Acute and chronic reactive peritonitis in peritoneal dialysis: neurogenic inflammation and citrate treatment

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ABSTRACT

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The prevalent problems associated with peritoneal dialysis (PD) are ultrafiltration failure and peritonitis. During PD the patient is sustained on a state of intraperitoneal inflammation, which over time impairs structure, and function of the peritoneal membrane, leading to loss of ultrafiltration efficacy. The aims of this project was: to establish whether neurogenic inflammation and mast cell activation are triggered by PD fluid exposure and to evaluate the effects of citrate as an additive to PD fluid in acute and chronic animal models.

The studies were conducted in rats, exposed to filter sterilised lactate or lactate/citrate buffered PD fluid with glucose (2.5 and 3.9 %) as osmotic agent through an implanted catheter. Acute studies were based on single exposure and long-term studies on daily exposures for a period of 5 weeks. Pharmacological intervention was used to study mast cell activation and the neurogenic inflammatory response.

Histamine was released into the peritoneal cavity within 30 minutes of infusion of standard PD fluid. Also osmotically neutral fluid triggered a histamine release from mast cells. Indirect evidence for the release of neuropeptides SP and CGRP suggested actions of a neurogenic inflammation. Mast cell activation was shown to be dependent on substance P stimulation of its receptor, NK-1. Inhibiting NK-1 significantly reduced vascular albumin loss from the blood to the peritoneal cavity by a mast cell independent mechanism. Blocking CGRP resulted in a significant increase in osmotic and net ultrafiltration. The classic trigger of neuropeptide release, the TRPV1 receptor was, unexpectedly, not responsible for neuropeptide actions in the present model.

Substituting 5-15 mM lactate with equal amounts of citrate gradually improved osmotic ultrafiltration (fluid transport) compared with lactate PD fluid, suggesting a dose-response relationship. Significantly improved net ultrafiltration (fluid gain) was the result of increased osmotic ultrafiltration, in response to 10 - 15 mM citrate substitution.

Long-term treatment with citrate-substituted PD fluid in rats did not significantly reduce fibrosis and angiogenesis of the peritoneal membrane compared with standard PD fluid. PD catheter patency was, however, significantly improved in animals treated with citrate substituted PD fluid. Macroscopic signs of fibrosis were also significantly reduced by citrate.

The clinical implications are that pharmacological intervention with the neurogenic inflammatory response and calcium chelation with citrate have potential to improve the efficiency of peritoneal dialysis.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to according to their roman numerals.

I. **Cavallini N, Wieslander A, Braide M.**
Substituting citrate for lactate in peritoneal dialysis fluid improves ultrafiltration in rats. *Perit Dial Int*. 2009 Jan-Feb; 29(1): 36-43.

II. **Cavallini N, Delbro D, Tobin G, Braide M.**
A neurogenic inflammatory response to PD exagerrates serum albumin loss and reduces ultrafiltration. 2009; to be submitted to *J Am Soc Nephrol*.

III. **Cavallini N, Braide M.**
Citrate substituted PD-fluid: effects on peritoneal integrity and catheter patency during 5 weeks of experimental PD in rats. 2009; submitted to *Perit Dial Int*

CONTENT

ABBREVIATIONS

APD	automated peritoneal dialysis
BSA	bovine serum albumin
CGRP	calcitonin gene related peptide
CAPD	continuous ambulatory peritoneal dialysis
C3	complement factor 3
C5	complement factor 5
CINC	cytokine induced neutrophil chemoattractant
DMSO	di-methyl-sulf-oxide
ECM	extracellular matrix
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assat
GDP	glucose degradation product
GM-CSF	granulocyte-monocyte colony-stimulating factor
ICAM	intracellular adhesion molecule
IgE	immunoglobulin E
IL	interleukin
MCP	monocytes chemoattractant peptide
MIP	macrophage inflammatory protein
NK-1	neurokinin 1
NKA	neurokinin A
NO	nitric oxide
NOS	nitrix oxide synthase
PAI-1	plasminogen activator inhibitor-1

PAF	platelet-activating factor
PAR	protease-activated receptors
PBS	phosphate buffered saline
PD	peritoneal dialysis
SP	substance P
TAT	thrombin-antithrombin complex
TF	tissue factor
TGF	transforming growth factor
TNFα	tumour necrosis factor alpha
TRPC	transient receptor potential cation channel
TRPV	transient receptor potential vanilloid
VACC	voltage activated calcium channels
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

INTRODUCTION

Peritoneal dialysis

Introduction to peritoneal dialysis

Peritoneal dialysis (PD), treatment for patients with uraemia resulting from kidney failure, is subdivided into continuous ambulatory dialysis (CAPD) and automated peritoneal dialysis (APD). PD is performed by repeatedly filling and draining the peritoneal cavity with a hypertonic PD fluid via a permanent catheter surgically implanted through the abdominal wall into the cavity. Depending on the method, CAPD or APD, the dwell varies in length. CAPD has 4-6 hours of dwell time, whilst patients on APD undergo several short dwells; a process aided by a machine and that takes in total 8-10 hours per cycle. PD is dependent on the peritoneum, a thin serous membrane with qualities similar to the artificial membrane used during haemodialysis, for the removal of water and waste products, such as urea, and creatinine.

PD was first used 1923 by Georg Ganter, but then it was far from being an accepted and commercially available treatment. During 1924-1938, numerous medical units in the U.S. and Germany made use of, and proved the method to be a passable short-term replacement for the kidneys. The main obstacle in these years was the lack of a safe and sterile method of accessing the patients' abdominal cavity, which is now done via a soft and pliable catheter. Henry Tenckhoff developed 1968 the first permanent catheter, which is still in use today. The development of a permanent catheter and a continuous use of PD brought another problem to attention: peritonitis. Up until 1978 PD fluids were stored and sterilised in glass containers, from which the patients filled the cavity. The high number of connections and disconnections to the same glass container involved a high risk of acquiring peritonitis. The development of disposable bags by Dimitrios Oreopoulos, and comprehensive calculations on dwell times and fluid quantities by Robert Popovich and Jack Moncrief signalled the age of CAPD. Although PD treatment has several advantages over haemodialysis, the predominant problems remain, i.e. peritonitis and ultrafiltration failure (Topley, 1998).

Peritonitis, accounting for 15-35 % of the hospitalisations in PD patients, is the foremost cause of transfer to haemodialysis (Fried *et al.*, 1999). Although peritonitis is most often bacterial (S. epidermidis and S. aureus) (Cameron, 1995) in its origin, this is not always the case. With better methodology, the proportion peritonitis caused by gram-negative bacteria, and non-bacterial inflammation is growing. Inflammation, bacterial or non-bacterial, is the probable cause of a major drawback in PD. Shortened technique survival as a result of ultrafiltration failure of the peritoneal membrane is associated with fibrosis and angiogenesis of the peritoneal membrane. Another common complication, probably related to inflammation is a reduced outflow caused by e.g. fibrin-clotting of the PD catheter.

Function and structure of peritoneal membrane during PD

PD is dependent on semi-permeable qualities of the peritoneal membrane. The peritoneal membrane is composed of a monolayer of mesothelial cells lining a thin basement membrane mainly composed of laminin, and a layer of connective tissue. Embedded in an extracellular matrix of proteoglycan gel interspersed with collagen fibres is an extended network of capillaries, lymphatics, and resident cells (fibroblasts, macrophages, and mast cells). The capillary membrane provides most of the semi-permeable properties that determine the exchange between the circulation, and thus provides the site for physiological interaction and exchange between blood, resident cells of the peritoneum, and the PD fluid during dialysis (De Vriese *et al.*, 2000).

The purpose of the blood circulation is to transport nutrients and waste products to and from tissues. This requires transport of water and dissolved molecules in both directions through the capillary membrane. Under normal circumstances water transport through the capillary wall is in balance as a result of two opposing forces: hydrostatic and osmotic pressure. Hydrostatic fluid pressure is the net result (24 mm Hg) of the pressure exerted by the fluid in the interstitial (-6 mm Hg) space and the pressure caused by the fluid in the capillaries (18 mm Hg), resulting in an outward filtration of water. Osmotic pressure is a direct result of the capillaries semi-permeable properties and solute concentration differences over the capillary wall. Water and small solutes can diffuse freely through the capillary wall but larger substances, e.g. proteins are restricted by their size, therefore causing osmotic effects at the capillary membrane. Plasma, due to its high concentration of protein, has an osmotic pressure of 28 mm Hg compared to 4 mm Hg developed by the interstitial fluid. Increased or decreased hydrostatic pressure in either the capillaries or in the interstitium, and increased or decreased osmotic forces, disturbs the equilibrium (Guyton, 1979).

