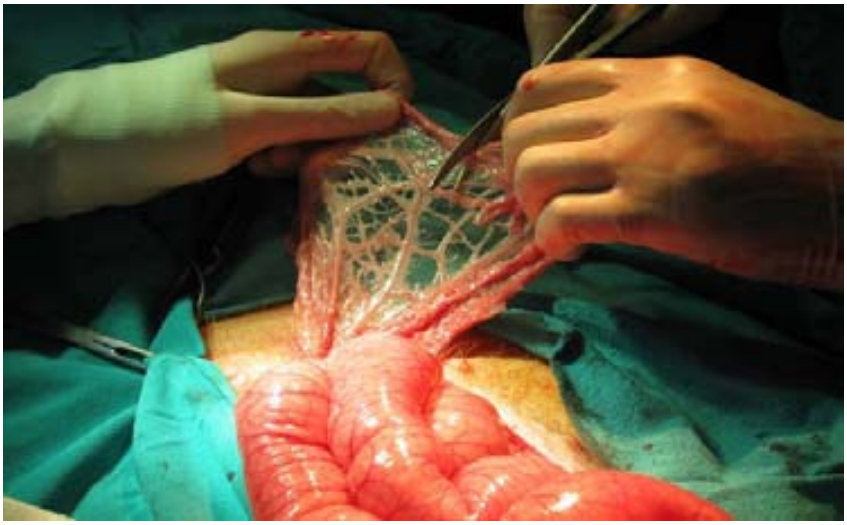


Figure 4.6 - Technique for mobilizing the omentum based on the epiploic arcade.

Post-operative period

All animals recovered from surgery without any complication and were allowed *ad libitum* activity. After the operation, the animals were monitored at least thrice-daily for subjects like daily activity, inquisitiveness, vocalization, gait, urinary and bowel habits, oral intake and examination of the abdomen wound incision.
Animals were gradually fed with a standard diet 12-24 hours postoperatively and given water *ad libitum*. Buprenorphine 20 μg/kg IM, was provided thrice-daily for postoperative analgesia. Antibiotic therapy consisted of 20 mg/kg cephalexin IV every 12 hours until endpoint day. Weights were obtained preoperatively, at midsurvival, and before end point.

To evaluate the occurrence of anastomotic leakage the following indicators were assessed:

(i) Clinical indicators (abdominal pain, abdomen distension, accumulation of gas or fluids and vomiting).

(ii) Biochemical and physiologic parameters or observation abnormalities (fever, tachycardia, hypopnea or shallow breathing, rapid breathing, low blood pressure, rapid pulse, polydipsia and increased white cell count).

b) Definitive study

Since the results obtained with the "sandwich technique" were not good enough (see Results section below), the "immersion technique" was chosen for definitive study purposes.

A total of 35 pigs (including those employed in the pilot study) were operated following the "immersion technique" for PRP gel application. From those 35 pigs, 15 pigs were assigned to evaluation of the intestinal healing through histological analysis, while 20 pigs were used for evaluation of the intestinal anastomotic strength.

4.3. Stage III. Evaluation of the intestinal healing

Evaluation of the intestinal healing following PRP application was done at different times by means of a macroscopic and histological examination and through determination of the breaking strength.
Thus, all the animals were sacrificed without pain by overdose with a lethal intravenous injection of Phenobarbital sodium 20 mg (Dolethal®) Vetoquinol- Madrid) preceded by a combination of ketamine 10 mg/kg IM, azaperone 2 mg/kg IM sedation, at respective times. All animals received care in accordance with the Spanish Code of Practice for the Care and Use of Animals for Scientific Purposes, published by Spanish Government Publishing Service, (Directiva CE 86/609- BOE RD 1201/05 - 21/11/05).

4.3.1. Macroscopic examination

In all operated animals (35 pigs), at appropriate endpoint day for each group respectively, macroscopic examination was performed.
Immediately after euthanasia, the previous incision was reopened, anastomosis was examined *in situ* and healing was visually assessed as normal or abnormal. The intestines were carefully exteriorized and the anastomotic sites identified, the tightness was checked for leakage occurrence. Abnormal healing was defined as the presence of stricture formation, anastomotic leakage, perianastomotic abscess formation, and anastomotic dehiscence or obstruction. Normal healing was defined as apparent anastomotic integrity with no evidence of leakage, perianastomotic collection, obstruction or distension of the bowel.

4.3.2. Histological examination

Histological examination was first performed in animals included in the pilot study in order to decide which surgical technique of PRP gel application was more suitable. Intestinal samples were evaluated at 24 and 48 hours.
After that, histological examination was performed in the definitive study, which included 15 animals divided in five groups of 3 animals each: group 1 was euthanized after 24 hours from surgery, group 2 after 48 hours, group 3 after 72 hours, group 4 after 96 hours, and group 5 after 7 days. Animals from to group 1 and group 2 belong to the pilot study using the "immersion technique" of PRP gel application.

Segments of approximately 10 cm in length containing the anastomosis in the middle were carefully resected. Intestinal samples were opened at the mesenteric side, gently washed with saline solution, and fixed in 10% neutral buffered formalin. Each intestinal sample was cut in 6-8 pieces and routinely processed for further histopathological study. Four μm sections routinely stained with hematoxylin and eosin (H-E) and with Masson trichrome were evaluated. Microscopic observations focused on healing of intestinal layers and thickness of granulation tissue formed.
Sections were then examined by the same trained pathologist who was blinded to the tissue source.

4.3.3. Anastomotic breaking strength

Unwounded intestinal samples were taken from the animals included in the pilot study in order to check the feasibility of the dynamometer (Instron Tensile Machine 1122 ™, Instron Corporation, MA) for evaluation of intestinal breaking strength.

For definitive purposes, 20 pigs were used for the anastomotic strength measurement. These animals were subdivided in two groups of 10 animals each: group 1 - In those animals the measurement of breaking strength was made at 3 days after surgery. Group 2 - The measurement of breaking strength was made at 7 days after surgery. For comparison purposes, 10 samples of unwounded intestine from the same animals were also taken and measured for breaking strength.
The intestines were carefully exteriorized and the anastomotic sites identified. Segments of approximately 10 cm in length containing the anastomosis in the middle were carefully resected, and adhesions were cut as far as possible without injury to the intestine. Intestines were opened at the mesenteric side and gently washed with saline solution. The segments were placed in the dynamometer, which provided a force of 10 kgf (0.1 kN) increasing at a rate of 300 mm/min (Fig. 4.7). The peak force necessary to induce disruption was taken as the breaking strength, as well as the site of rupture (within or outside the anastomotic line) was recorded.

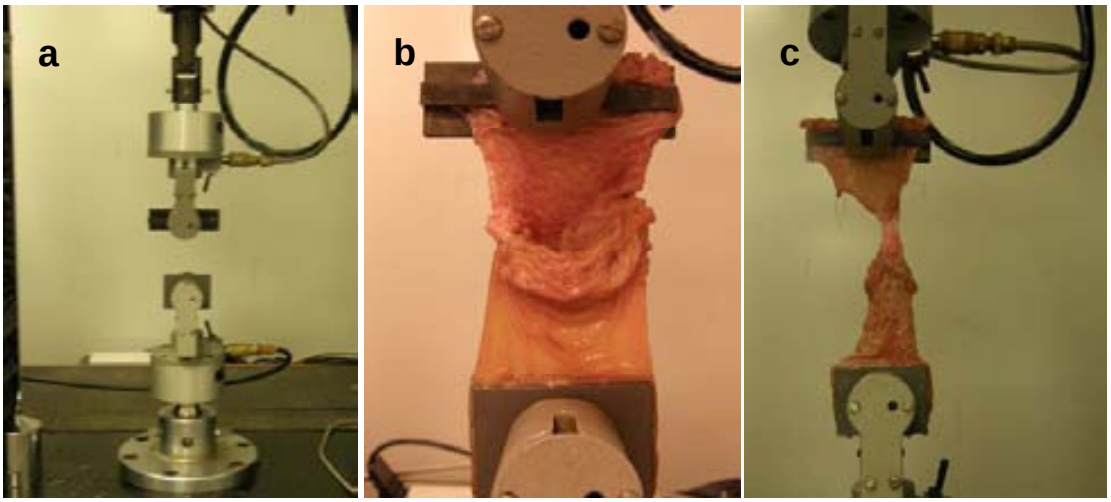


Figure 4.7- Anastomotic breaking strength evaluation. a) Instron Tensile Machine 1122 ™; b) The specimen was attached to the gripping component; c) Distraction of the tissue until rupture.

4.4. Statistical analysis

Data were analysed using a statistical computer software program (SPSS v15.0). Nonparametric tests were selected for all variables because they were either not normally distributed. All quantitative measurements were described using summary statistics (number, mean, and standard deviation, minimum, maximum). All duplicate values were within 10% of each other, and the mean of duplicates was used for data analysis. The results are presented as mean±SD, unless stated otherwise, *n* represents number of animals.

The means of platelets number in arterial blood, means of platelets number in PRP, means of the enrichment percentages, and means of hematological values, means of MPC values on PRP and whole blood; means of TGF-β1 concentrations, on PPP, PRP and PRP gel, of experimental groups were compared, using Wilcoxon statically analysis to compare means. Significance in all instances was set at a value $p<0.05$.

A paired Student's t-test statistical analysis was employed to compare breaking strength between PRP and control groups. Breaking strength at 3 days, 7 days and unwounded intestine was compared using a one- way ANOVA. Significance in all instances was set at a value $p<0.05$.

5. Results

5.1 Stage I: PRP obtention and quality evaluation

5.1.1. PRP obtention

After the realization of different trials, it was concluded that the PRP obtention technique in pigs consisting in a tube method, using a double spin centrifugation (786g for 10 minutes for first spin, and 526g for 10 minutes for second spin, and resuspending the final pellet in 500 μL), yielded the best results. PRP obtention, using that technique, was prepared in approximately 30-40 minutes.

5.1.2. Evaluation of PRP quality

Erythrocytic parameters, leukocytic series, and platelet parameters, including monitorization of MPC, on whole blood and PRP samples of the 35 pigs submitted to the surgical procedure were assessed. Calculation of the quality parameters was also performed. The same parameters were evaluated in the 12 animals used for TGF-β1 levels determination, as well as concentration of this growth factor in the different fractions (PPP, PRP, PRP gel). Mean, standard deviation (SD) and range values in statistical analyses were used.

a) Erythrocyte, platelet and leukocyte count, and quality parameters

The values of erythrocytic and platelet parameters, before and after centrifugation on operated animals are resumed on table 5.1. Statistically significant differences ($P<0.05$), were observed between erythrocytic parameters on whole blood, when comparing those parameters on PRP. However, on some platelet parameters MPV, MCV, MPM and LP no significant differences ($P<0.05$) were observed. In contrast, in other platelet parameters (PDW) significant differences were observed on whole blood, when comparing with (PDW) count on PRP.

The leukocytic series (neutrophils, lymphocytes, monocytes, eosinophils and basophils) values in $10^3/\mu L$ and %, before and after centrifugation on operated animals, are resumed on table 5.2 and 5.3, respectively. Statistically significant differences ($P<0.05$), were observed between leucocytic series count on whole blood and PRP.

	Citrated whole blood			Platelet - rich plasma		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
RBC [a] ($10^3/\mu L$)	5,7	0,4	4,8-6,6	0,01	0,04	0,01-0,02
Hemoglobin (g/dL)	9,7	0,9	8,1-11,6	0,01	0,07	0-0,4
Hematocrit (%)	30	2,8	24,1-35,1	0,09	0,02	0-0,1
MCH [b] (pg)	16,9	0,9	14,6-18,7	0	0	0
MCHC [c] (g/dL)	32,5	1,1	30,2-36,9	0	0	0
RDW [d] (%)	16,7	1,2	15,3-20,3	26,2	6,64	17,3-43,5
HDW [e] (g/dL)	1,9	0,09	1,73-2,24	3,46	0,86	2,4-6,56
MPV [f] (fl)	8,96	0,74	7,1-10,2	8,44	0,69	6,8-9,3
MCV [g] (fl)	51,94	2,5	46,6-56,1	50,5	4,72	35,1-64,5
MPM [h] (g/dL)	1,72	0,12	1,51-1,84	1,56	0,14	1,31-1,76
PDW [i] (%)	56,4	5,32	48,5-69,4	42,58	3,5	36,6-50,4
PCT [j] (%)	0,72	0,09	0,26-0,69	1,3	0,43	0,69-2,34
LP [l] ($10^3/\mu L$)	13,37	4,37	8-26	16,3	10,1	3-49

Table 5.1 - Mean, SD and range values of erythrocyte and platelet parameters on citrated whole blood and PRP of operated animals (n=35).
a, RBC-red blood cells; **b**, MCH-mean corpuscular hemoglobin; **c**, MCHC-mean corpuscular hemoglobin concentration; **d**, RDW-red blood cell distribution width; **e**, HDW-hemoglobin distribution width; **f**, MPV-mean platelet volume; **g**, MCV-mean corpuscular volume; **h**, MPM-mean platelet mass; **i**, PDW-platelet distribution width; **j**, PCT-Plateletcrit; **l**, LP-large platelets.

	Citrated whole blood			Platelet - rich plasma		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
WBC ($10^3/\mu L$)	15,33	3,45	9,02-23,63	1,83	1,03	0,37- 4,29
Neutrophils ($10^3/\mu L$)	4,34	1,56	2,03-7,99	0,06	0,04	0,01-0,24
Lymphocytes ($10^3/\mu L$)	9,47	2,77	3,12-16,18	1,64	0,94	0,32-3,95
Monocytes ($10^3/\mu L$)	0,97	1,28	0,27-8,11	0,08	0,06	0,01-0,3
Eosinophils ($10^3/\mu L$)	0,24	0,2	0,03-1,18	0,01	0,01	0-0,1
Basophils ($10^3/\mu L$)	0,2	0,08	0,08-0,48	0,01	0,02	0-0,1
LUC ($10^3/\mu L$)	0,26	0,29	0,03-1,7	0,03	0,04	0-0,2

Table 5.2 - Mean, SD and range values of leucocytic series ($10^3/\mu L$) on citrated whole blood and PRP of operated animals (n=35).
LUC - large unstained cells; WBC- white blood cells

	Citrated whole blood			Platelet - rich plasma		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Neutrophils (%)	28,3	7,71	13,2-43	4,13	5,1	0,4-28,9
Lymphocytes (%)	62,5	7,71	48,5-79,3	89,34	5,6	64,7-94,7
Monocytes (%)	4,9	1,63	2,5-10	4,02	1,8	1,2-8,4
Eosinophils (%)	1,5	0,97	0,2-5,5	0,92	1,2	0,1-5,1
Basophils (%)	1,4	0,46	0,7-2,9	0,42	0,2	0-0,8
LUC (%)	1,5	1,0	0,2-4,4	1,14	0,04	0,01-0,25

Table 5.3 - Mean, SD and range values of white blood cells (%) on citrated whole blood and PRP of operated animals (n=35).

The values of erythrocytic and platelet parameters, before and after centrifugation on animals used for TGF-β1 levels determination are resumed on table 5.4. Statistically significant differences ($P<0.05$) were observed between erythrocytic parameters on whole blood, when comparing those parameters to PRP. However on some platelet parameters (MPV, MCV, MPM and LP) no significant differences ($P<0.05$) were observed. In contrast, in other platelet parameters (PDW) significant differences were observed on whole blood, when comparing with PRP.

The leukocytic series (neutrophils, lymphocytes, monocytes, eosinophils and basophils) values in $10^3/\mu L$ and %, before and after centrifugation, on animals used for TGF-β1 levels determinations are resumed on table 5.7 and 5.8, respectively. Statistically significant differences ($P<0.05$) were observed between leucocytic series count on whole blood, when comparing to PRP.

	Citrated whole blood			Platelet - rich plasma		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
RBC [a] ($10^3/\mu L$)	5,76	0,48	5,08-6,58	0,01	0,01	0-0,02
Hemoglobin (g/dL)	10,45	0,95	9,2-11,9	0	0	0
Hematocrit (%)	32,08	7,4	26-54	0,05	0,05	0-0,1
MCH [b] (pg)	16,82	4,77	1,95	0	0	0
MCHC [c] (g/dL)	34,9	1,31	32,5-37	0	0	0
RDW [d] (%)	16,39	0,82	15-17,8	43,5	10,8	22,8-56,1
HDW [e] (g/dL)	2,17	0,19	1,84-2,53	6,02	1,27	4,32-8,91
MPV [f] (fl)	8,4	0,88	6,9-9,5	7,65	0,74	5,8-8,8
MCV [g] (fl)	48,8	10,85	15-54,6	51,5	4,66	44,9-59,2
MPM [h] (g/dL)	1,7	1,12	1,51-1,84	1,56	0,14	1,31-1,76
PDW [i] (%)	57,6	4,22	51,8-63,1	45,6	2,86	39,4-48,8
PCT [j] (%)	0,26	0,05	0,13-0,33	0,75	0,28	0,37-1,15
LP [l] ($10^3/\mu L$)	8,33	3,82	3-14	9,58	6,81	2-24

Table 5.4 - Mean, SD and range values of erythrocyte and platelet parameters on citrated whole blood and PRP of animals used to determinate TGF- β1 (n=12). (For figure legends, see table 5.1)

	Citrated whole blood			Platelet - rich plasma		
Parameter	Mean	SD	Range	Mean	SD	Range
WBC ($10^3/\mu L$)	19,9	3,12	14,6-24,5	5,57	3,73	1,81-13,1
Neutrophils ($10^3/\mu L$)	6,54	2,65	3,87-10,9	0,31	0,51	0,03-1,89
Lymphocytes($10^3/\mu L$)	11,98	2,42	8,18-16,5	4,9	3,2	1,68-12,2
Monocytes ($10^3/\mu L$)	0,74	0,35	0,3-1,54	0,27	0,32	0,03-1,19
Eosinophils ($10^3/\mu L$)	0,25	0,12	0,11-0,56	0,06	0,11	0,01-0,4
Basophils ($10^3/\mu L$)	0,35	0,46	0,09-1,8	0,03	0,03	0-0,12
LUC ($10^3/\mu L$)	0,15	0,13	0,04-0,54	0,03	0,03	0-0,09

Table 5.5 - Mean, SD and range values of white blood cells and leukocytic series on citrated whole blood and PRP of animals used to determinate a TGF- β1 levels (n=12).

	Citrated whole blood			Platelet - rich plasma		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Neutrophils (%)	32,55	10,4	17-45,6	4,49	4,36	1-16,2
Lymphocytes (%)	60,47	9,58	46,9-72,6	89,45	6,86	76-96,4
Monocytes (%)	3,63	1,49	1,7-6,8	4,1	4,1	1,2-16,3
Eosinophils (%)	1,33	0,72	0,5-3	0,78	0,87	0,2-3,4
Basophils (%)	1,22	0,37	0,6-1,8	0,46	0,28	0,1-0,9
LUC (%)	0,75	0,73	0,2-3	0,68	0,74	0,2-2,9

Table 5.6 - Mean, SD and range values of white blood cells (%) on citrated whole blood and PRP of animals used to determinate a TGF- β1 levels (n=12).

The parameters used to evaluate the tube method and double spin centrifugation to obtain PRP on operated animals, and on animals used for TGF-β1 levels determination, are resumed on tables 5.7 and 5.8, respectively. Statistically significant differences ($P<0.05$) were observed between platelet count on whole blood, when comparing platelet count on PRP.

Variables	Mean	SD	Range
Platelets in whole blood ($10^3/\mu L$)	424,97	107,54	192-814
Platelets in PRP ($10^3/\mu L$)	1620,4	536,69	894 -3173
Increase in PLT concentration (%)	388	88	254 -547
PLT increase over baseline (%)	2,8	0,94	1,5 - 4,5
Collection efficiency (%)	38,4	9,4	25,4 -54,8

Table 5.7 - Mean, SD and range values of platelet yields on citrated whole blood and PRP of operated animals (n=35).
PLT: platelet.

Variables	Mean	SD	Range
Platelets in whole blood($10^3/\mu L$)	308,2	70,4	145 - 402
Platelets in PRP($10^3/\mu L$)	991	343,5	461- 1436
PLT increase baseline (%)	3,16	0,61	2,2 - 3,9
PLT increase over baseline (%)	2,2	0,61	1,2 - 2,9
Collection efficiency (%)	31,6	6,1	21,8- 39,8

Table 5.8 - Mean, SD and range values of platelet yields on citrated whole blood and PRP of animals used to determinate a TGF- β1 levels (n=12).

b) Determination of platelet activation by flow cytometry

The platelet activation evaluation on operated animals and on animals used for TGF-β1 levels determination, by means of MPC assessment on PRP and whole blood, are represented on table 5.9 and 5.10, respectively.

Mean platelet component (MPC)- g/dL		
	Whole blood	**PRP**
Mean	21,30	20,03
SD	1,58	1,39
Min	19,2	18,1
Max	24,8	24,2

Table 5.9 - Mean, SD and range values of mean platelet component (MPC) on citrated whole blood and PRP of operated animals (n= 35).

Mean platelet component (MPC) - g/dL			
	Whole blood	**PRP**	**PPP**
Mean	22,4	21,88	20,78
SD	1,59	1,68	1,95
Min	18,9	19,4	18,8
Max	25	25	18,8

Table 5.10 - Mean, SD and range values of mean platelet component (MPC) on citrated whole blood, PRP and PPP determinate by citometry (n= 12).

In the operated animals, statistically no significant differences ($P<0.05$) were observed between whole blood values, when comparing to PRP values.

In the animals used for TGF-β1 determination, statistically no significant differences ($P<0.05$) were observed between whole blood values, when comparing to PRP values. However, statically significant differences ($P<0.05$) were observed between whole blood values, when comparing with PPP values.

c) Determination of TGF-β1 levels

The TGF-β1 concentrations on the different fractions (PPP, PRP, PRP gel) are resumed on table 5.11. Statistically significant differences ($P<0.05$) were observed between TGF-β1 levels in all PRP fractions.

Transforming growth factor beta- TGF- β1 (pg/mL)			
	PPP	**PRP**	**PRP gel**
Mean	3715	24312	15504
SD	2188	21755	11279
Min	210	3521	5092
Max	8336	67815	36345

Table 5.11 - Mean, SD and range values of TGF-β1 on PRP, PPP, and PRP gel (n=12). PPP - platelet poor plasma after second centrifugation; PRP - platelet rich plasma Frozen and thawed once; PRP gel - calcium chloride activated platelet rich plasma.

5.2. Stage II: Surgical procedure

Any animal died during the surgery either on pilot or the definitive study. Heart rate, peripheral oxygen saturation, and body temperature remained within the normal limits. All pigs survived after surgery and recovered quickly from the operation, resulting in normal postoperative behaviour already after a few hours with no postoperative complications. Pigs drank water immediately after recovery from general anesthesia. The solid meal was gradually restored 24 hours after surgery.

Any animal presented classic features of peritonitis characterized by abdominal pain, pyrexia, leucocytosis, dehiscence or other complications.

5.3. Stage III: Evaluation of the intestinal healing

5.3.1. Macroscopic examination

Macroscopically, the dissected bowel appeared thickened in PRP treated animals as compared with control animals, in both pilot and definitive study. The anastomosis line was completely healed and without visible perforation since 24 hours after surgery until 7 days after surgery on PRP and control anastomoses samples. In all observed days, the anastomosis line was completely closed. (Fig. 5.1) shows the macroscopic appearance of the intestinal healing at 24h and 7 days after the immersion technique of PRP gel application.

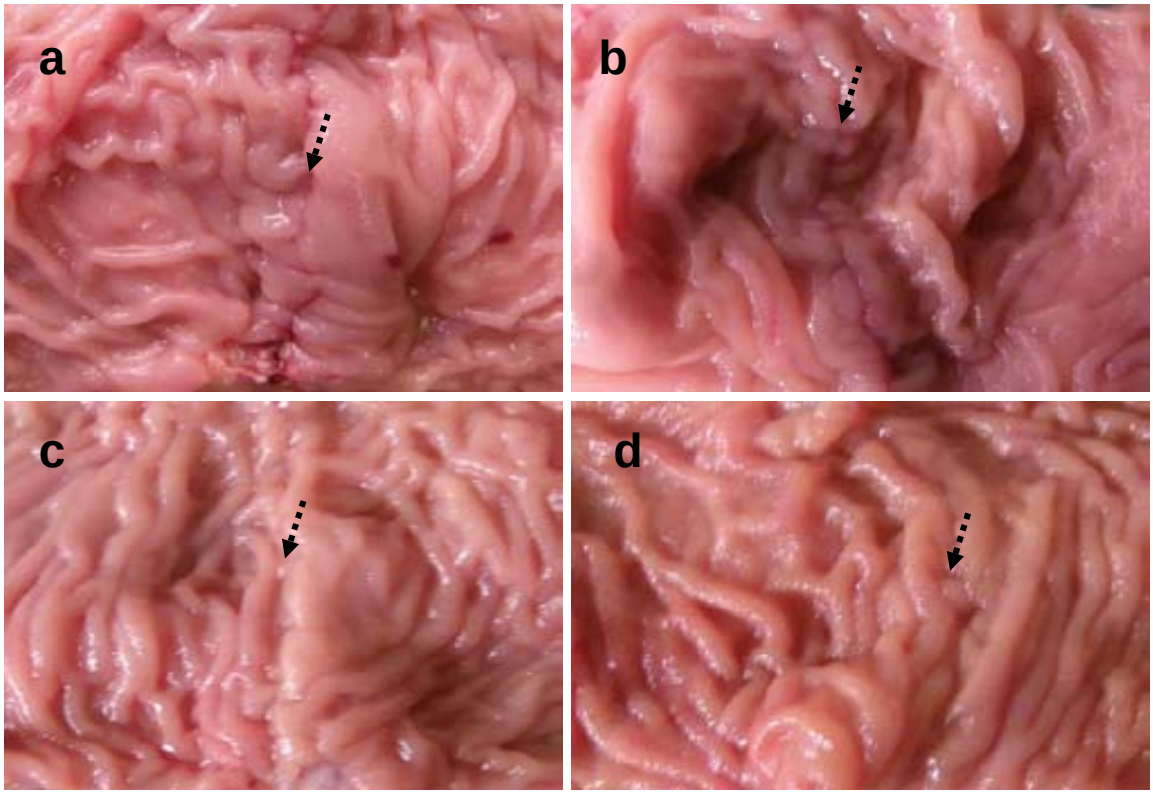


Figure 5.1- Pig small intestinal mucosa. a) PRP at 24 hours; b) Control at 24 hours; c) PRP at 7 days; d) Control at 7 days. (Arrows indicate the anastomosis line)

5.3.2. Histological examination

a) Pilot study

Histological examination of both PRP (Fig. 5.2.a) and control (Fig. 5.2.b) anastomosis section from "sandwich technique" at 48 hours showed edema, increasing inflammation without evident granulation tissue in the submucosal layer. PRP gel acts as a barrier between intestinal edges.

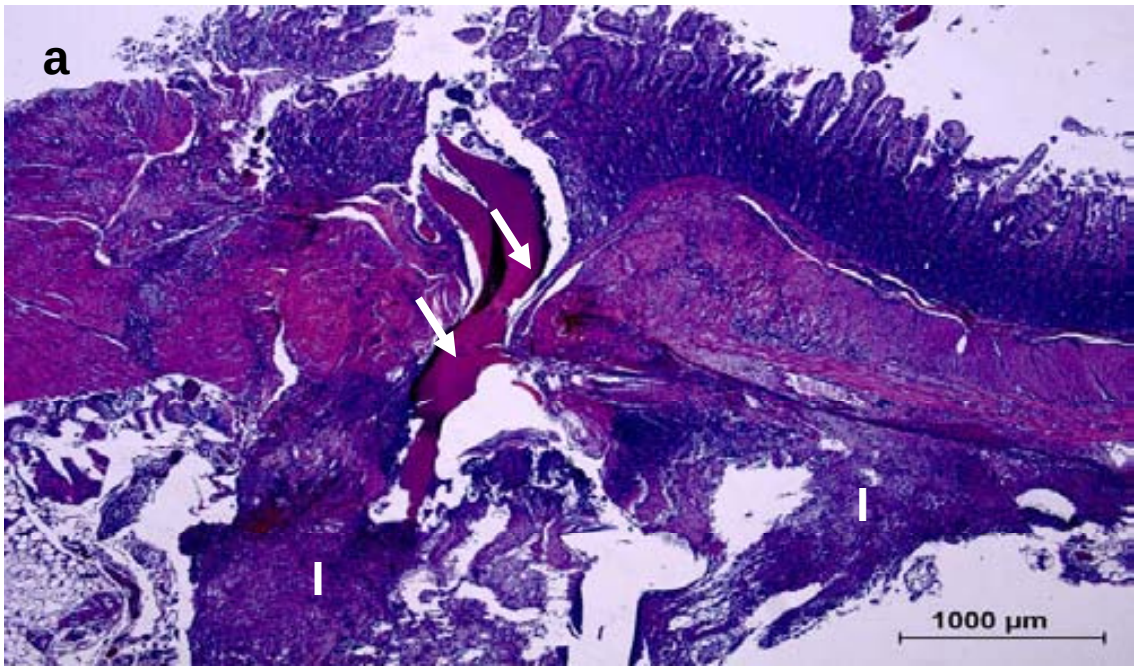


Figure 5.2.a. HE. PRP - 48h "Sandwich technique". The PRP gel is seen between intestinal edges as a homogeneous eosinophilic material (white arrow). I - inflammation.