The capillary membrane is a smooth structure consisting of flat endothelial cells. In the junctions between cells there are two types of pores, small pores (radius: 4-5 nm) and large pores with a radius of 25 nm. Water and solutes, e.g. ions, urea, and glucose, with a radius smaller than 4-5 nm can pass by diffusion without restriction; larger molecules, e.g. proteins pass through large pores. Small pore area corresponds to 99.5 % of the total pore area, and large pores add 0.3 % of the total pore area; small and large pores contribute 90 and 5-8 % respectively of the water transport during normal circumstances. However, a third pore type, aquaporines, exists in the endothelial cell membranes. Aquaporines have very small radius (0.3 nm) and are thus selective for water but contribute only 2 % to the water transport (Rippe, 1993).

During PD, two litres of lactate buffered fluid with glucose as osmotic agent is inserted into the abdominal cavity, resulting in an increased osmotic pressure. In PD, the movement of water, from surrounding capillaries, into the peritoneal cavity is referred to as ultrafiltration.

Glucose has little osmotic effect on small pores. However, because aquaporines are selective water channels, glucose here achieves maximum osmotic effect. During a dwell, water will be redirected, and 40-50 % of the water will pass through aquaporines compared to 2 % in the normal situation (Rippe, 1993). The high concentration of

glucose in PD fluid maintains an osmotic ultrafiltration of water into the peritoneal cavity during the PD dwell. Water transport in the opposite direction is referred to as reabsorption and the difference between osmotic ultrafiltration and reabsorption is named net ultrafiltration and corresponds to the actual yield of fluid provided by the dialysis. Towards the end of the dwell, the combined osmotic and hydrostatic pressure gradient may favour reabsorption leading to a negative net ultrafiltration and thus a loss of fluid.

The peritoneal inflammatory response

Inflammation in general

The innate, non-specific, immune defence protects the body against foreign cells or substances without recognising specific identities. Inflammation is a part of the innate systems defence mechanisms and acts in response to local infections and trauma. The inflammatory process serves to eliminate, either by removal or inactivation, foreign matter, and initiate tissue repair.

The general sequence of events in a typical non-specific inflammatory response is similar, whether caused by an infection or a trauma, and includes: 1. vasodilation, 2. increased capillary permeability, 3. recruitment of inflammatory cells, 4. phagocytosis of foreign cell and bacteria by mainly neutrophils and macrophages, and 5. tissue repair. Those events are reflected by redness, heat swelling, and pain, which are normally referred to as the cardinal signs of inflammation.

In response to an inflammatory stimulus, resident cells of the peritoneal membrane (mast cells, macrophages) are activated, initiating a local inflammation. Mast cells and macrophages are located in the submesothelial connective tissue included in the peritoneal membrane, and also suspended in the fluid of the intraperitoneal space. These cells play an important role in the initiation of the peritoneal defence. Mesothelial cells lining the peritoneum are important in the defence and activation of a peritoneal inflammation. They are directly involved in the trafficking of leukocytes via the production of interleukins and adhesion molecules (Nagy, 1996). Also, the protease cascades, e.g. the coagulation and complement systems, dissolved in plasma and in the intraperitoneal fluid are important during the initial stages of an acute inflammation. Sensory neurons located in the vicinity of the small blood vessels are believed to initiate a neurogenic reaction during inflammation, normally referred to as neurogenic inflammation.

Within minutes after inflammation is triggered there is an increased vasodilation and vascular permeability (Goldsby *et al.*, 2003). Vasodilation increases local blood volume and blood flow, causing an increased blood perfusion and a leakage of plasma from the blood vessels. Changes to local capillaries are subsequent effects of activation of macrophages and mast cells, which release, e.g. bradykinin, histamine, interleukin (IL)-1, IL-8, and tumour necrosis factor alpha (TNFα). Activation of the complement system also takes place during the first minutes (Goldsby *et al.*, 2003). Further, vascular epithelial cells induce an increased production and expression of E- and P-selectins,

which are necessary for leukocyte attachment and migration. Thrombin and histamine induce increased expression of P-selectin. Cytokines such as IL-1 and TNFα induce elevated levels of E-selectin. Within hours of the onset of inflammation recruited leukocytes, mainly neutrophils and macrophages, start to increase in quantity in the peritoneal tissue.

If the inflammatory response is maintained over time, it will transform into chronic inflammation characterised by fibrosis and angiogenesis. Chronic inflammation may result from a persistent acute inflammation or by other mechanisms. An acute inflammation will become chronic if the immune system is unable to remove the underlying cause, or if the agent is constantly able to re-enter the body. Chronic peritoenal inflammation in PD is most likely the result of recurring dwells over time, repeatedly causing an acute inflammation. Over time the cell population will be modified, and growth factors such as vascular endothelial growth factor (VEGF) can be found in increasing concentration.

A key feature of chronic inflammation is collagen production. Excessive collagen formation leads to the condition known as fibrosis. Fibroblasts recruited to the inflamed (and those present) site are able to produce various cytokines, and collagen, which is necessary to replace the damaged tissue during long term inflammation. Fibroblasts are can also express VEGF, a proagniogenic factor, in response to IL-8, TGFβ (Kellouche *et al.*, 2007), and thrombin (Huang *et al.*, 2001).

Cascade systems

The coagulation system

The coagulation system is an enzymatic cascade triggered by damage to blood vessels. It is also speculated that the coagulation cascade can be initiated by disturbing the balance of pro- versus anticoagulation factors. Mesothelial cells are closely involved in the regulation of this balance. During the cascade thrombin is yielded, which acts on soluble fibrinogen in tissue and plasma producing fibrin and fibrinopeptides. Fibrin is the main component in the creating of clots, which serve as barriers. The fibrinopeptides are inflammatory mediators, acting on the vascular bed, inducing increased permeability and neutrophil chemotaxis. Thrombin is also a pro-inflammatory mediator acting on peritoneal resident cells and recruited leukocytes. Thrombin ca stimulate angiogenesis via the stabilisation of VEGF (Huang *et al.*, 2001).

The coagulation system is mainly in place to seal damaged vessels and initiate healing, via the formation of clot or thrombus. A clot occurs locally around a platelet plug, and consists primarily of the protein fibrin. The events surrounding coagulation, the forming of a clot, are initiated when the endothelium of a vessel is disrupted and blood is able to come in contact with the underlying tissue (Vander *et al.*, 2001). Contact initiates a cascade of chemical reactions, in which an inactive factor is converted to a proteolytic enzyme, which then catalyses the conversion of a subsequent inactive factor. Extensive deposition of fibrin can lead to the formation of fibrous connective tissue and adhesion formation Disruption of the fibrinolytic activity of mesothelial cells in the peritoneum by

infusion of PD fluid could lead to catheter encapsulation. Mesothelial cells can also produce tissue factor, and it is possible that disruption of their normal function during PD can initiate the coagulation cascade and fibrin deposition without damage to blood vessel endothelial cells (Nagy, 1996). Coagulation can therefore be of importance when evaluating the effects of PD fluid.

The clotting cascade consists of two sequential pathways, intrinsic and extrinsic, which are linked at the step previous to the prothrombin-thrombin reaction (Fig. 1). Either pathway can theoretically initiate the cascade. However normally, clotting is initiated at the extrinsic pathway (Vander *et al.*, 2001), when blood comes in contact with tissue factor located on the plasma membrane of various tissue cells situated outside the endothelium of the walls of blood vessels. The plasma protein factor VII binds to tissue factor and it becomes activated forming a tissue factor VII complex, which then catalyses the activation of factor X. In addition, it catalyses the activation IX, which amplifies the activation of more factor X via the intrinsic pathway (Fig. 1).

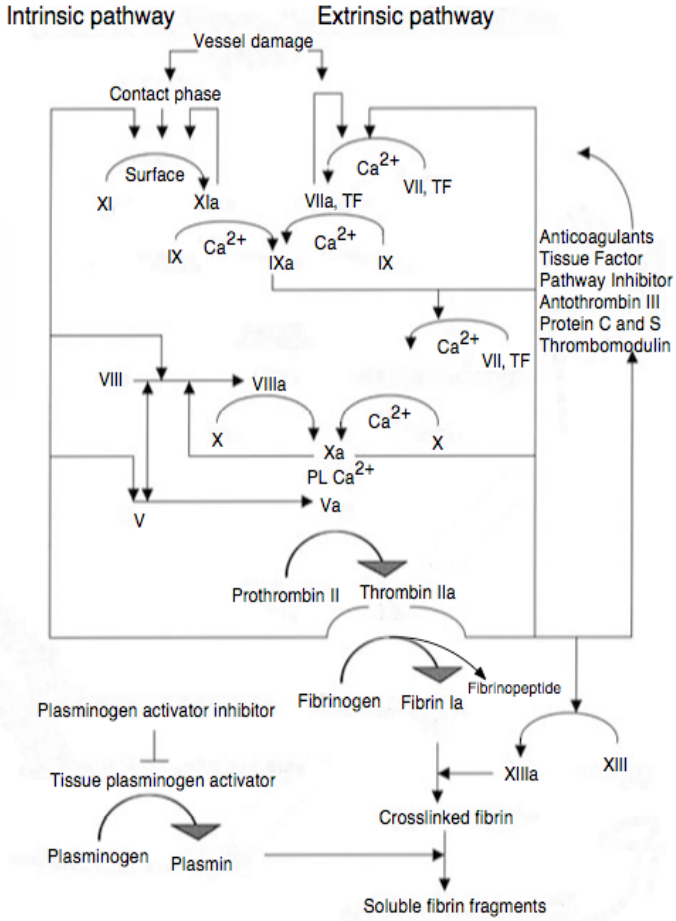

Figure 1. Overview of the coagulation cascade Originally from (Auguste *et al.*, 2004)

The first plasma protein in the intrinsic pathway is called factor XII, and becomes activated to XIIa when it comes in contact with, e.g. collagen, which is underlying the vessel endothelium. Factor XIIa in turn activates XI to XIa, which then activates IX to IXa, which is the factor that converts prothrombin to thrombin.

Thrombin, the end product of both pathways, can activate factor X and VIII but more importantly factor XI. Although the pathways seemingly act in parallel this is rarely the case; the extrinsic pathway initiates the clotting cascade, triggering activation of the intrinsic pathway through self-amplifying mechanism (Vander *et al.*, 2001). The amount of thrombin activated by the extrinsic pathway is too small to sustain coagulation. However, the quantities are large enough tot trigger activation of factor XI and VIII activating the intrinsic pathway independently of factor XII. Moreover, thrombin also facilitates the prothrombin-thrombin step by activating factor V and platelets (Fig. 1). Activated platelets are necessary because several of the reactions take place on their surface.

Thrombin is not only active in the coagulation cascade; it also acts extensively on various cells involved in inflammation, neutrophils, mesothelial (Mandl-Weber *et al.*, 2002), and mast cells (Szaba and Smiley, 2002), stimulating the release of histamine and cytokines. Stimulation of inflammatory active cells is mediated via membrane-bound protease-activated receptors (PARs) (Dugina *et al.*, 2003).

The complement system

Similar to the coagulation system, complement activation progresses through a cascade of chemical enzymatic reactions. The complement system can be activated through three distinct pathways: the classic pathway, alternative pathway, and the lectin-mediated pathway. The alternative pathway, as part of the innate immune defence is triggered by elements of bacterial cell surfaces or foreign substances, and is therefore the initiating mechanism for complement activation during peritoneal non-microbial inflammation. According to theories, the alternative pathway is activated by surfaces absent of complement inactivators. The PD catheter is one such apparent structure present during PD, thus a potential trigger of complement activation

All three pathways result in the formation C3 convertase, an enzyme responsible for the cleavage of C3 through hydrolysis to its active products, C3a and C3b. One of C3b's more important functions is to propel the cascade further, causing the hydrolysis of C5 to C5a and C5b (Fig. 2).

C3a and C5a induce inflammation by recruiting inflammatory cells into the area of complement activation and escalate the inflammation by direct activation of endothelial cell, macrophages, and mast cells. In addition, C5a acts as a chemoattractant (Haynes *et al.*, 2000) directing migration of leukocytes.

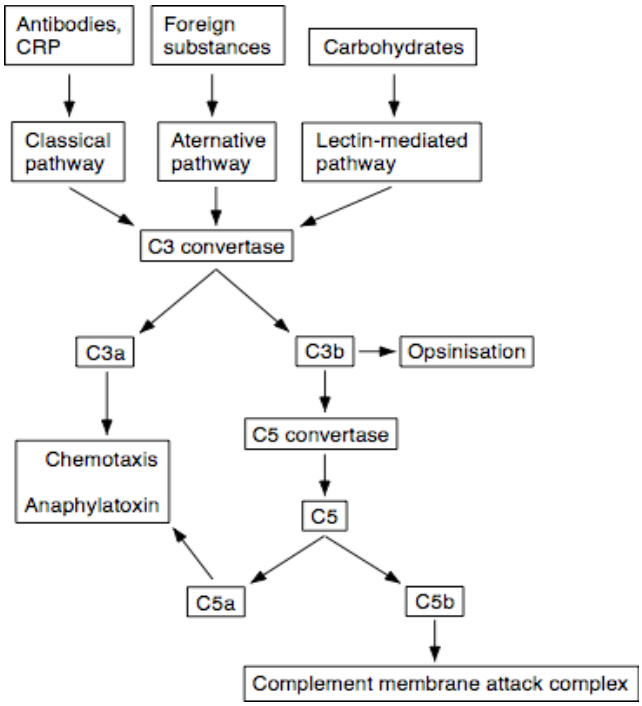

Figure 2. Overview of the complement cascade

The coagulation and complement systems have structural and functional similarities. Also, they are activated by the same stimuli, e.g. endothelial damage. Recent data has led to the hypothesis that both systems originate from a common ancestral development-immune cascade existing before the divergence of protostomes and deuterostomes. It seems that activation of coagulation and complement cascades occur simultaneously and interact during several cascade phases, e.g. C3a activates platelets, enhancing their aggregation and adhesion; C5a can enhance blood thrombogenicity through the up regulation of TF, and PAI-1 expression. C5b may also interact with platelets through the complex C5b-9, which is incorporated into the platelet membrane

inducing an increased surface area on which clotting can occur. Notably, thrombin can cleave C3 and C5 to generate their active components, thus amplifying the activation of complement.

Resident cells of the peritoneum

During peritoneal dialysis the peritoneal membrane may be defined as the set of cellular and extracellular components encountered between the peritoneal capillaries and the peritoneal cavity. The peritoneal membrane is composed of three distinct layers: a single mesothelial layer, a continuous basal membrane, and furthest from the peritoneal cavity a layer of connective tissue rich in extracellular matrix (ECM). In the connective tissue, capillaries, and a population of cells can be found; fibroblasts, mast cells, and macrophages are the most abundant. The capillaries mark the starting-point of the transperitoneal transport route between blood and the intraperitoneal fluid space

Mesothelial cells are involved in many important aspects of PD. They contribute to the transport of water and solutes and the structural integrity of the mesothelium, via various types of junctions and non-junctional cell-matrix adhesions respectively; they excrete surface-active material, composed of a mixture of phospholipids, which lubricate the peritoneum. Mesothelial cells are also able to respond too and propel an inflammatory reaction. These cells can express adhesion molecules, e.g. intercellular adhesion molecule (ICAM)-1 and 2, which are involved in the attachment and trafficking of leukocytes. Further, these cells express various cytokines during inflammation, e.g. IL-6, IL-8 and TNFα (Nagy, 1996), which can cause disturbance in the fibrinolytic/anti-fibrinolytic, and anticoagulative state of the peritoneum. Mesothelial cells can express PAI-1 and -2, inhibiting fibrinolytic activity, thus tipping the balance in favour of adhesion and fibrosis. Evidence also suggest that these cells epress TF during peritoneal inflammation, which can change the peritoneal condition towards a procoagulant state (Nagy, 1996).

Fibroblasts that are found in the ECM of the connective tissue produce the macromolecules that make up the ECM, mainly collagen. Via secretion of IL-6 and 8 they also contribute to the recruitment of leukocytes and other pro-inflammatory cells. Via the production of fibroblast growth factor, fibroblasts are important in the development of new blood vessels.

Tissue macrophages located in the vicinity of blood vessels in the peritoneum are a source of various cytokines, such as transforming growth factor beta (TGFβ), and TNF. Thus macrophages are important in e.g. recruitment of leukocytes and in the propulsion of fibrois. As part of the inflammatory response a new macrophage population, known as “inflammatory macrophages” is recruited from the blood. In the present studies, we have focused on another resident cell, the mast cell.

Mast cells

Mast cell precursors are formed in the bone marrow by haematopoiesis as undifferentiated cells, which do not differentiate until they leave the blood. Mast cells

can be found in many different tissues, including the skin and connective tissue. They are characterised by their large number of cytoplasmic granule, in which many proinflammatory mediators are stored. Mature mast cells are found throughout the body, habitually in close proximity to blood vessels and nerves.