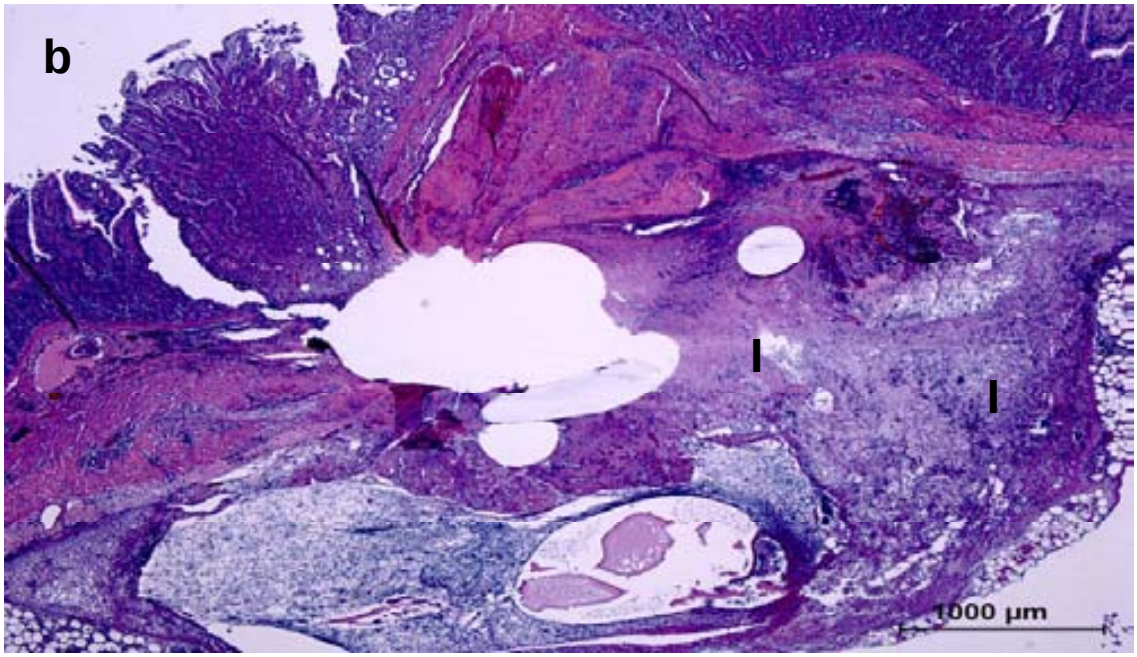


Figure 5.2.b. HE. Control - 48h "Sandwich technique". I - inflammation.

Since PRP gel application in a "sandwich technique" acts as a physical barrier, blocking the contact between both intestinal wound edges, and thus preventing a rapid healing, this technique was ruled as a method for PRP gel positioning.

Results of the histological examination of the "immersion technique" PRP gel application in the pilot study at 24 and 48 hours are included below, in the definitive study.

b) Definitive study

Based on the results obtained with the sandwich technique, the "immersion technique" was used for definitive purposes.

Microscopically, the lumen surface of the anastomotic line at 24 hours (Fig. 5.3) was covered by the epithelium. At 48 hours PRP (Fig. 5.4.a) and control (Fig. 5.4.b) intestinal anastomosis samples showed hemorrhage and inflammation, this was replaced by an immature granulation tissue at 72 hours.

On PRP intestinal anastomosis samples at 72 hours high production of immature granulation tissue with neoangiogenesis, immature fibroblast and intense inflammation (Fig. 5.5.a and Fig. 5.6.a) were observed, whereas on control intestinal anastomosis samples discreet presence of granulation tissue and low inflammation (Fig. 5.5.b and Fig. 5.6.b) have been appreciated. With Masson`s stain for collagen fibers, at 72 hours on PRP (Fig. 5.7.a) and control (Fig. 5.7.b) intestinal anastomosis samples, more fibroblasts and less collagen fibres were observed, without evident differences between control and PRP anastomosis sample.

Histological examination of PRP (Fig. 5.8.a) and control (Fig. 5.8.b) intestinal anastomosis samples at 96 hours also revealed an increased in immature granulation tissue, with less neovascularization and inflammation.

At 7 days, PRP (Fig. 5.9.a) anastomosis samples showed a marked increase of thickness of intestinal wall wereas a control samples showed a low thickness of intestinal wall (Fig.5.9.b). Presence of mature granulation tissue with fibrosis and deposition of collagen was observed in both PRP (Fig. 5.10.a) and control (Fig. 5.10.b) intestinal anastomosis samples without evident differences. With Masson`s stain for collagen fibers, at day 7 showed a collagen deposition but no evident differences between PRP (Fig. 5.11.a) and control (Fig. 5.11.b) intestinal anastomosis samples were observed. In line with this is the observation that the amount of collagen increases rapidly between days 3 and 7.

On histological examination of PRP anastomosis samples from "immersion technique" at 24 hours (Fig. 5.3), the lumen surface of the anastomotic line was covered by the epithelium. No granulation tissue has appeared yet in the submucosal layer. Images from control samples are not shown because not evident differences were appreciated compared to the PRP samples.

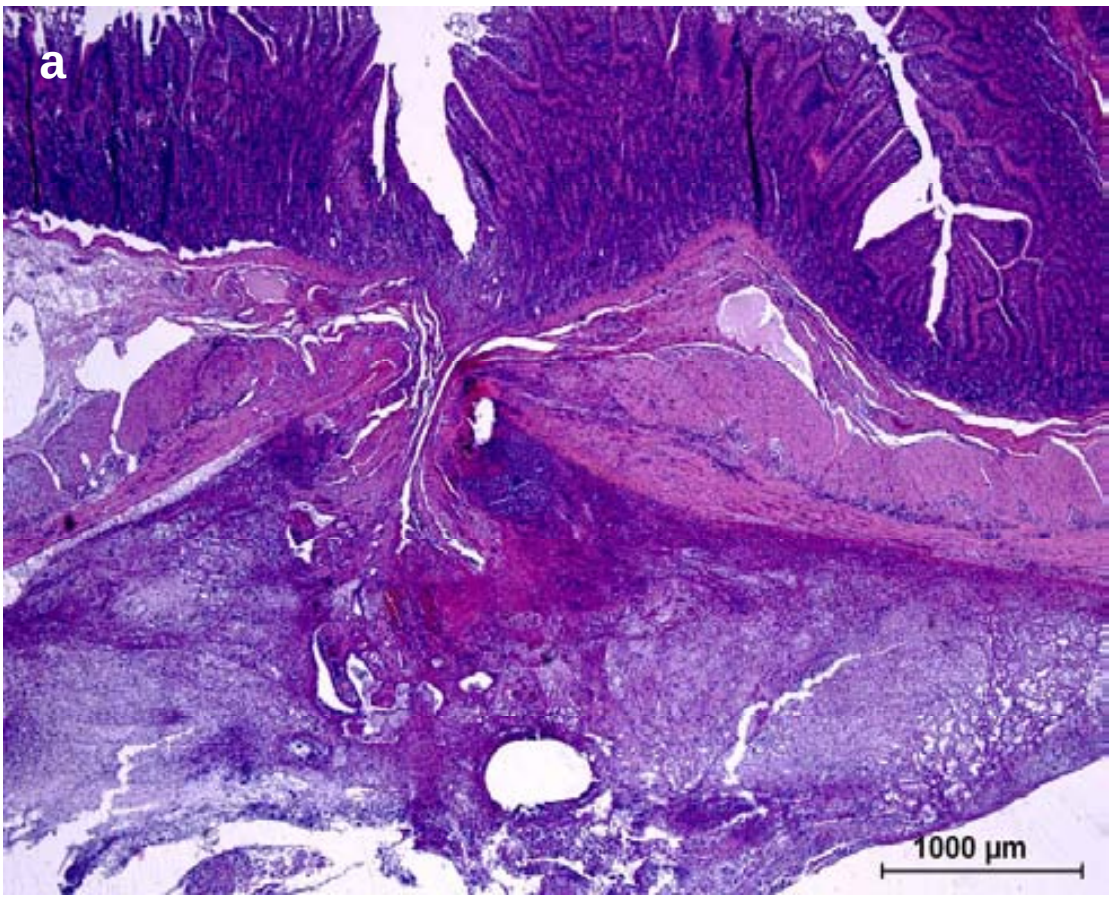


Figure 5.3. HE. PRP - 24 h.

On histological examination of both, PRP (Fig. 5.4.a) and control anastomosis (Fig. 5.4.b) samples at 48 hours, showed hemorrhage and inflammation whithout evident differences, no granulation tissue has appeared yet in the submucosal layer.

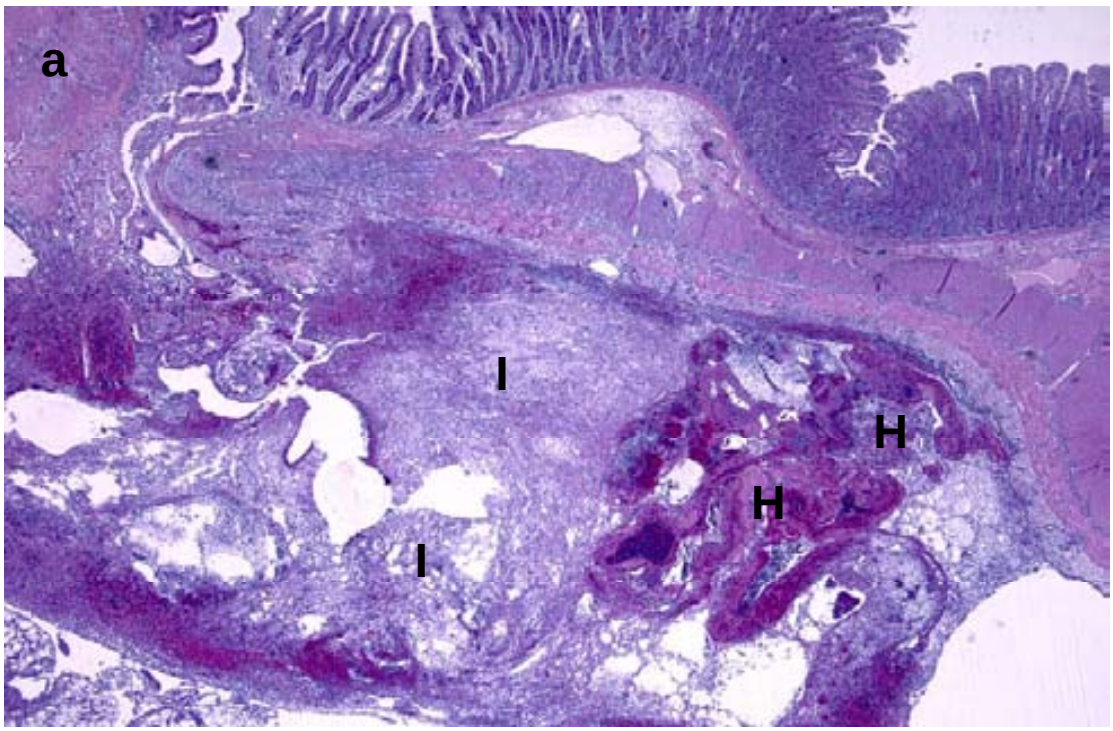


Figure 5.4.a. HE. PRP - 48 h. (I- inflammation, H- hemorrhage)

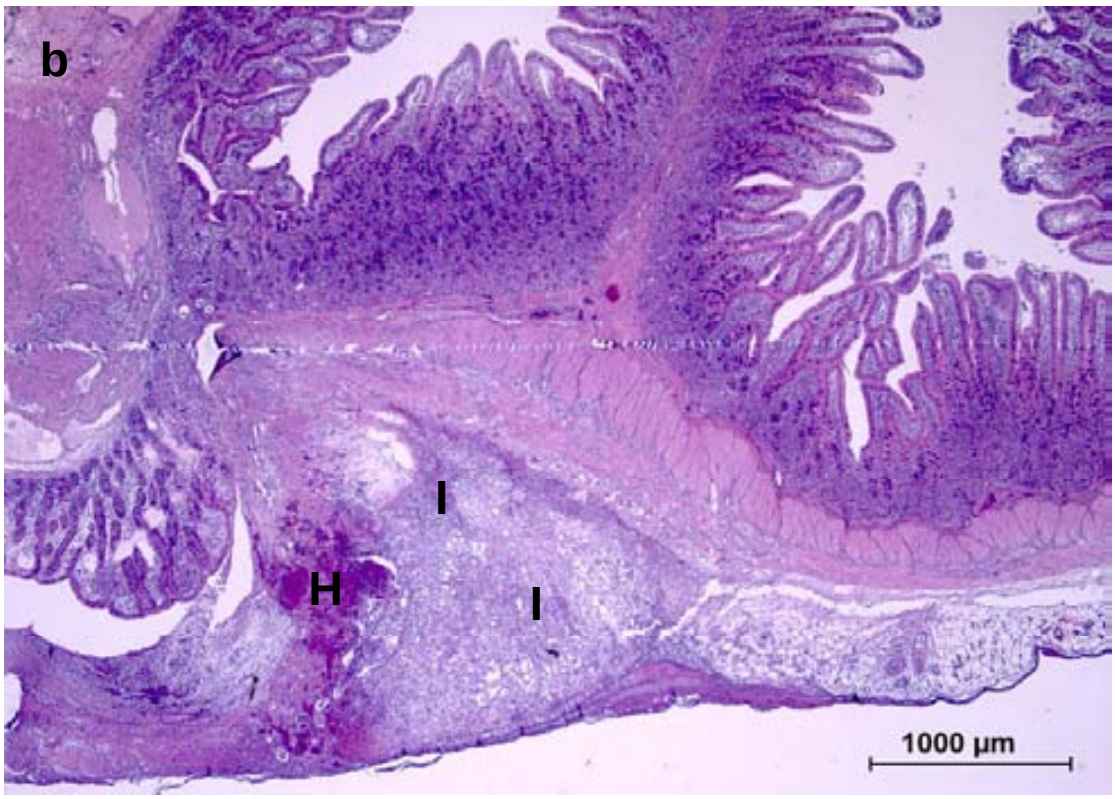


Figure 5.4.b. HE. Control - 48 h. (I- inflammation, H- hemorrhage).

Histological assessment of the PRP samples at 72 hours revealed a much more extended inflammatory activity at the anastomotic site (Fig. 5.5.a), with marked increase of thickness in intestinal wall, while a lower thickness of the healing reaction was observed in the control samples (Fig. 5.5.b) at the same time period.