There are two major subsets of mast cells: mucosal, which are highly dependent on T-cells, and connective tissue mast cells, which exhibit little T-cell dependency. Mucosal mast cells dependency suggests that they are involved in T-cell and IgE-dependent immediate hypersensitivity reactions. Much suggests that mast cells are not set in their phenotype and that phenotype changes and adaptation occur in different microenvironments, in response to cytokines and growth factors (Abbas and Lichtman, 2005).

Molecules released upon activation mediate the effector functions of mast cells: degranulation and secretion of preformed contents stored in granules like histamine; synthesis and secretion of lipid mediators like leukotriens; synthesis and secretion of cytokines. The effector release may vary depending on the signal triggering a mast cell response.

Although mast cell degradation is generally initiated by allergen cross linkage of bound IgE, numerous other factors can trigger mast cell activation, including C3a, C5a, chemokines, and cytokines (Goldsby *et al.*, 2003). Substance P (SP) (Cao *et al.*, 1999), and vasoactive intestinal peptide (VIP) (Kulka *et al.*, 2008) can cause histamine release. C5a, monocytes chemoattractant peptide (MCP-1), and macrophage inflammatory protein (MIP) 1α are especially potent mast cell activators in the non-IgE-mediated pathway (Bird and Walker, 1998). The non-IgE mediated pathways are different in that they are not dependent on Ca^{2+} influx. Mast cells may also be activated by osmotic stimuli (Silber *et al.*, 1988, Eggleston *et al.*, 1990), although the mechanisms are not entirely clear it is possible that the expression of TRP channels (Turner *et al.*, 2007) can explain their sensitivity to changes in osmolarity.

Mast cell mediators

Histamine

Many of the mast cell effects are mediated by biogenic amines, e.g. histamine, released from intracellular granules. Although histamine is removed from the extra cellular environment shortly after release by an amine-specific transport system it affects its environment strongly. It stimulates enhanced proliferation of mesothelial cells and fibroblasts, induces increased permeability of nearby blood vessels, and facilitates the recruitment of leukocytes from the blood. Histamine acts mainly by interacting with and activating the target receptors H1, H2, and H3.

Activation of H1 receptor causes the formation of IP_3 by the breakdown of phosphatidylinositol in many cell types resulting in the release of calcium from cytosolic locations, including other mast cell, thus creating a positive feedback-loop. Cytoplasmic calcium is vital in many cells for cell signalling. Activation of the H1 receptor on vascular

endothelial cells causes increased permeability (Nakahara *et al.*, 2000). Stimulation of H2 receptor is established to cause mesothelial cell and fibroblast proliferation, whether calcium is required need confirmation. H1 receptors are also numerous in the central nervous system (Ter Laak *et al.*, 1993). The H3 histamine receptor provides feedback inhibition of histamine and release as well as the inhibition of some neurotransmitter release. The H3 receptor is found on mast cells and sensory neurons, however the H3 receptors in the brain are believed to be of another subtype (Leurs and Timmerman, 1998).

Serine proteases

Serine proteases, including tryptase and chymase are stored in cytoplasmic granules. Although their function is not entirely understood it is hypothesised that tryptase cleaves fibrinogen and activates collagenase, thereby causing tissue damage. Chymase can convert angiotension I to angiotension II, degrade epidermal basement membrane, and stimulate mucus secretion. Tryptase is only found within mast cells and is therefore often used a marker for mast cell activation. Also protoglycans such as heparin are stored within the granules. Protoglycans serve in the granules as protective storage, preventing enzymes such as proteases, access to the rest of the cell.

Lipid-derived mediators

A slower and more complex process involves the synthesis and release of lipid-derived mediators that affect blood vessels and leukocytes. The most important lipid-derived mediators are probably cyclogenase and lipoxygenase metabolites of arachidonic acid. One such metabolite produced by the cyclogenase pathway is prostaglandin D_2, which is involved in vasodilation and neutrophil chemotaxis. Leukotrines are produced by the lipoxygenase pathway and can act potently on the cardiovascular system, intestinal tract, and central nervous system. A third and important lipid mediator is called platelet-activating factor (PAF), which in addition to its involvement in the coagulation cascade causes increased permeability, vasodilation, and adhesion of neutrophil to endothelial cells (Macconi *et al.*, 1995).

Cytokines

Mast cells produce many different cytokines that contribute to initiation of inflammation, whether mast cell activation is IgE-dependent or not. The cytokines include TNFα, IL.1, IL-4, IL-5, IL-6, MIPs (MIPα and 1β), and granulocyte-monocyte colony-stimulating factor (GM-CSF). On cell activation, transcription and synthesis is induced producing these cytokines, with the exception of TNF, which can be stored within granules. MCP-1 and MIPα are involved in the recruitment of monocytes and lymphocytes. TNFα and IL-1 activate mesothelial cells and fibroblasts (Bird and Walker, 1998). TNFα can also stimulate expression of ICAM-1 and vascular cell adhesion molecule (VCAM-1) in endothelial cells. Mast cells have been shown to produce IL-8 in carcinoma cells (Aoki *et al.*, 2003), whether they have the ability to do it during other circumstances is not entirely known. IL-8 is also a strong chemoattractant for mast cells and neutrophils (Jiang *et al.*, 2001).

Neurogenic inflammation

Nociceptive neurons

Neurogenic inflammation refers to the activation of subset primary afferent neurons, mainly polymodal nociceptors: unmyelinated C- and myelinated Aδ-fibres, which can be activated in response to heat, cold, osmolarity, chemical, and mechanical stimuli. The inflammatory responses triggered by activation of these neurons is mediated by calcitonin gene related peptide (CGRP), SP, and neurokinin A (NKA). C- and Aδ-fibres express on their plasma membrane a large number of excitatory and inhibitory receptors and channels, which have the ability to trigger a neurogenic inflammatory component. Transient receptor potential vanilloid-1 (TRPV1) was one of the first receptors identified.

Many diseases like allergic arthritis, asthma, inflammatory bowel disease, and migraine are thought to include a neurogenic component. Neurogenic inflammation can cause many of the cardinal symptoms of inflammation, e.g. pain, redness, and swelling, either direct or indirect. Redness, heat, and swelling during inflammation are caused by changes to the vascular arteriolar and capillary systems. Vasodilation, the relaxation of smooth muscle cells within the large arteries, veins and arterioles leads to a decreased blood pressure, and an increased blood flow, resulting in the two cardinal signs heat and redness. Capillary permeability is the result of endothelial cell contraction, and a subsequent increased leakage of water, proteins, and leukocytes leading to a swelling of the inflamed tissue. Arteriolar dilation and increased blood flow potentiates leakage trough the capillaries. Modern research in neurogenic inflammation suggests that there is a neurogenic component in several inflammatory diseases, such as asthma (Rogers, 1997) (Butler and Heaney, 2007). There is recent evidence that neurogenic vasodilation, triggered by CGRP is a major underlying mechanism in migraine (Geppetti *et al.*, 2005), possibly through . An established connection between mast cells and SP further suggest that a neurogenic component is potentially important in many inflammatory diseases where mast cells are involved, e.g. asthma, and coronary inflammation (Theoharides and Kalogeromitros, 2006).

TRPV1

The TRPV1 receptor, recognized as a trigger of neurogenic inflammation, is a member of a family of polymodal receptors and calcium channels (TRPV1-4) that form nociceptive subgroup of the TRP receptors (Levine and Alessandri-Haber, 2007). Once activated the neurogenic response can quickly propagate via dorsal root reflexes (Lin *et al.*, 2007) and axonal reflexes (Kiernan, 1975). Activation of members of the TRPV family is shown to initiate interleukin release from cells outside the nervous system via the actions of released neurotransmitters such as substance P and CGRP (Massaad *et al.*, 2004).

Neuropeptides

Substance P

Although it is discussed whether SP can trigger mast cell degranulation *in vivo*, it has been a hot topic since the discovery that SP-containing nerve fibres and mast cells are in close proximity, (Zhao *et al.*, 1997) (Hagforsen *et al.*, 2000) (Batbayar *et al.*, 2003) and since SP has been shown to release histamine from mast cells *in vitro* experiments. SP acts at least via two different pathways, stimulation of neurokinin-1 (NK1) receptor (Wick *et al.*, 2006) or directly in a receptor-independent manner. During the receptor independent mechanism, the basic N-terminal of SP interacts with a sialic acid residue (Foreman *et al.*, 1983). NK1 receptors are common on capillary endothelial cells, and activation by SP increases vascular permeability (Cao *et al.*, 1999). Additionally, SP can activate mast cells via the NK1 receptor (Lilly *et al.*, 1995) or via a receptor-independent pathway (Devillier *et al.*, 1989), further potentiating vascular permeability but also causing vascular vasodilation. Activation of mast cells by SP can cause the release of histamine, proinflammatory cytokines (IL-1β, IL-6, TNFα and NGF) (Massaad *et al.*, 2004), and vascular endothelial growth factor (VEGF), which may affect leukocytes, angiogenesis, and fibrosis. SP can also induce nitric oxide (NO) mediated vasodilatation (Brock and Joshua, 1991).