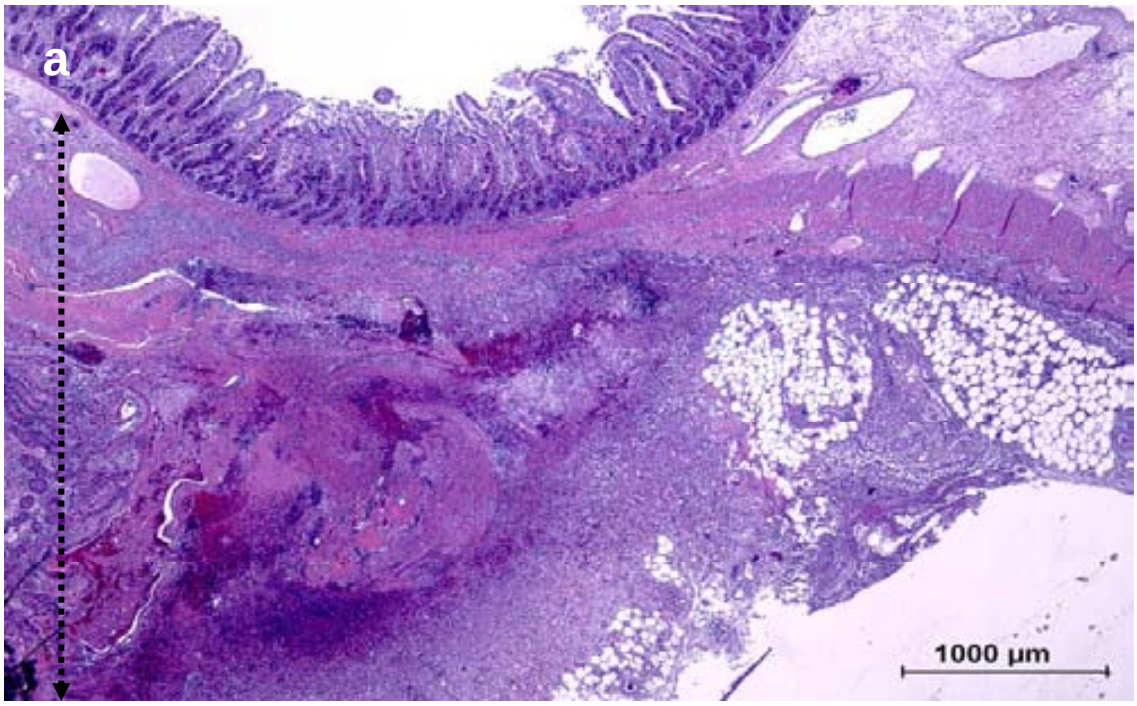


Figure 5.5.a. HE. PRP - 72 h. Thickness of the healing reaction is shown (dotted line).

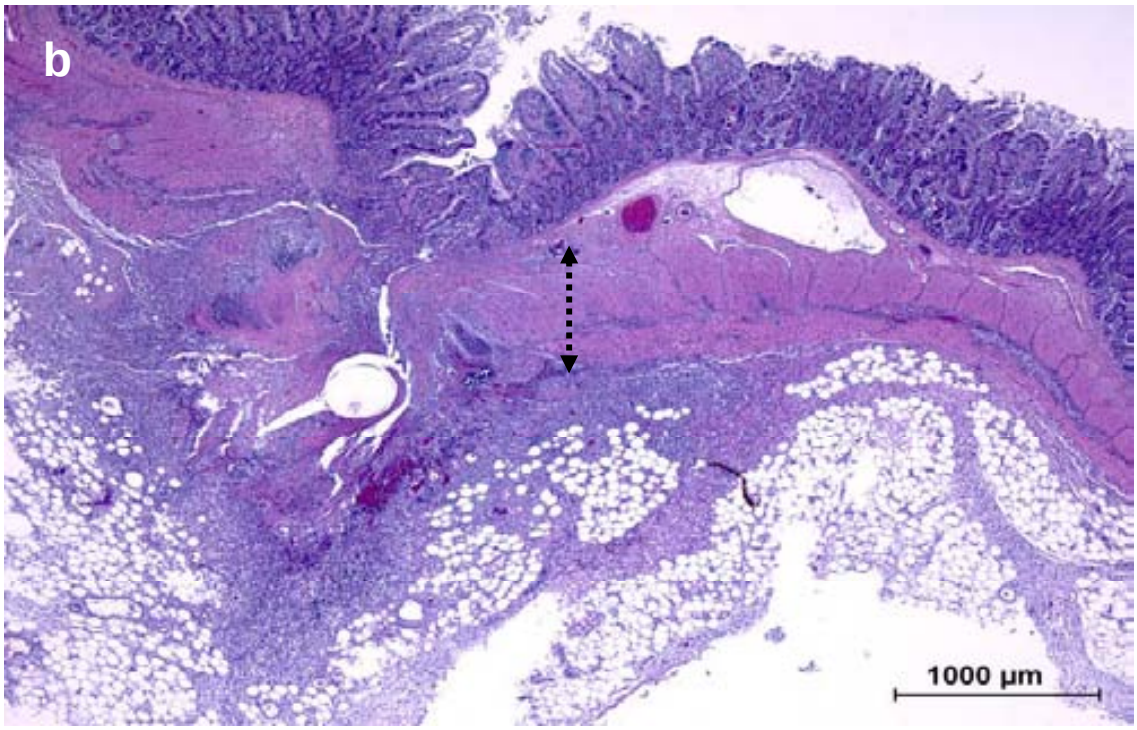


Figure 5.5.a. HE. Control - 72 h. Thickness of the healing reaction is shown (dotted line).

Higher magnification images of the granulation tissue in both, PRP and control samples at 72h are shown in figures 5.6.a and 5.6.b.

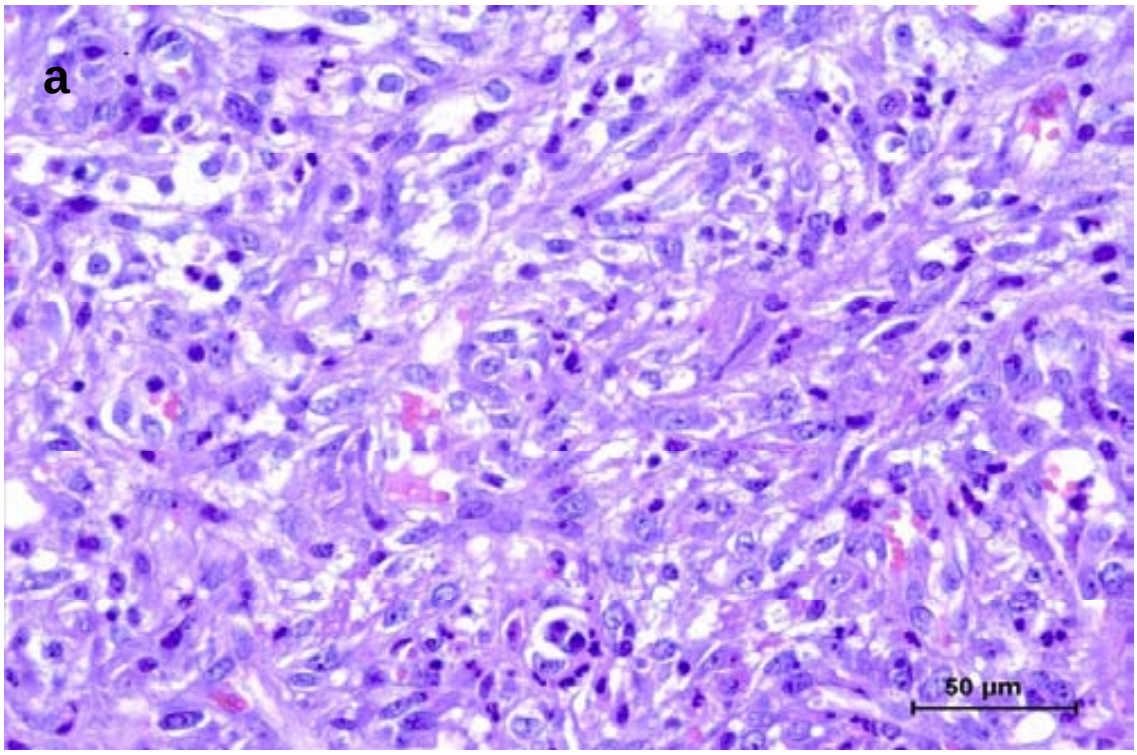


Figure 5.6.a. HE. PRP - 72 h.

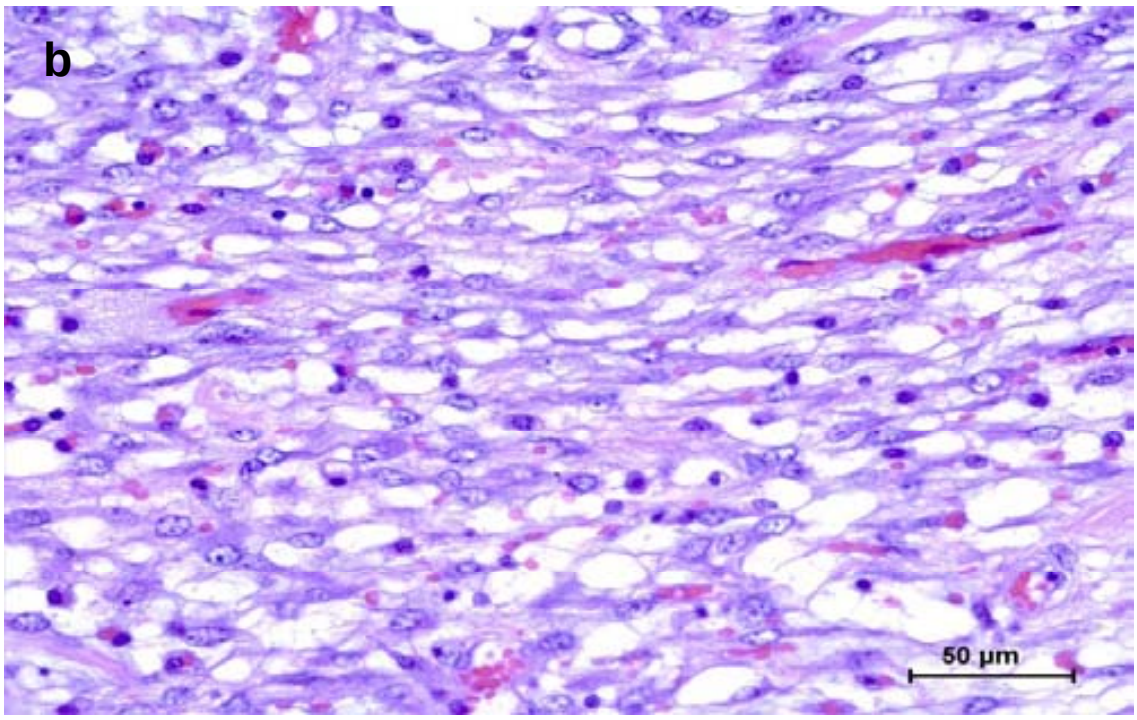


Figure 5.6.b. HE. Control - 72 h.

On Masson's trichrome stain sections at high magnification intestinal anastomosis showed evident neovascularisation and fibroblast proliferation and only few collagen fibers.

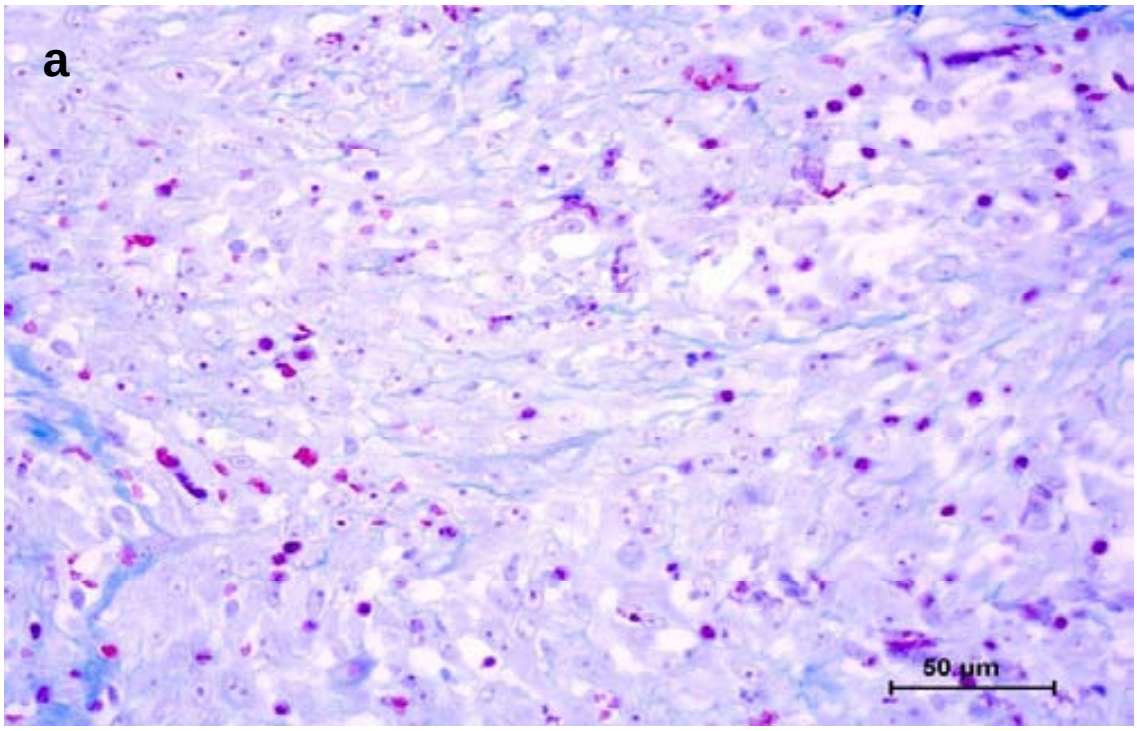


Figure 5.7.a. Masson's trichrome. PRP - 72 h.