CGRP

CGRP is a potent vasodilator (Geppetti *et al.*, 2005) that acts on at least two receptor subtypes, $CGRP_1$ and $CGRP_2$. Classification is based on both agonist and antagonist relative potency and affinity (Wisskirchen *et al.*, 1998). In humans, two forms exist, αCGRP and βCGRP; αCGRP is most abundant and found mostly in the central and peripheral nervous system. βCGRP is primarily found within enteric nerves (Geppetti *et al.*, 2005). Location of activity and receptor specificity may differ between species.

CGRP is released during neurogenic inflammation, activated by TRPV1. It can either be stored co-localised with SP or stored separately. CGRP actions of mechanism are contrary to SP mediated actions NO-independent. However, it has been suggested that CGRP release is indeed NO-dependent (Brain *et al.*, 1993). These results are not unanimous agreed upon (Merhi *et al.*, 1998). In recent research it has been found that NO-donating compounds did not trigger CGRP release (Eltorp *et al.*, 2000). An explanation to this discrepancy in results may be differences in species and vascular regions examined. CGRP has in addition to its vasoactive function, the ability to potentiate the vascular permeability increase mediated by SP and histamine (Brain and Williams, 1985).

In human plasma, CGRP has a relatively short half-life, 7-10 minutes (Struthers *et al.*, 1986). However, in rabbit it has been noted that CGRP has a long duration of vasodilator action, 40 minutes before its activity is reduced (Brain and Williams, 1985). The long duration suggests a slow metabolising mechanism although it may be that the smaller peptides still retain activity. However, a recent study by Arulmani *et al.* (2004) on the effects of CGRP on haemodynamics shows that CGRP is not involved in the normal regulation of heart and vascular systems.

The effects of single and repeated instillations of PD fluid into the peritoneal cavity

Acute effects of PD fluid

Exposure to PD fluid induces an acute inflammatory reaction in rat models of PD. In rats, the acute inflammation during a single dwell is characterised by recruitment of neutrophils to peritoneal cavity (Bos *et al.*, 1989), increased levels of C3a-des-Arg, thrombin-antithrombin (TAT), and cytokine induced neutrophil chemoattractant (CINC)-1 (Bazargani, 2005). The occurrence of CINC-1 suggests mast cell activation (Ramos *et al.*, 2003). However, the triggers and mechanisms behind an acute peritoneal inflammation are unknown, and are one of the main focuses in our studies. The PD fluid has properties, e.g. hyperosmolarity and low pH, which may hypothetically activate peritoneal inflammation (Jonasson, 2004), possibly through activation of coagulation, and complement cascade, mast cell, and sensory neurons. Mast cells are sensitive to changes in osmolarity (Silber *et al.*, 1988) (Eggleston *et al.*, 1990). High osmolarity also enhances the production of TNFα by peripheral blood mononuclear cell (Cendoroglo *et al.*, 1998), and TRPV receptors on C-, and Aδ-fibres are also sensitive to changes in osmolarity (Levine and Alessandri-Haber, 2007).

Introducing PD fluid into the peritoneal cavity causes a potent dilution of resident cells, proteins, and opsonins. After four hour dwell time, cell concentration has increased but is still low, only 1-10 % of normal concentrations (Cameron, 1995). Also plasma protein levels are still greatly decreased, about 2-4 % of plasma levels (Cameron, 1995) compared to 14 % in healthy people. Decreased levels of cells and proteins are probably a combined result of dilution and washout.

Chronic effects of PD fluid

Long-term CAPD is plagued by the washout of inflammatory and mesothelial cells and proteins, and cytokines. Described changes include loss of microvilli, interstitial fibrosis and thickening, remodelling of omental tissue, and vascular changes.

During peritoneal dialysis induced inflammation a constant state of neutrophil recruitment (Flanigan *et al.*, 1985), cytokine production, (Lin *et al.*, 1993, Zemel *et al.*, 1994) maintenance of coagulation, and complement cascades (Homma *et al.*, 2002, Reddingius *et al.*, 1995, Young *et al.*, 1993) leads to changes in the peritoneum, suggesting a chronic inflammatory state (Dobbie, 1993 and Honda *et al.*, 1996 and Wilson and Bonta, 1986). Distinctive changes to peritoneum over time include angiogenesis and fibrosis, considered to be the related to increased reabsorption, and loss of ultrafiltration seen in long-term patients.

PD fluid can trigger the formation of collagen I and III via the up regulation of PAI-1 from fibroblasts. Further, evidence suggests that infusion of PD fluid and subsequent inflammation, and mast cell activation is responsible for fibrosis and angiogenesis; supported by increased mast cell quantity in the omentum during continuous dialysis

(Zareie *et al.*, 2006). Mast cells express collagen and have been shown to be a major factor in the development of fibrosis (Levick *et al.*, 2009) in several conditions. Several characteristic mast cell components, e.g. tryptase, TGF-β, and chymase, are able to induce fibroblasts collagen synthesis and proliferation in various tissues (Garbuzenko *et al.*, 2002, Cairns and Walls, 1997, Zhao *et al.*, 2008). TGF-β is closely associated with adhesion formation; (Holmdahl *et al.*, 2001) expression is increased in peritoneal mesothelial cells and fibroblasts in adhesion formation (Saed *et al.*, 2004), which is extensive during PD in animal models. In PD patients, adhesion are rare; they show peritoneal fibrosis and a small number of patients suffer from sclerosing peritonitis.

Angiogenesis during chronic PD

The chronic response of the peritoneum during dialysis involves angiogenesis, somehow induced by inflammatory cytokines, e.g. IL-1 and TNFα, and the interaction between the resident cell population: macrophages, mesothelial cells, and mast cells. One of the earlier responses during PD entails the activation of TNFα and IL-1 (Margetts *et al.*, 2002). What follow is a complex interaction, activation, and recruitment of cytokines, chemokines, leukocytes, prostaglandins, nitric oxide, and adhesion molecules. Angiogenesis is an area of great importance since it seems to be closely related to increased glucose transport and ultrafiltration failure. Data from animal models correspond well with prospective patient-studies; loss of ultrafiltration is accompanied by an increased diffusion of small solutes (kreatinine, urea, and glucose (Heimburger *et al.*, 1999). However, ultrafiltration in patients is generally stable for significantly longer than in animal models.

VEGF secretion has been connected to PD-induced angiogenesis in humans as well as in animals. IL-1 and TNFα induce early expression of VEGF. Data suggests that TNFα induces a rapid and transient induction of VEGF and collagen deposition, whilst IL-1 seems to induce a more long-lasting expression of VEGF and angiogenesis (Margetts *et al.*, 2002). Mast cells are a known source of TNFα and IL-1, (Aoki *et al.*, 2003) suggesting involvement in angiogenesis. VEGF can be released from mast cells (Aoki *et al.*, 2003) along with other angiogenic factors such a basic fibroblast factor, and TGFβ (Margetts *et al.*, 2002, Aoki *et al.*, 2003), possibly creating an endocrine feedback mechanism. VEGF is also released from peritoneal mesothelial cells (Selgas *et al.*, 2000) in response to thrombin (Mandl-Weber *et al.*, 2002). IL-8, a proinflammatory cytokine, released from mast cells is able to interact with endothelial cells, (Aoki *et al.*, 2003) thus creating a casual connection between mast cells and mesothelial cell in the process of angiogenesis. TGFβ, released from activated mast cells and mesothelial cell is likely the main growth factor for extracellular matrix proliferation along with collagen deposition (Zweers *et al.*, 1999), further hinting at a complex endocrine mechanism involving many cell types and mediators in the process of angiogenesis and fibrosis.

The calcium dependence of inflammation and the possible beneficial therapeutic effects of citrate

Calcium regulation during inflammation is critical

Calcium signalling pathways have been implicated in the regulation of several cellular, and non-cellular processes, including complement, and coagulation activation, proliferation and rearrangement of cellular structures, e.g. mesothelial, and vascular endothelial structures. Calcium also seems to be involved in the activation of pro-inflammatory cells such as mast cells and sensory neurons.

Activation of the complement and coagulation systems takes place during the first minutes of PD-induced inflammatory response.

During the coagulation cascade thrombin is yielded, which acts on soluble fibrinogen in tissue and plasma producing fibrin and fibrinopeptides, promoting increased permeability and neutrophil chemotaxis. Factor IX in its active form (IXa) is responsible conversion of prothrombin to thrombin. Activation of IX to IXa requires calcium, as almost all steps in the coagulation cascade. Calcium is not only important in the activation of IX but is also important in its stabilisation. Four calcium ions are required to stabilise the active omega loop of IX; stabilisation increases activity and its lifespan (Kim *et al.*, 2009).

Thrombin is as many inflammatory mediators multifunctional; it is active in the coagulation cascade, it promotes activation of e.g. monocytes, neutrophils, and mast cells, which are necessary in the peritoneal defence. One important function of thrombin is the interaction with complement, potentiating this cascade.

Activation of the complement cascade, either via the classical or the alternative pathway requires calcium. C1s, the enzymatic subunit of C1 which cleaves C2 and C4 during activation of the classical pathway, requires binding Ca^{2+} to its NH_2-terminal in order to interact with other subunits (Busby and Ingham, 1990). Although the alternative pathway mostly requires Mg^{2+}, calcium is needed further down the chain. It is speculated that Ca^{2+} stabilises C9 (Thielens *et al.*, 1988), which is active downstream.

Activated mast cells and endothelial cells produce VEGF and TGFβ, two major mediators of angiogenesis and fibrosis, which require calcium. The intracellular mechanisms by which VEGF induces angiogenesis are not entirely understood, however recent evidence proposes stimulation of endothelial cells, including calcium entry, possibly via transient receptor potential cation channel (TRPC) (Hamdollah Zadeh *et al.*, 2008), a member of the conserved TRP family. Inhibition of VEGF has been shown to inhibit an increase in intracellular levels of calcium and activation of nitric oxide synthase (NOS), (Hamdollah Zadeh *et al.*, 2008), (Bauer *et al.*, 2000) thereby reducing endothelial proliferation. There are many forms of NOS, some which require calcium. An increase in NOS results in the production of NO. Recent studies show an association between NO and VEGF. VEGF can up regulate NO levels by increasing intracellular levels of calcium (Bauer *et al.*, 2000). However, NO also induces VEGF production, thereby creating a relationship between calcium, VEGF, NOS, and NO, and a subsequent angiogenesis (Bauer *et al.*, 2000).

Recent studies further enhance the hypothesis that calcium is a key factor in angiogenesis and fibrosis. Inhibition of calcium channels has shown a reduction in collagenolytic activity, endothelial cell migration and proliferation, and capillary outgrowth (Kohn *et al.*, 1995), key processes in angiogenesis.

Nociceptive neurons regulate their calcium levels, by calcium-selective pores at two sites: intracellular calcium stores, e.g. mitochondria, endoplasmatic reticulum, and channels in the cell membrane. These calcium channels are receptors belonging to the TRP family; the TRPV subgroup modulates pain in response to various stimuli and is thought to be important during inflammatory processes.

Activation of TRPV1 increases calcium by entry of calcium from the extra cellular space and/or by calcium release from intracellular stores (Hagenacker and Busselberg, 2007). But TPRV1 activation not only increases calcium levels, in fact calcium itself modules TRPV1 activity (Hagenacker and Busselberg, 2007). Different subtypes of voltage activated calcium channels (VACC) are expressed on the cell membrane of nociceptive neurons. The activation of TRPV1 receptors modulates calcium entry through these channels (Hagenacker *et al.*, 2008) (Hagenacker and Busselberg, 2007). Further, activation of calcineurin, an intracellular phosphatase, which inhibits TRPV1 activity is activated by high calcium levels thereby creating an autocrine self-regulatory mechanism.

Clearly, the complex and self-regulatory mechanisms underlying a peritoneal inflammation require calcium, both intra, and extra cellular calcium during many critical steps. The requirement of calcium in many of the processes underlying the induction and propulsion of the peritoneal inflammation during PD hints at a viable target in improving PD. A reduction in inflammation via inhibition of calcium could possibly reduce capillary alteration, angiogenesis, and fibrosis, increasing PD technique survival.

Citrate, a possible pharmacological additive

During recent years the implementation of additives to PD fluids has been of some interest in the ways of retaining peritoneal membrane integrity, thus increasing PD technique survival. Recent efforts are put into increasing the biocompability of the solutions used during PD by reducing glucose degradation products and neutralising pH to better preserve peritoneal membrane function, and reduce loss of dialysis efficacy. New solutions under investigation contain additives to protect the peritoneal membrane in order to minimise degradation of efficiency over time resulting in increased solute clearance, and ultrafiltration loss. Efforts include trials with heparin and sulodexide, which could improve ultrafiltration via anti-inflammatory mechanisms. Counteracting inflammation has been a common mechanism for such interactions.

Heparin has been shown to reduce plasma CRP and delay IL-6 release (Sjoland *et al.*, 2005), suggesting a reduction in local and systemic inflammation. Heparin has also been shown to inhibit angiogenesis. (Norrby and Ostergaard, 1996, Norrby and Ostergaard, 1997), and possibly complement (Matzner *et al.*, 1984, Ekre *et al.*, 1986). Sjoland *et al.*

(2005) have shown a significant correlation between appearance rates of IL-6 and creatinine dialysate-to-plasma ratios, but not with sings of systemic inflammation, thus indicating that transperitoneal transport is closely related to local inflammation. Intraperitoneal application of low molecular weight heparin on 21 patients in a crossover study resulted in increases to ultrafiltration and D/D0 glucose ratio (Sjoland *et al.*, 2004).

An important factor to take into consideration when looking for a pharmacological active substance is heat resistance since commercial manufacturing of PD fluids depends on heat-sterilization. Some calcium chelators such as sodium citrate are therefore of interest. Sodium citrate is currently used in haemodialysis (Moran and Ash, 2008, Cointault *et al.*, 2004) as anticoagulant and supplementary data demonstrate a significant inhibition of the complement system (Kadar *et al.*, 1992) in the concentration used as anticoagulant during dialysis, however, inhibition was not complete (Fiorante *et al.*, 2001, Ish *et al.*, 1993). The anticoagulant effect of citrate depends on its ability to bind calcium, thereby interacting with many processes during inflammation. Citrate has been shown to reduce fibrosis (Szaba and Smiley, 2002), which could potentially have great impact on long-term PD. Citrate is metabolised in the liver and therefore is not dependent on renal excretion.

AIMS OF THE STUDIES

General aim

To evaluate the effects of citrate as an additive to PD fluid in an acute and a chronic animal model, and study to two calcium dependent components of the inflammatory response: neurogenic inflammation in reaction to PD, and mast cell degranulation during acute peritoneal inflammation in an animal model.

Specific aims:

- To establish a practicable citrate dose as lactate substitution in an animal model
- To study the pharmacological effects of citrate substitution on transperitoneal transport in acute, and long-term experiments
- To study fibrosis, angiogenesis and the development of ultrafiltration failure over time
- To characterise the impact of mast cell degranulation on peritoneal transport during PD
- To investigate involvement of a neurogenic inflammatory component in response to PD fluid exposure, and establish mechanisms of action
- To characterise the neurogenic component in relation to peritoneal transport

MATERIALS AND METHODS

Experimental protocols

The study protocols were approved by the Göteborg ethical committee, and the NIH Guide for the Care and Use of Laboratory Animals was adhered too.

Paper I – Evaluation of citrate in single PD dwells

Animals were exposed to PD fluid by infusion through a previously implanted catheter. Citrate was substituted for lactate as buffer at concentrations 5 - 15 mM and compared to a standard (not substituted) PD fluid buffered with 40 mM lactate only. Glucose concentration was unchanged at 2.5 %. During single 4-hour dwells, animals were exposed to either standard PD fluid or citrate at concentrations 5 mM (n=10), 10 mM (n=8), and 15 mM (n=6). All groups were included in a dose response evaluation. The 10 mM citrate PD fluid and the standard PD fluid were further compared to evaluate the effects of citrate substitution. ^{125}I albumin was added to the PD fluids as intraperitoneal volume marker.

PD fluid samples were collected at 0, 2, and 4 hours, and analysed for ultrafiltration volume, glucose concentration, citrate concentration, cell quantity, and thrombin-antithrombin complex (TAT) concentration. The samples (PD fluid and blood) were supplemented with sodium EDTA at a final concentration of 10 mM. Samples were used directly for cell counting and after centrifugation the cell-free supernatant was stored frozen for the remaining analyses.

In a second experiment, 10 mM (n=10) citrate substitution and lactate PD fluid (n=10) were compared further in 1-hour dwells. Samples were collected at 0, 30, and 60 minutes, and evaluated for glucose and urea transport. Intraperitoneal kinetics of citrate and calcium were related to coagulation, estimated from TAT concentrations.