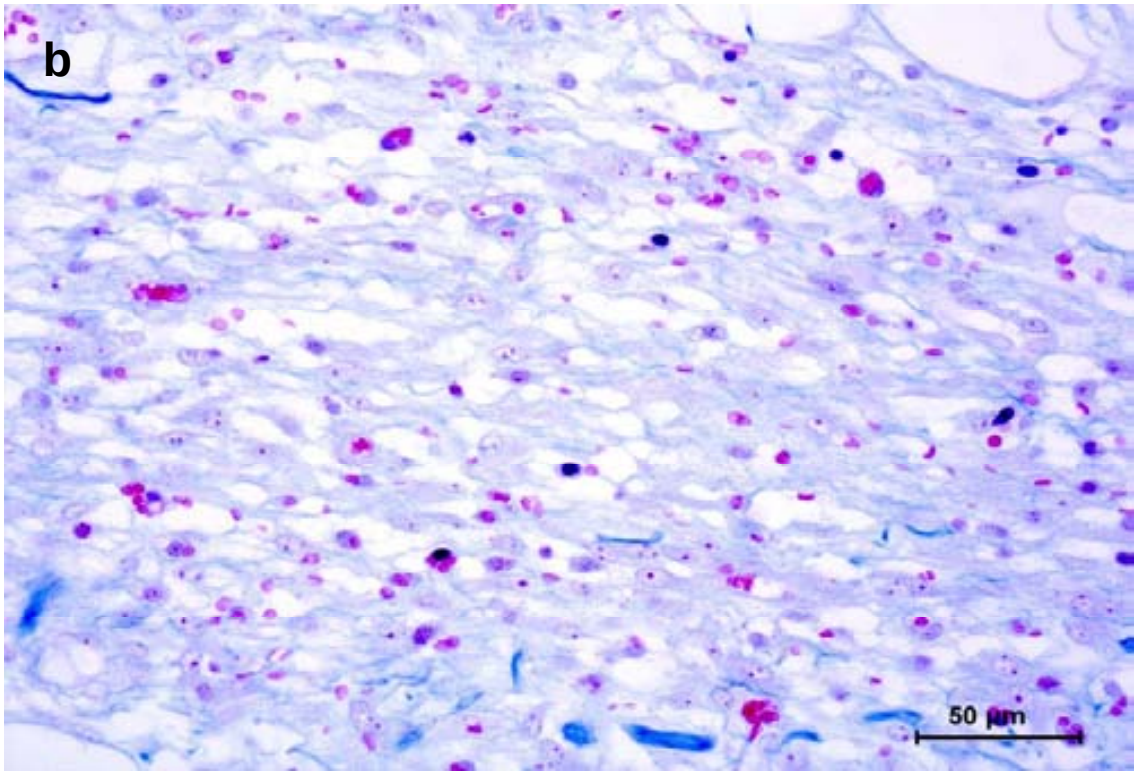


Figure 5.7.b. Masson's trichrome. Control - 72 h.

At 96 hours, PRP anastomosis samples (Fig. 5.8.a.) showed high thickness of the healing reaction consisting on an immature granulation tissue with immature fibroblasts, less neoangiogenesis and inflammation.
Control anastomosis samples (Fig. 5.8.b.) showed the lower thickness of the healing reaction is shown with the dotted line.

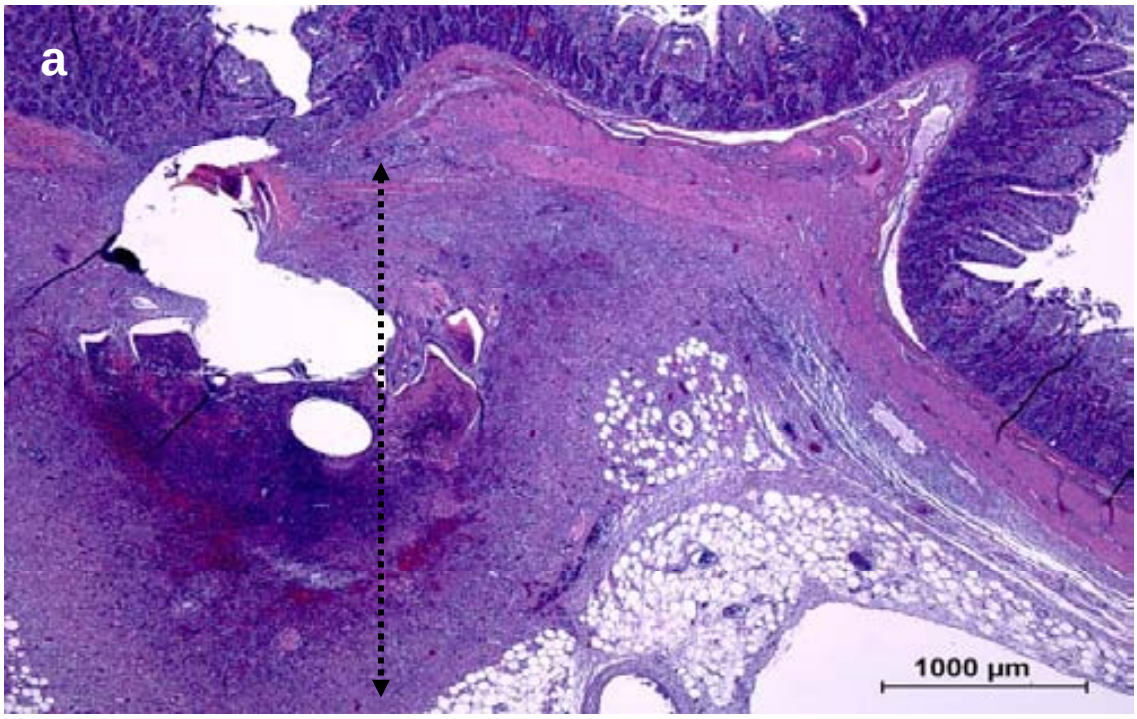


Fig. 5.8.a.. HE. PRP - 96 h. Thickness of the healing reaction is shown (dotted line)

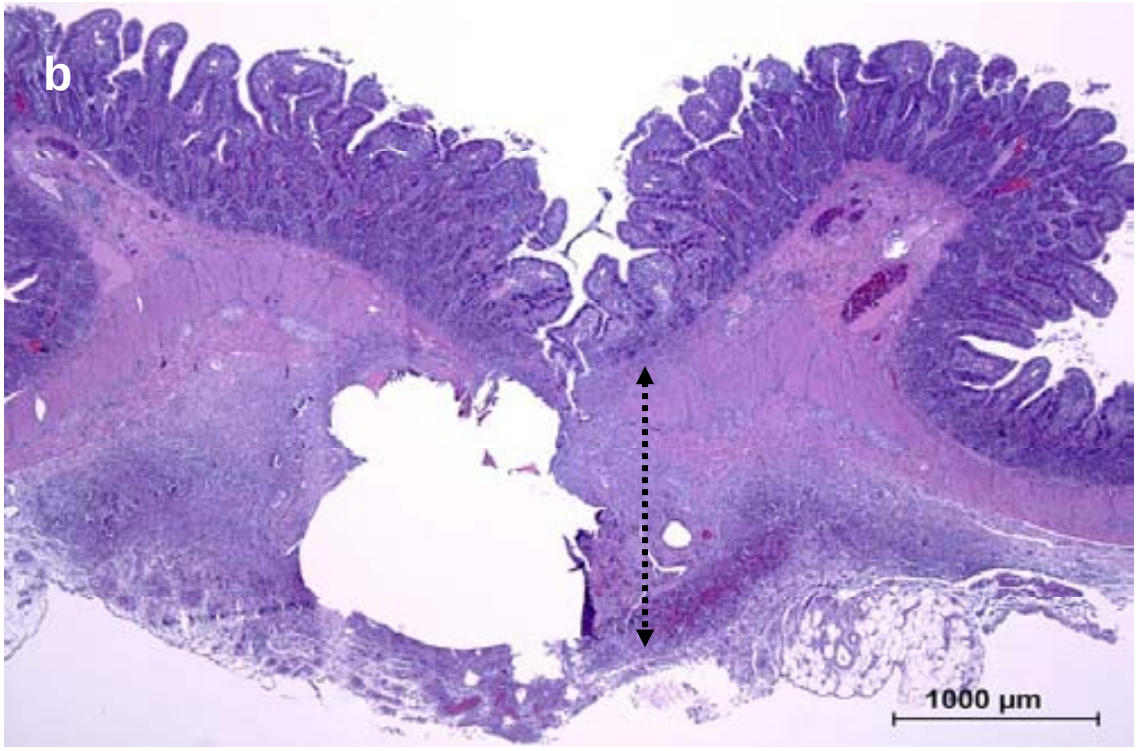


Fig. 5.8.b. HE. Control - 96 h. Thickness of the healing reaction is shown (dotted line)

At 7 days, PRP anastomosis samples (Fig. 5.9.a.) showed more mature granulation tissue that control anastomosis samples (Fig. 5.9.b.)

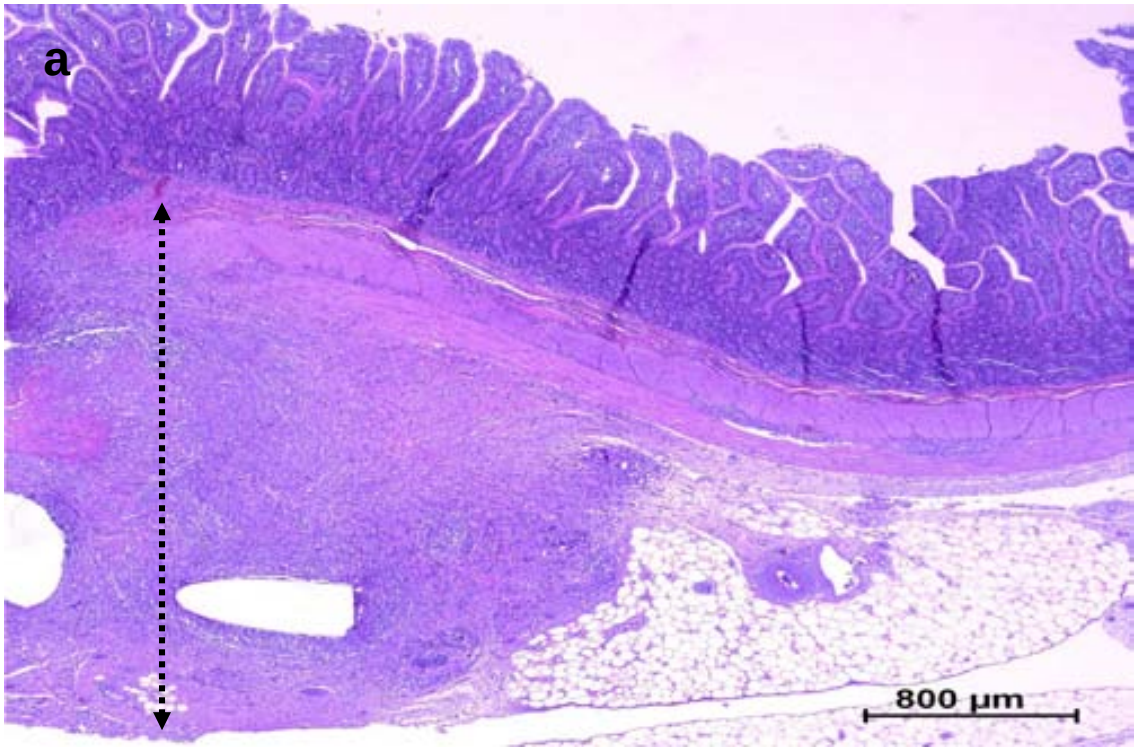


Fig. 5.9.a. HE. PRP - 7 days. Thickness of the healing reaction is shown (dotted line).

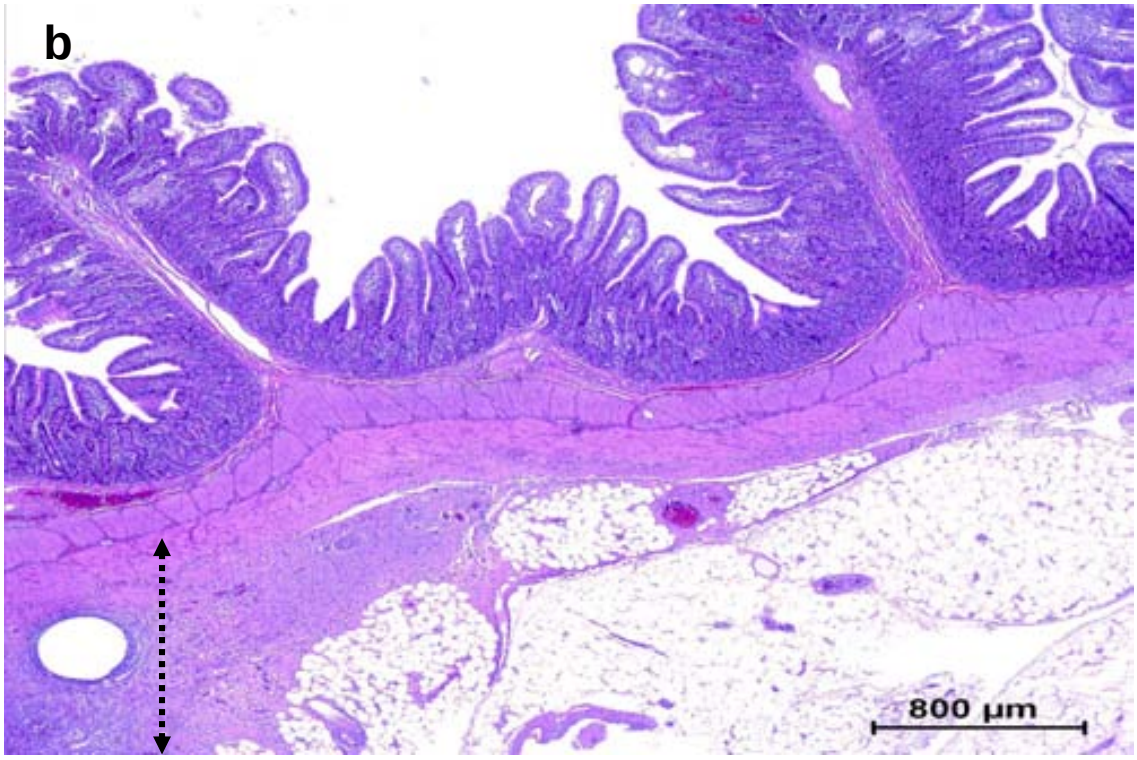


Fig. 5.9.b. HE. Control - 7 days. Thickness of the healing reaction is shown (dotted line).

Higher magnification images of the granulation tissue in both, PRP and control samples at 7 days are shown in figures (5.10.a and 5.10.b) Both, PRP and control anastomosis samples showed mature granulation tissue with collagen fibers.

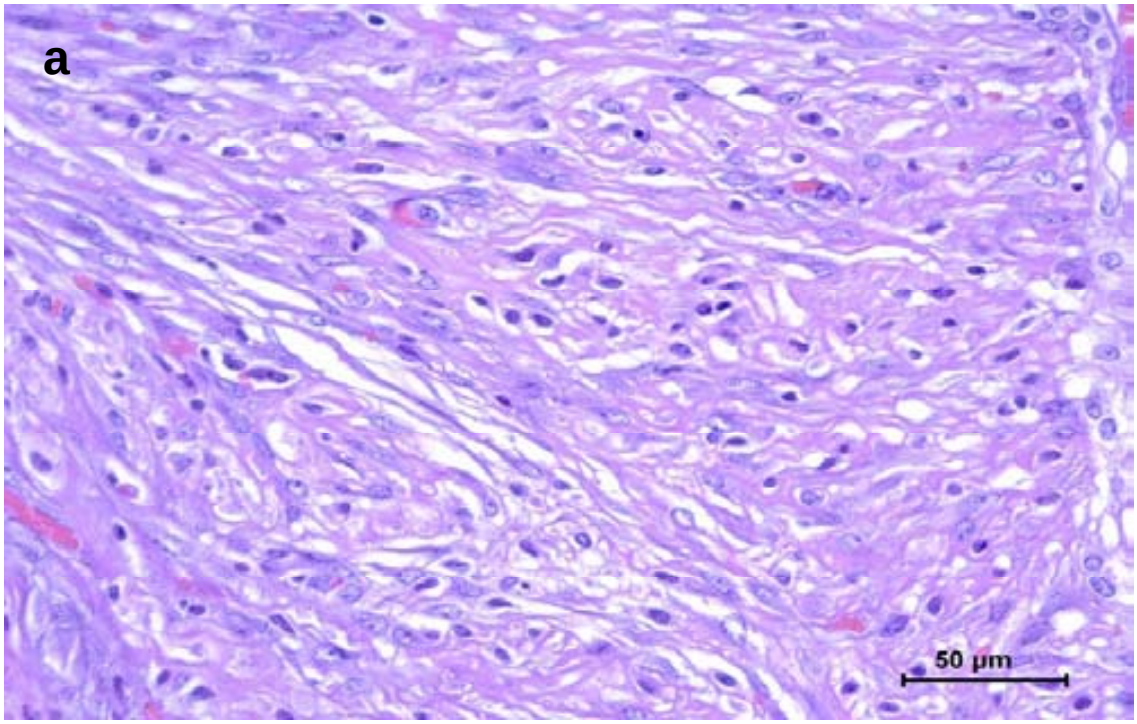


Figure 5.10.a. HE. PRP - 7 days.

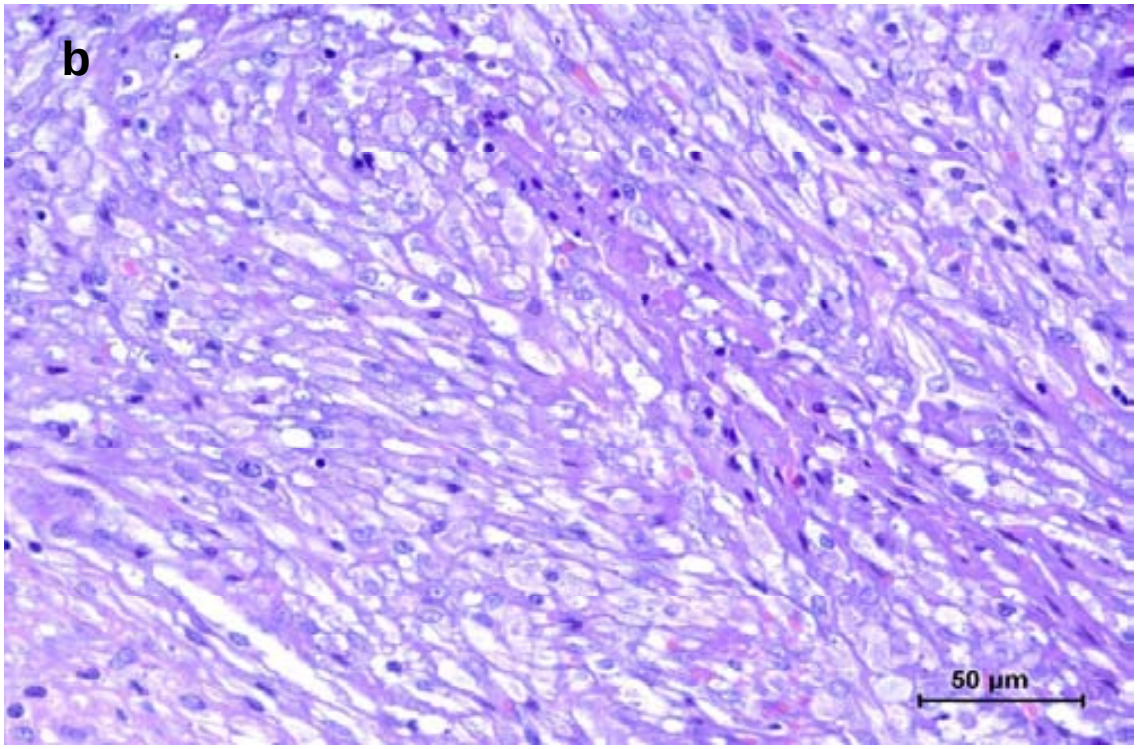


Figure 5.10.b. HE. Control - 7 days.

On Masson's trichrome stain sections at high magnification, at 7 days both, PRP (Fig. 5.11.a) and control (Fig. 5.11.b) samples showed a pattern of collagen deposition.

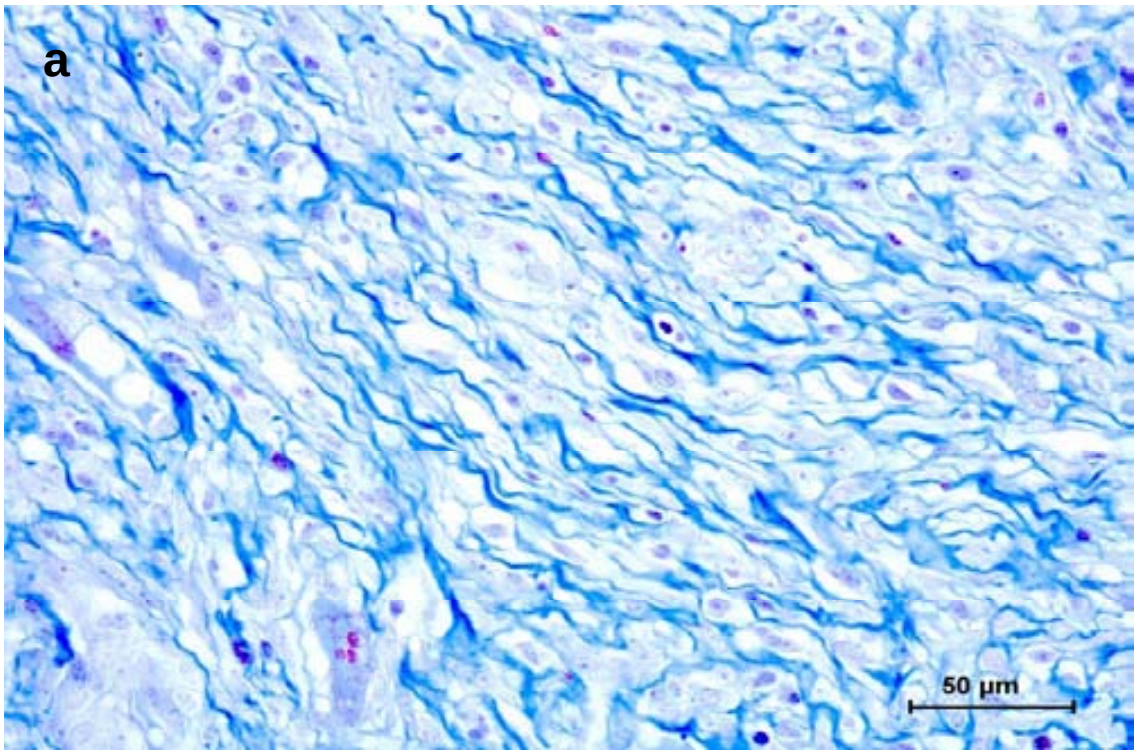


Figure 5.11.a. Masson's trichrome. PRP - 7 days.

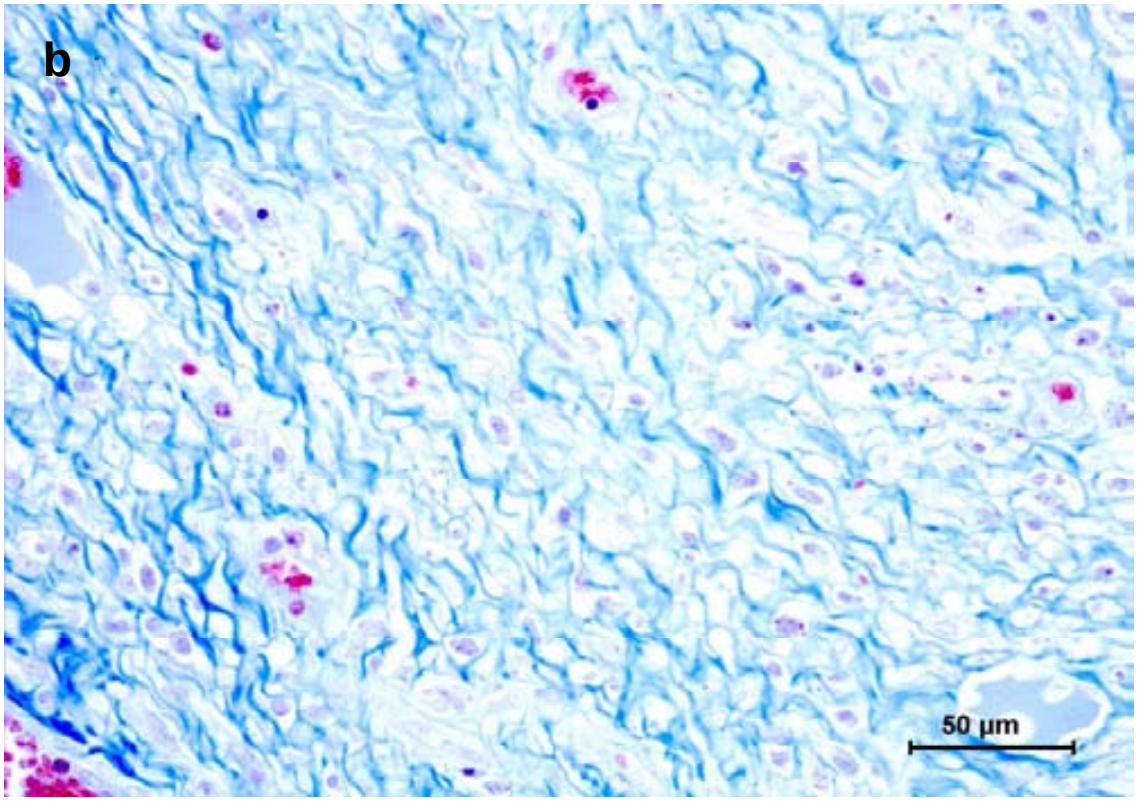


Figure 5.11.b. Masson's trichrome. Control - 7 days.

5.3.3. Determination of the breaking strength

Trials on breaking strength of unwounded intestine using the Instron Tensile Machine 1122 yielded satisfactory results (data not shown). Based on this, the dynamometer was employed for definitive purposes.
The results of anastomotic breaking strength at 72 hours, 7 days and those of the unwounded intestine are represented on table 5.12. The lowest values were obtained in control samples, while the highest were observed in PRP samples. Differences in breaking strength between control and PRP samples at 72 hours and 7 days were not significant. Breaking strength values of control and PRP samples never reached those of the unwounded tissue with statistically significant differences ($P<0.05$).

	Mean ± SD (kgf)	Range (kgf)
Control 72 hours	1,21± 0,27	0,64 - 1,65
PRP 72 hours	1,34± 0,22	1,02 - 1,83
Control 7 days	1,10± 0,27	0,56 - 1,56
PRP 7 days	1,08± 0,41	0,44 - 1,83
Unwounded sample	1,89±0,18	1,57 - 2,15

Table 5.12 - Mean, SD and range values of breaking strength of anastomotic wound on PRP and control samples at 72 hours, 7 days and unwounded tissue.

Tearing of the tissue breaking site occurred within the anastomoses (Fig 5.12.a), except for 2/10 PRP samples at 72 hours, and 2/10 PRP samples and 1/10 control samples at 7 days, where tearing occurred outside the anastomotic site (Fig 5.12.b).

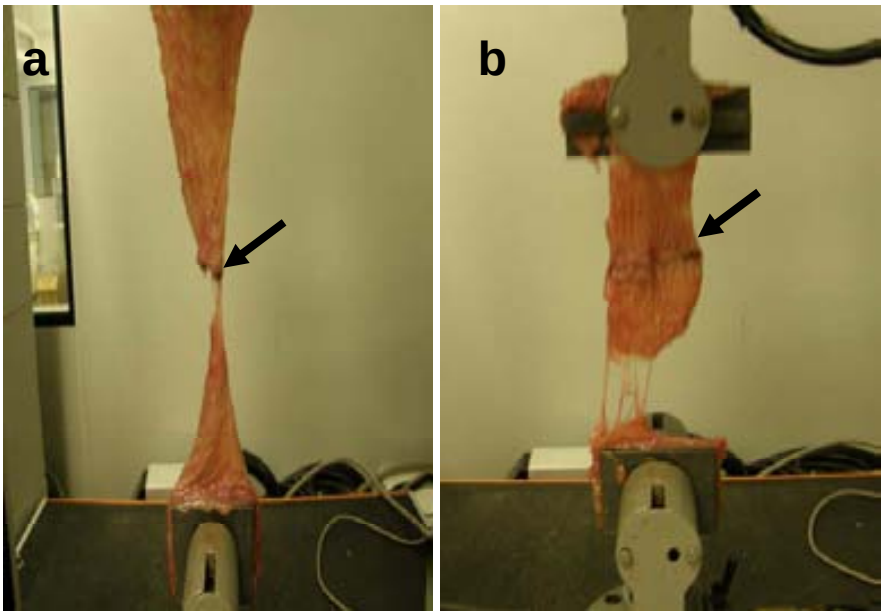
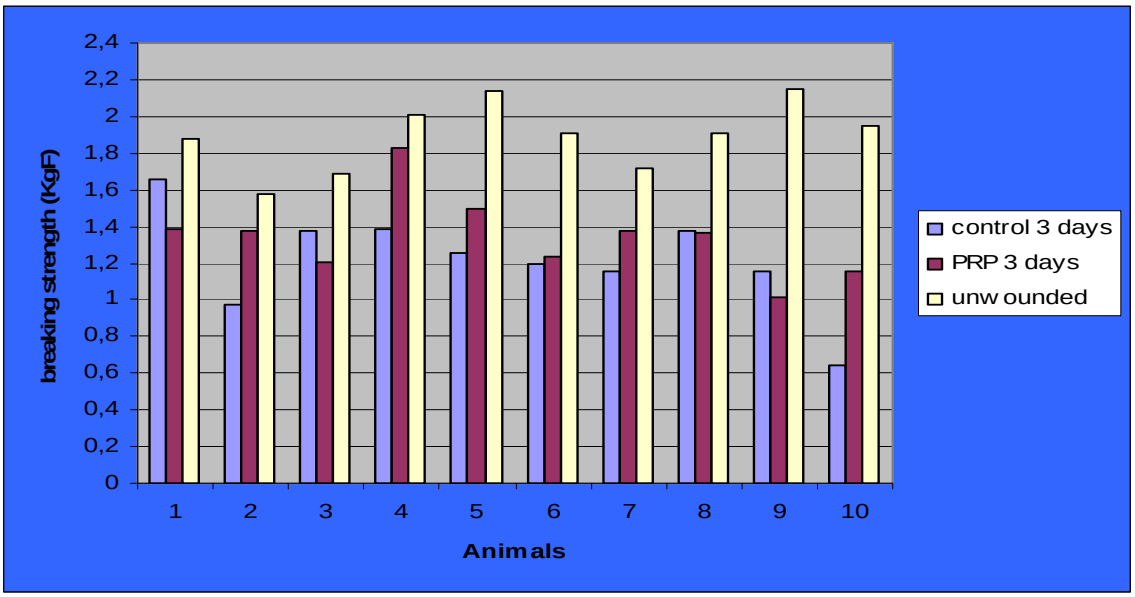