Paper II - Evaluation of neurogenic inflammation and histamine release

Neurogenic inflammation and histamine release was studied in single 2-hour dwells. Animals were exposed to PD fluid by infusion through a previously implanted catheter. The effects of seven different interventions were compared with a control group treated with, standard lactate buffered PD fluid. Treatments were based on 20 ml; 3.9 % glucose; 483 mOsm/kg with the excepting two, which were composed of an isotonic PD fluid (20 ml; 0.5 % glucose; 294 mOsm/kg) and a low volume of standard PD fluid (14 ml; 3.9 % glucose; 483 mOsm/kg) respectively. Standard PD treatment was compared with low volume treatment, and isotonic fluid treatment. In addition 5 different pharmacological treatments were used to create positive and negative controls for mast cell degranulation and to block TRPV1 receptors and neuropeptides. Drugs were injected or added to the PD fluid (table 1). ^{125}I albumin was added to the PD fluids as intraperitoneal volume marker and ^{131}I albumin was injected i.v. at the beginning of the experiment in order to trace albumin transport from blood to PD fluid.

Substance	**Dose**	**Action**	**Administration**	**References**
SB366791	0.5 mg/kg	TRPV1 blocker	i.v. 30 min before PD	(Varga *et al.*, 2005)
Spantide	300 µg/kg	SP blocker	i.v. 20 min before PD	(Inoue *et al.*, 1995)
CGRP8-37	30 µg/kg	CGRP blocker	i.v. 20 min before PD	(Lin *et al.*, 2007)
C48/80	0.6 mg/kg	Mast cell degranulator	Added to the PD fluid	(Gaboury *et al.*, 1995)
Doxantrazole	30 mg/kg	Mast cell stabilizer	s.c. 1h before PD	(Ohashi *et al.*, 2007)

Table 1. Summary of the pharmacological treatment protocols. Doses (presented per kg body weight) were based and chosen for maximum activity according to references from the literature.

During the first 30 minutes of the dwell, 0.5 ml PD fluid samples were collected every 5 minutes via the PD catheter. Remaining samples were collected at 60 and 120 minutes dwell time. Blood was collected before and after injection of PD fluid.

Samples were collected in chilled tubes, containing EDTA, protease inhibitor cocktail, and PMSF, and centrifuged. Analysis of retrieved fluid included quantification of intraperitoneal and intravascular volume markers, histamine and neuropeptides. From acquired data, ultrafiltration, reabsorption of PD-fluid from the intraperitoneal cavity, clearance of albumin from blood to dialysate, and secretion of histamine and neuropeptides was calculated.

Paper III – Evaluation of citrate in a 36-day continuous PD fluid exposure

Long-term evaluation of citrate was performed in two separate experiments. In the first experiment catheter patency was evaluated using the same type of silicone rubber catheters as in the previous study of single dwells (Paper I). Animals were exposed five days per week to 20 ml PD fluid for 36 days. Catheter patency was compared between standard PD fluid (lactate) (n=10) and citrate substituted (10 mM) PD fluid (n=10). Untreated animals with implanted PD catheters were used as controls (n=7). At the end of the 36-day period, animals were evaluated macroscopically according to a scoring system (score 0-2).

In a second experiment, animals were treated with equivalent PD fluids, over the same obersarvation time but in order to ensure catheter patency, heparin-coated polyurethane catheters were used (Zareie *et al.*, 2004). Of the initial 26 rats controls: n=8; citrate: n=9, lactate: n=9), 3 dropped out due to post surgical complications.

Transperitoneal fluid transport was measured from single dwells at the beginning and at the end of the 36-day observation period using the intraperitoneal volume marker ^{125}I albumin; morphological evaluation of angiogenesis and fibrosis was done after ending the experiment.

Animals, surgical procedures, fluids, and anaesthesia

Animals

In the acute studies, male Sprague-Dawley rats with a weight between 250 and 300 g were used. In the long-term study animals were initially weighing between 250 and 275 g. The rats were kept 4 by 4 in cages, and had free access to standard food (pellets) and water. Animals followed a 12-hour days/night cycle.

General anaesthesia was induced and maintained by inhalation of Isofluran Baxter (Baxter Medical AB, Kista, Sweden) in room air. During the experiments, the animals were anaesthetised during the intravenous injection of albumin, the PD fluid infusion. The rats were subjected to short durations of anaesthesia during sampling with the exception of paper II where animals were anaesthetised for the first 30 minutes of dwell time and sampling. At the end of the experiment animals were killed by cutting the thorax and heart

Surgical procedures

rats (male) weighing between 300 and 400 grams were used in the experiments. A 7 French silicone catheter (Renasil® SILO8O; Braintree Scientific Inc., Braintree. MA, USA) was implanted under sterile conditions and general anaesthesia one week before the experiment. A midline incision was made through the abdominal skin, taking care not to cause any bleeding, and a hole was pierced through linea alba with a 3 mm diameter tapered needle. After inserting 2.5 cm of the tip through the hole, the catheter was sutured to the superficial abdominal muscle fascia and the rest of the catheter was tunnelled subcutaneously to the neck region and mobilised through the skin. After injecting 5 ml of saline, a stainless clip was used to close the catheter and the wounds were closed with agraffes. No antibiotics were administered.

Preceding the long-term experiment, the animals were implanted with a catheters and subcutaneous injection ports (Rat-O-Port #ROP-5NC; Access Technologies, Skokie, Il, USA) were connected in the neck region and sutured to the subcutaneous fascia before closing the wounds.

Fluids and additives

The PD-fluids used to expose the animals were laboratory made and filter sterilised (Nalgene® 0.2 UM SFCA 150 ml Nalgene NUNC International, New York, USA). They were based on a solution of 5.4 g/L NaCl, 0.051 g/L $MgCl_2x6H_2O$ and 0.198 g/L $CaCl_2x2H_2O$ buffered with 40 mM lactate (7.5 g/L 60 % sodium lactate syrup) or with citrate + lactate mixtures where 5, 10, or 15 mM of the lactate was substituted by an equal concentration of citrate (10 mM = 2.94 g/L Na-citrat). Glucose was used as osmotic agent in all fluids. The isotonic PD fluid used in paper II (294 mOsm) was created by adding 5.0 g/L d-glucose (0.5 %), all other treatment protocols in this study were performed with a 3.9 % PD fluid. In paper I, and III the PD fluids contained 2.5 % glucose.

In all experiments EDTA (Sigma®, # ED4SS) was added to the samples at a final concentration of 10 mM to prevent coagulation. In paper II and 3, PMSF (Fluka BioChemika, # 78830) and Protease Inhibitor Cocktail for General Use (Sigma®, # P2714-1BTL) was added to the samples in addition to EDTA to prevent activation of inflammatory systems and to minimize the effect of proteases.

In paper II various drugs were added to the PD fluid or administered intravenously before the experiments as indicated by Table 1.

Measurements

Determination of ultrafiltration volume and albumin clearance

Radiolabelled albumin was used as intraperitoneal volume marker to allow measurements of ultrafiltration and PD-fluid reabsorption. Thus, 10 kBq ^{125}I labeled human serum albumin (GE Healthcare, Kjeller, Norway) was added to the PD-fluid in combination with 1 mg of unlabeled bovine serum albumin that blocked surface adsorption. In paper II a second marker, I^{131} conjugated human serum albumin, was injected intravenously at the beginning of the experiment in addition to the volume marker added to the PD fluid. The concentration of these volume markers was determined by gamma counting of plasma and PD-fluid samples.

At the end of the dwell, 10 ml of PD-fluid without additives was injected through the catheter after the final PD fluid sample had been collected. The dilution of tracer, induced by this injection, was used to calculate the final intraperitoneal fluid volume. Osmotic ultrafiltration, net ultrafiltration and fluid reabsorption were calculated by combining data from tracer dilution during the dwell with the measured final intraperitoneal fluid volume and the known total activity of tracer infused. Fluid reabsorption was assumed to occur at a constant volume flow rate during the dwell and measured as the clearance of intraperitoneal volume marker from the peritoneal cavity. This is a common way to measure reabsorption in experimental PD but it implies a more restricted definition of reabsorption than that presented in the “INTRODUCTION” of this thesis. There, the term reabsorption was referring to all fluid transport from the peritoneal cavity to the intravascular space. Net ultrafiltration as defined as the net volume gain at 2 hours (paper II and III) or at 2 and 4 hours (paper I) and osmotic ultrafiltration was calculated from osmotic ultrafiltration = net ultafiltration + fluid reabsorption. In paper II, ^{131}I albumin clearance was calculated in order to quantify the diffusion of albumin from plasma to dialysate. Clearance was determined from the measured concentrations of ^{131}I in plasma at the beginning and end of the dwell and from a straight line best fit of the ^{131}I concentration over time in the PD-fluid samples obtained during the dwell.