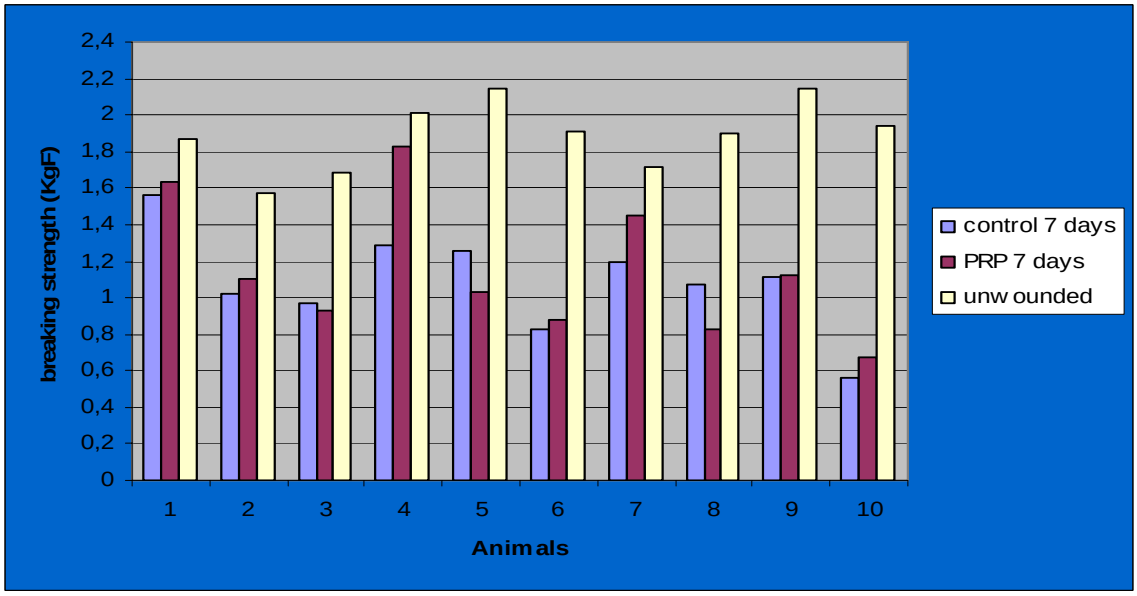
Figure 5.12 - Tearing of the tissue breaking site at 72 hours and 7 days (black arrows show the anastomotic line).

At 72 hours (3 days), the lowest values of the breaking strength were obtained in control samples, while the highest were observed in PRP samples. However, breaking strength values of control and PRP samples never reached those of the unwounded tissue (Graphic 1).



Graphic 1- Breaking strength on control, PRP, and unwounded samples at 72 hours.

At day 7, the lowest values of breaking strength were obtained in control samples, while the highest were observed in PRP samples. However, breaking strength values of control and PRP samples never reached those of the unwounded tissue (Graphic 2).



Graphic 2- Breaking strength in control, PRP and unwounded samples at 7 days.

6. Discussion

Nowadays strategies to accelerate wound healing have recently included the use of PRP. PRP is a highly concentrated form of autogenous platelets, providing a rich and readily obtainable source of diverse growth factors. The pig is being increasingly used in research projects that require new surgical techniques for the experiments to be performed, including the use of PRP on hard and soft tissue (Henderson *et al.,* 2003; Akeda *et al.,* 2006; Murray *et al.,* 2007; Nordentoft *et al.,* 2007). Porcine has been widely used as a biomedical model for humans (McKenzie, 1996). This is because the physiology and metabolism of most organ systems are very similar to man. Besides its broad similarities to humans, the size of the pig, unlike that of rabbits and rats, allows the use of clinically routine monitoring techniques and equipment and the frequent collection of blood samples. These common characteristics made swine blood an appropriate model for the initial development of PRP obtention. However, even similitude on blood composition between pig and man, the human tube centrifugation protocol (460g/8 min) described by Anitua (1999) was not applicable for pigs platelets. So in this study, we performed new PRP protocol appropriate for pigs.

At the beginning, preliminary studies were performed to find a best technique to concentrate platelets on PRP. This initial study included the amount of blood collected the centrifugal forces which were decided to perform the double spin centrifugation, and the final volume of the obtained PRP. These parameters were optimized for each bioassay in order to obtain effective concentration of around 1000 x 10^3 platelets on PRP and around 3-fold above baseline as postulated by Marx *et al.* (1998).

In this study double spin centrifugation was performed. Early, Marx *et al.* (2001) stated that a double-centrifugation technique is necessary to truly concentrate platelets from autologous blood. The first spin separates the red blood cells from the plasma, which contains the platelets, white blood cells, and clotting factors. The second spin finally separates the platelets and white blood cells, along with a few red blood cells, from the plasma. This "soft spin" produces a PRP free from the obstruction due to a large number of red blood cells. Marx *et al.* (2001) stated that obtaining PRP with a single spin technique would not produce a true PRP, but rather a PRP - PPP mixture with a disappointingly low platelet count. Different techniques of preparation have been known to yield substantially different amounts of cells, i.e., platelets and leukocytes, as well as different levels of growth factors (Zimmermann *et al.*, 2001; Weibich and Kleis, 2002; Weibrich *et al.*, 2003; Wiltfang *et al.*, 2004).

While evaluating arterial blood and PRP cell counts, blood parameters that are specific for pigs were taken into consideration. The results obtained from those all large white pigs provided important hematologic and serum biochemistry reference intervals. Although age and gender can affect reference interval results (Kaneko *et al.*, 1997; Thorn, 2000), in this study all sample results from both male and female pigs were combined in order to acquire a number of animals enough for adequate statistical evaluations. In our study, differences observed between any hematologic parameters were not statistically significant, when comparing male and female large white pigs, so that is why we used both gender. Hematological and serum biochemistry values obtained here were similar to those reported for domestic pigs (Kaneko *et al.*, 1997; Thorn *et al.*, 2000).

Numerous alterations in erythrocyte morphologic features were reported in routine hematologic preparations in the pigs, especially in younger pigs. These alterations include

Howell-Jolly bodies, polychromasia, poikilocytosis, crenation, and rouleaux formation. Although anisocytosis is also seen in adults, is more prominent in young pigs (Thorn, 2000). In our study, crenation was evident on whole blood smear.

In the present experiment pigs showed a hematocrit of 30 ± 2.8% (mean±SD) and an initial platelet count of 420 ± 105 x 10^3 cells/μL (range: 292-814 x 10^3cells/μL), which is slightly higher than that among humans. The amount of citrated whole blood was about 60 mL and yielded a harvest of 6 mL of PRP; this volume is 10% of the whole blood volume drawn from each pig, as proposed by Marx *et al.* (2000). In the PRP samples, the platelet concentration was significantly higher than that of native blood ($p<0.05$), being the mean platelet count in PRP of 1620,6 x 10^3cells/μL. This concentration is comparable to the effective concentration of 1000 x 10^3 reported in the literature (Weibrich *et al.,* 2004).

With PRP tube method used in this study, concentration of 383% (3,8-fold) mean and range of 250 to 550% (2,5 to 5,5-fold) were obtained. These results are compared with the pig values reported for Curasan system 410% (4,1-fold) and buffy coat system 390% (3,9-fold) (Schlegel *et al.,* 2004; Wiltfang *et al.,* 2004; Thorwarth *et al.,* 2005; Akeda *et al.,* 2006). The average increase in platelet increase above baseline was 280% (2,8-fold) mean and range of 150 to 450% (1,5 to 4,5-fold).

The number of white blood cells on whole blood was 15,33 ± 3,45 x 10^3 cells/μL (range: 9,02 to 23,63 x10^3 cells/μL). In the PRP samples, the number of white blood cells was significantly lower than that of citrated whole blood ($p<0.05$), the mean count of white blood cells was 1,83 cells x10^3/μL (range: 0,37 to 4,29 cells x 10^3 cells/μL). This values are lower than those reported by other authors (Li *et al.,* 2004; Thorwarth *et al.,* 2005) on pig model using Symphony II (29,5 x10^3 cells/μL); PCCS (14,7±68 x 10^3 cells/μL), and Curasan (24,8±8,9 x 10^3 cells/μL).

As occurs with Anitua's tube technique (Weibrich *et al.,* 2005), in the technique described here a very low quantity of leukocytes are concentrated in comparison with the buffy coat and tube methods (pig and human). The real role of the leukocytes present in human and pig PRP prepared for wound healing has never been defined very well, and their true effect is completely unknown because there is lack of reliable research data (Weibrich *et al.,* 2005).

To evaluate the double spin centrifugation method to prepare PRP we also calculated an enrichment percentage which was 38,4 ±6,1% (range: 25,4 to 54,8%). This values are within the range reported in human PRP concentrate systems 19,0±16,6% (laboratory system), 41,9±9,7% (Curasan system) and 49,6±21,0% (PCCS) (Appel *et al.,* 2002), and 30-39% using the manual systems for platelet concentrate (Marx *et al.,* 2001). This parameter confirms the ability of the method to collect platelets within a certain volume of plasma.

Generally, PRP clot are formed by mixing autologous PRP and bovine thrombin dissolved in calcium chloride solution. Those substances are added to PRP to initiate fibrin polymerization and to release platelet growth factors and cytokines, depending on the surgical requirements (Landsberg *et al.,* 2000). But for some purposes only calcium chloride is used (Anitua, 1999; Dugrillon *et al.,* 2002). In this study, to produce a PRP clot sterile 10% calcium chloride ($CaCl_2$) solution was added. For this purpose Anitua's (1999) procedure was used (50 μL per 1mL of PRP, and the time of forming PRP clot was approximately 4 minutes and ranged between 3-5 minutes).

The PRP obtention tecnhique used in this study was analyzed not only for cellular content but also for platelet activation during PRP procedure, and content of the growth factors, especially TGF-β1.

In our study, the viability and structural changes of the pig platelets were investigated by assessing the platelet parameter supplied by a flow cytometry hematology system, such as MPC. It is known that this parameter, are correlated with platelet activation in human beings (Macey *et al.,* 1999) dogs (Stanworth *et al.,* 1999) and cats (Zelmanovic and Hetherington, 1998). We monitored MPC values on citrated whole blood and on PRP (a decrease in the MPC value occurs when platelets are activated and degranulated *in vitro*). The MPC values were not statistically significantly different ($p<0.05$) on citrated whole blood 21,3±1,58 g/dL (range:19,2-24,8 g/dL) when compared to PRP 20,3±1,39 g/dL (range:18,1-24,2 g/dL) values. In our opinion, this finding might be the result of the non activation of platelets during PRP preparation process.

TGF-β1 is produced by a variety of cells including platelets that are normally recruited to an injury site. TGF-β1 regulates cellular differentiation, proliferation, chemotaxis, and synthesis of many ECM components (Roberts *et al*., 1988; Kane *et al*., 1991). *In vivo* studies have confirmed that exogenous TGF-β1 increases granulation tissue, collagen formation, and wound tensile strength when applied locally in animal models (Ashcroft et al., 1999). Because it was found to be pivotal on intestinal wound healing (Ciacci *et al*., 1993; Dignass *et al*., 1993; Beck *et al*., 2003), we analyzed the amount of TGF-β1 by enzyme-linked immunosorbent sandwich assay (ELISA) on PRP (obtained after double spin centrifugation), PPP (supernatant after second centrifugation) and PRP gel (obtained by mixing fresh PRP and calcium chloride).

In our study, concentrations of TGF-β1 on PRP fractions were different. The concentration of TGF-β1 on PRP (24312 pg/mL) was significantly increased compared to PPP (3715 pg/mL). The concentration of TGF-β1 was 6,5-fold increased on PRP compared to PPP. This concentration is higher than that obtained by Akeda *et al.* (2006) on mini pigs using the Symphony II Platelet Concentration System (DePuy Spine, Raynham, MA) in which they obtained TGF-β1 concentrations two-fold higher than PPP values.

It is important to point out that if platelets become activated by the preparation before a final centrifugation step, growth factors may be lost with a discarded supernatant finding high concentrations of growth factors in the PPP while low concentrations in the activated PRP. In our study, the concentration of TGF-β1 on supernatant (PPP) was low, and after a freeze/thawing activation, concentration of TGF-β1 were high. This supposes that the centrifugation method used in this study does not activate the platelets to release their growth factors which are also in accordance with the results obtained by the MPC values.

As freezing and thawing activates platelets (Sekido *et al.,* 1987; Weibrich *et al.,* 2003; Zimmerman *et al.,* 2003), the TGF-β1 levels in calcium chloride activated PRP (gel) are expected to be the same or higher than those in frozen and thawed PRP. However, on this study, the concentration of TGF-β1 was significantly increased on frozen and thawed PRP (24312 pg/mL) than in calcium activated PRP (15504 pg/mL). Two possible hypotheses could explain the results obtained. On one hand, during platelet activation by calcium chloride, a clot or gel formation occurs and released growth factors are recruited on this gel, resulting in a few growth factors left into the surrounding liquid which will be analysed (Tsay *et al.,* 2005). On the other hand, because growth factors are not stable in plasma,

growth factors released by calcium chloride activation and then frozen and thawed may be degraded by this overall process and thus not detected.

Nevertheless, we obtained higher TGF-β1 levels on PRP (24312±21755 pg/mL) and in PRP gel (15504±11279 pg/mL). *In vitro* concentrations of 5000 pg/mL TGF-β1 are known to induce chondrocyte proliferation and an increase of cartilage ECM synthesis (Fortier *et al.*, 1997; Carmona *et al.,* 2007). Perhaps supraphysiologic concentrations of TGF-β1 can be found in these PRP, and could control catabolic and inflammatory processes occurring during intestinal wound healing.

TGF-β1 has been found, *in vitro*, to exert potent effects on the intestinal epithelium, which appear to stimulate production of extracellular matrix components. Furthermore, TGF-β1 has potent effects on many other cell populations present in the intestinal mucosa that could dominate the overall outcome of mucosal injury including lymphocytes, macrophages, fibroblasts, neutrophils. TGF-β1 has also been proposed to play a key role in regulating intestinal epithelial cell migration and wound repair (Ciacci *et al.*, 1993; Dignass *et al.*, 1993; Beck *et al.*, 2003).

Despite ongoing improvements in surgical technique, anastomotic leakage remains a relatively frequent complication of gastrointestinal surgery (Pickleman *et al.*, 1999). This complication is much feared, because it has a profound negative impact on clinical outcome as shown by the significant increase in morbidity and in mortality. Experimental studies have shown that in the intestine the immediate postoperative anastomotic strength is low and decreases even further during the early postoperative period (Hogstrom and Haglund, 1985; Hendriks and Mastboom, 1990; Siemonsma *et al.*, 2003). After the third day, when the lowest levels are encountered, wound strength increases rapidly. This postoperative pattern occurs even when the anastomosis is constructed under ideal circumstances and has been attributed to physiologic changes in matrix metabolism during this period.

In the immediate postoperative period, wound strength is mainly determined by the suture holding capacity of the submucosa, in which collagen is the major structural protein. After a few days, newly formed matrix deposited in the anastomotic area will restore tissue strength. The capacity to synthesize collagen is increased in anastomotic tissue soon after operation (Martens and Hendriks, 1991). However, matrix degradation, e.g., to remove damaged tissue and to permit vessel formation, also is an intrinsic feature of wound repair. In the intestine, localized and limited degradation of the existing submucosal matrix may lead to transient loss of anastomotic strength during the first postoperative days. Therefore, it seems reasonable to promote intestinal healing to reduce the dehiscence-risk in this period as much as possible.

Nowadays basic clinical research on gastro-intestinal anastomosis impaired wound healing has focused on the application of growth factors. Platelet concentrates such as platelet-rich plasma (PRP) have been used in a variety of clinical applications based on the premise that higher growth factor content should promote better healing (Fréchette *et al.,* 2005). Autologous platelets as a source of healing factors have been shown to enhance bone regeneration and soft tissue maturation in the fields of orthopedics, periodontal and maxillofacial surgery, urology and plastic surgery (Marx *et al.*, 1998; Anitua, 1999; Anitua *et al.,* 2005; Fréchette *et al.,* 2005; Murray *et al.,* 2007).

The use of PRP is based on the premise that the large number of platelets in PRP releases significant quantities of growth factors. However, the amount of basic research that endorses PRP's ability to promote intestinal healing is limited. To our knowledge only one study is available in colonic rats with promising results (Yol *et al.*, 2008). In a review of the current literature, we found few animal studies that substantiate PRP's ability in this respect. The present experimental study was performed to investigate the ability of PRP to enhance intestinal (end-to-end anastomosis) wound healing in pigs. The study compared PRP treated with untreated anastomosis, using as main outcome measures: the operative findings, the mechanical strength and the histological findings.

Controversies exist in the literature regarding the added benefit of PRP application. Variations in some key properties of the PRPs, including the platelet concentration, the type of clot activator, the leukocyte content and the time that the fibrin scaffold is put into place around the tissue after clotting has started, can markedly influence the different biological effects (Anitua *et al.,* 2006).

PRP works via the degranulation of the α-granules in platelets after their activation; these granules contain the synthesized and prepackage growth factors. PRP can be obtained by either manual or automated methods, and for both approaches, PRP must be calcium-activated before being applied over the surgical site (Tamimi *et al.,* 2007). Addition of calcium chloride as a clot activator promotes the formation of native thrombin, mimicking the physiological clotting process and enabling a more sustained release of growth factors, which might be crucial to proper tissue repair and wound healing (Anitua *et al.,* 2006). It is important that the platelet gel applied *in vivo* can deliver viable growth factors. This ability decreases when PRP becomes activated *in vitro* before the platelet gel is delivered to tissues (Everts *et al.,* 2006). The active secretion of these growth factors is initiated by the clotting process of blood and begins within 10 minutes after clotting. More than 95% of the presynthesized growth factors are secreted within 1 hour. Therefore, PRP must be developed in an anticoagulated state and should be used on the graft, flap, or wound within 10 min of clot initiation (Marx, 2004).

On this experiment we performed preliminary studies to identify the more appropriate technique to apply a PRP gel on the anastomosis. For these purpose 6 animals divided in 2 groups of 3 animals each, were used for surgery and "sandwich technique" of PRP application was performed. Another six pigs, divided into the same manner, were treated in a similar fashion, but PRP gel application technique was modified, as an "immersion technique" was performed.
However, the "sandwich technique" used for PRP application on the anastomosis was unfortunately not good enough because the platelet gel served as a barrier between both intestinal edges, consequently making difficult the contact between both edges and wound healing. After these unsatisfactory results, we used an "immersion technique" for definitive purposes.

In this technique the PRP gel was applied before end-to-end anastomosis was performed, the intestinal edges were immerged immediately into calcium choride activated PRP and maintained in place for at least 10 minutes in order to ensure the intestinal contact with the initial burst of PRP-related growth factors. After this initial burst, the platelets synthesize and secrete additional growth factors for the remaining 7 days of their life span (Marx, 2004). Platelet damaged or nonviable during the PRP processing will not secrete bioactive growth factors and this may result in disappointing outcomes (Marx, 2004).

Our procedure, despite the advantage of ensuring the initial burst of growth factors release, may be associated with drawbacks, such as PRP-gel contamination with blood and/or intestinal contents during immersion of the intestinal edges. Although intestinal content contamination did not happen in any of the enterectomies performed, blood contamination from the intestinal edges is less controllable. Blood contamination could dilute growth factors in activated PRP, thus decreasing its clinical efficacy (Jensen *et al.*, 2004).

In general, application of PRP in slow rate healing tissues or low healing capability tissues, such as the musculoskeletal tissues, induces cell proliferation and promotes the synthesis of angiogenic factors, thus enhancing the healing process.

Indeed, some studies in oral and maxillofacial surgery (Marx *et al.,* 1998) suggested that PRP addition accelerated the rate and the degree of bone formation in a bone graft through at least the first 6 months. It has also been reported that PRP application accelerates microcirculatory angiogenesis after surgical injury, which subsequently leads to soft tissue repair (Lindeboom *et al.,* 2007). Intestinal healing, however, is *per se* a very rapid mechanism, and probably any enhancement in the healing process by the PRP addition is less noticeable. The intestinal epithelium is a highly specialized cell population undergoing continuous rapid turnover. In generally, when injury occurs, rapid restoration of the continuity of the epithelial barrier and ultimately normal mucosal architecture is essential (Beck *et al.,* 2003).

Intestinal healing is characterized by 3 overlapping phases of healing named the lag phase, the proliferative phase, and the maturation phase. The lag phase occurs during days 0-4 and is associated with inflammation and edema of the healing intestine, and formation of a fibrin seal. Although the fibrin clot contributes to wound strength, during this phase most of the wound strength is attributed to sutures. During the lag phase, macrophages are important in wound debridement and the production of growth factors that modulate fibroplasia and angiogenesis (TGF-β, PDGF, EGF, cytokines) (Hedlund, 2002).

The third and fourth days after an intestinal anastomosis, the anastomotic strength is at his lowest because of fibrinolysis and collagen deposition, and therefore dehiscence most commonly occurs during this period (Hendriks and Mastboom, 1990; Hedlund, 2002; Siemonsma *et al.,* 2003; Posma *et al.,* 2007). The proliferative phase occurs between days 3 and 14. Fibrous repair occurs accompanied by a rapid gain in wound strength. The strength of the repair site approximates that of intestine 10 and 17 days after surgery. The maturation phase occurs between 10 and 180 days. Collagen is reorganized and remodelled during this phase (Hedlund, 2002). The first postoperative period after construction of an intestinal anastomosis is characterized by transiently lowered wound strength, even if the operation is performed under optimal conditions (Siemonsma *et al.,* 2003). The frequency of this transiently lowered wound strength, increases if surgery has to be performed in the presence of adverse conditions to wound repair.

During the lag phase, after PRP application in healthy intestinal anastomosis, increased inflammation and presence of granulation tissue has been observed, showing a higher level of angiogenesis and more fibroblasts than in the control anastomosis. So, application of PRP on the compromised intestinal healing could be beneficial in this earlier phase.
At early stages of the proliferative phase more mature granulation tissue is observed, but inflammatory reaction is not superior to the control sample. This is in agreement with Yol *et al.* (2008), where less inflammation was observed after 7 days of PRP application as a film

layer to the anastomotic line. However, in the latter study, the histopathological examination and bursting pressure was only evaluated on postoperative day 7, and effects of PRP were not determined during the lag phase.

The increased granulation tissue present in PRP samples at 72 hours may lead to a higher fibrous tissue formation and thus to a major gain in wound strength. These facts may reflect a higher anastomotic resistance in PRP samples compared to control samples during this phase. Indeed, these results were in accordance with the results in breaking strength. Although differences were not significant and dehiscence most commonly occurs 3-4 days after intestinal surgery, breaking strength have been found to be highest at 72 hours after PRP application. At 7 days breaking strength measurements demonstrated that, rupture occurred primarily within anastomosis line in the control groups whereas on 2/10 tested samples occurred outside the wound area in PRP treated animals which indicates that at this point, the anastomosis had grown stronger than the adjacent bowel wall. In this case the highest values were observed on treated samples but statistically the differences were not significant.

Thus, although differences were not significant, PRP application on intestinal anastomosis seemed to increase the wound strength. This is in accordance to the study reported by Yol *et al.* (2008) in rats, where topical PRP gel applied to the anastomotic line as a film layer obtained a better anastomotic strength than the group subjected to colonic anastomosis only. However, in contrast to the latter study, the higher anastomotic strength on PRP samples observed in the present study is speculated to be caused by the increased granulation tissue and fibrosis caused by the PRP application.

The findings of the present study are based on macroscopic and microscopic analysis of tissue sections. This approach enables a more accurate study of the various cell types and extracellular matrix components involved in the repair process as it has previously proposed by Verhofstand *et al.* (2001). We used the classic haematoxylin-eosin stain to evaluate the overall architectural completion of the anastomosis (healing of intestinal layers and thickness of granulation tissue formed), and Masson's trichrome stain to visualize collagen fiber organization in all tissue sections at 3 and 7 days.

Despite we did not evaluate growth factor content neither metalloprotease matrix activity, an increase on thickness of the intestinal wall with high presence of granulation tissue were observed on PRP treated animals on 72 hours, 96 hours and 7 days. This result is in agreement with Yol *et al.* (2008), who considered that growth factors in PRP are responsible for better anastomotic strength and wound healing. However, they did not evaluate the inflammatory reaction and granulation tissue during the first days of intestinal healing (lag phase), so we do not compare the initial inflammatory reaction caused by PRP application observed in our study with Yol *et al.* (2008)'s investigation.

An anastomosis should be able to withstand both intraluminal and longitudinal forces caused by movements of the intestine (Posma *et al.,* 2007).
Anastomotic bursting pressure and breaking strength are valid criteria for assessing anastomotic strength and integrity during its healing (Rabau *et al.,* 1998). Breaking strength measures the resistance of the intestinal wall to longitudinal forces and evaluates the biological aspect of the healing process; while bursting pressure determines the resistance of the intestinal wall to intraluminal pressure and can evaluate the overall anastomotic integrity (Hendriks and Mastboom, 1990; Rabau *et al.,* 1998; Ikeuchi *et al.,* 1999). However, Ikeuchi *et al.* (1999) reported that the MITS (minimal tensile strength) appears to be a

better standard to evaluate the biological aspect of anastomotic healing including suture-holding capacity. In the present study, measurements performed by the Instron machine approached the minimal tensile strength.

Several studies have been performed on improvement of intestinal healing based on the factors suspected of influencing anastomotic healing (Yagci *et al.,* 2006). Although the effects of growth hormone and growth factors applied systemically and topically on healing of bowel anastomosis are currently being evaluated (Christensen *et al.,* 1991; Christensen *et al.,* 1993; Christensen *et al.,* 1995; Gomez de-Segura *et al.,* 1995; Egger *et al.,* 1998; Dinc *et al.,* 2002), the effects of PRP application have been poorly documented (Yol *et al.,* 2008). The present investigation evaluates the effects of PRP on intestinal healing during the lag phase. However, it is a short-term study that does not address long-term effects or complications. Additionally, further investigations on different PRP application techniques on intestinal anastomosis (rather than the immersion technique) as well as evaluations at longer time intervals are required.

Although differences were subtle, PRP application on intestinal anastomosis seemed to slightly improve the anastomotic resistance due to an increase in inflammatory infiltrates and fibrosis. PRP use on intestine does not appear to have obvious disadvantages. Whether PRP application in intestinal anastomosis can reduce the frequency of anastomotic leakage needs to be evaluated in future studies.

7. Conclusions

From the results obtained in this experimental study we may conclude:

1. The protocol for PRP obtention using a double spin centrifugation procedure employed in this study results in a high platelet concentrate and maintains a high quality biological product as measured by MPC values and TGF-β1 levels.

2. The anti-human TGF-β1 antibodies for TGF-β1 detection by enzyme linked immunosorbent assay (ELISA) have been proved to be suitable for the detection of porcine TGF-β1.

3. The immersion technique for the application of PRP in the intestine seems to be easy to perform and may guarantee the initial burst of growth factors.

4. The dynamometer Instron 1122 seems useful as a method for breaking strength evaluation during the lag phase as it better measures the suture-holding capacity.

5. Microscopic study revealed more inflammatory infiltrate and tissue granulation formation in PRP samples.

6. Although differences were not significant, PRP samples showed a higher breaking strength values in agreement to microscopic findings, especially in the lag phase.

7. At 7 days the breaking strength values, neither of PRP or control samples, never reached those of the unwounded intestine.

8. The slightly improvement of intestinal resistance by PRP gel application on healthy intestinal anastomoses does not seem to compensate for the technical and surgical effort required for PRP obtention and application. However, future studies in compromised intestine healing will be required.

8. References

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