Paper I

Analysis of coagulation

Coagulation was determined from measurements of the thrombin-antithrombin complex concentration (Enzygnost® TAT micro #OWMG 15; Dade Behring, Marburg GmbFl, Marburg, Germany). TAT levels were expressed as total intraperitoneal quantities.

Measurements of glucose, urea and citric acid

To determine glucose concentration, the samples were diluted 1:10 with sterile water in order to obtain glucose concentrations within the measurement range. Glucose Hexokinase II Reagent kit (ADVIA® Chemistry, # 04903429; Bayer Healthcare LLC, UK) was adapted to be used with 96-well plates in a plate reader. The method has a typical reproducibility of ca ±5 %.

For citric acid measurement the samples were diluted 1:10 with sterile water in order to obtain concentrations within the kits analysis range. (Citric acid: UV-method, #10139076035, R-Biopahrm AG, D-64293 Darmstadt, Germany). The method, originally designed for 1 cm cuvettes, was adapted for 96-well plates.

Urea was measured using Quantichrom™ Urea Assay Kit (#DIUR-500; BioAssay Systems, Hayward, CA, U.S.A.)

Measurements of calcium ions

Free (ionized) calcium was determined from calibrated readings of a calcium ion-selective electrode from Thermo Electron Corporation (Orion 97-20 ionplus®).

Paper II

Measurement of Histamine, SP and CGRP

Concentrations of Histamine, SP and CGRP were determined using commercially available ELISA kits (IBL Histamine ELISA, # RE59221; Assay Designs Substance P EIA kit # 900-018; Cayman Chemical CGRP EIA kit # 589001).

Paper III

Peritoneal biopsies

At the end of the experiment the abdominal cavity was fixed in situ under general anesthesia using roughly 15 ml 99.5 % alcohol for 15 minutes. Mesenteric windows, used to evaluate angiogenesis, were prepared as described earlier (Albrektsson *et al.*, 2006) and kept frozen until evaluated.

Biopsies from the ventral part of the diaphragm were taken, fixated and kept in 4 % neutral buffered formaldehyde until prepared for histological determination of peritoneal fibrosis.

Evaluation of PD catheter patency and peritoneal macroscopic morphology

PD catheter patency time was defined as the day for the last successful infusion of 20 ml of PD-fluid. 20 ml syringes were used for the infusions, and when excessive pressure had to be applied fluid was seen to leak from the access port into the surrounding subcutaneous space of the neck, the infusion was classified as unsuccessful and the catheter patency time was up. The animals were evaluated macroscopically at the end of the observation period and a scoring system (scores 0 - 2) was used to classify fibrotic contraction of the mesentery.

The scoring system was based on adhesions of the omentum and intestines, and fibrotic contraction of the small intestine mesentery. A 0 score represented no adhesions and normally sized mesenteric windows, a 1 score represented reduced mesenteric window size and a 2 score represented adhesions involving omentum and/or intestines and fibrotic contraction resulting in a partial or complete loss of mesenteric windows throughout the mesentery.

Immunofluorescence staining

All samples were stained for angiogenesis using RECA-1 (mouse anti-Rat Endothelial Cell Antigen 1; Serotec #MCA970GA) as a marker of endothelial cells. Windows were first incubated with the primary antibody 1:333 in PBS + 1 % BSA + 0.02 % Triton X100 overnight in 4°C or for three hours at room temperature. Incubation with goat anti-mouse Alexa-fluor 594 (Invitrogen #A11032) 1:1000 in PBS + 1 % BSA+0.02 % Triton X100 for 2 hour in room temperature was used to detect RECA-1 immunoreactivity.

Microscopic evaluation of fibrosis and angiogenesis

Angiogenesis was evaluated from micrographs of mesenteric windows at 2.5 magnification in fluorescence-microscopy. From each window, micrographs were captured along the entire window margin, together covering a 5 mm wide marginal zone. The micrographs were analyzed interactively and blinded, using ImageJ® image analysis software, for total mesenteric window area, vascularized area, and intervascular distance. Vascularized area was expressed as fraction of total observed window area. Vascular density was expressed as 100/intervascular distance in µm.

Fibrosis was evaluated interactively and blinded from micrographs of paraffin-embedded, cross-sectioned biopsies from the diaphragm stained with Masson Trichrome staining. Submesothelial tissue was defined as the tissue segment between the mesothelial cell layer and the muscle fibers and average mesothelial tissue thickness in µm was used to quantify peritoneal fibrosis.

Statistics

In paper I

Nonparametric statistics were used to evaluate differences between treatments (Mann-Whitney U-test) and correlations between variables in groups of animals (Spearman Rank test). The chosen level of significance was p = 5 %. Measured variables are presented in the text as mean ± SEM.

Paper II

Student's t-test was used to evaluate differences between treatments. The chosen level of significance was p≤5 %. Multiple comparisons were compensated for by a sequentially rejective Bonferroni procedure. Measured variables are presented in the text as mean ± SEM.

Paper III

Student's unpaired t-test was used to evaluate differences between treatments and Students paired t-test was used to evaluate changes over time for each treatment. The chosen level of significance was p≤5 %. Multiple comparisons were compensated for by a sequentially rejective Bonferroni procedure. Measured variables are presented in the text as mean±SEM.

RESULTS

Paper I - Substituting citrate for lactate in peritoneal dialysis fluid improves ultrafiltration in single dwells in rats.

In a dose-response evaluation, lactate was substituted with sodium citrate (0-15 mM) resulting in a significant correlation between osmotic ultrafiltration and citrate concentration. 10 and 15 mM substitution resulted in a significant increase in osmotic and net ultrafiltration (at 2 and 4 hours). At the concentration 10 mM citrate substitution, osmotic (at 2 hours) and net (at 2 and 4 hours) ultrafiltration were significantly increased. Increases in ultrafiltration were accompanied by an increase in glucose retention (Fig. 3 and 4). At 4 hours the increase in osmotic ultrafiltration corresponded to 10 %, and the gain in net ultrafiltration correspond to 20 % of the infused Pd fluid volume.

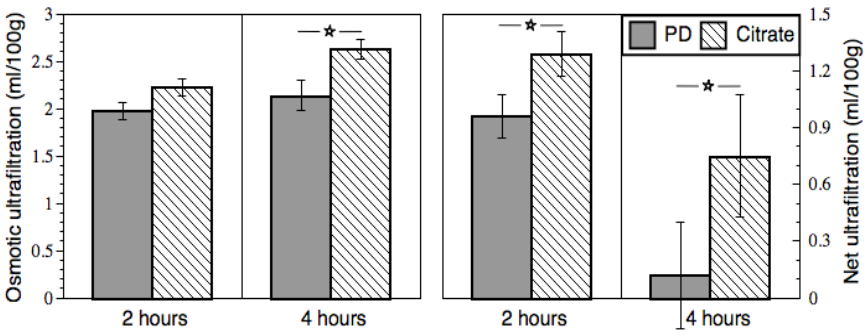

Figure 3. Osmotic and net ultrafiltration (ml/100 g body weight; mean±SEM) measured from albumin dilution. During substitution of 10 mM citrate for lactate significantly increased osmotic and net ultrafiltration at four hours; at two hours net ultrafiltration, but not osmotic ultrafiltration (p = 0.06) increased significantly. Citrate = 10 mM citrate; PD = 0 mM citrate. P ≤ 0.05*.

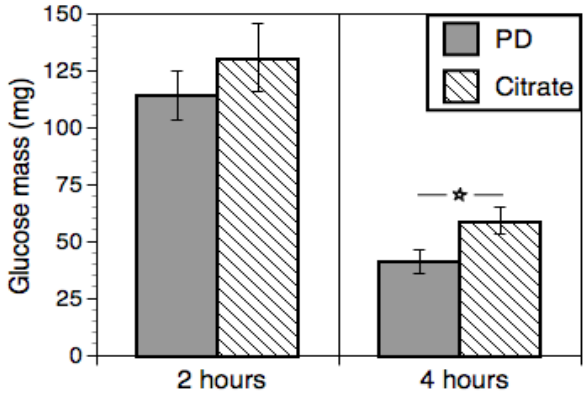

Figure 4. Intraperitoneal glucose mass was used to assess glucose transport from the peritoneal cavity. Treatment with 10 mM citrate for lactate substitution reduced glucose transport rate, as indicated by a significantly increased intraperitoneal glucose mass at 4 hours. Citrate = 10 mM citrate; PD = 0 mM citrate. p≤0.05*.

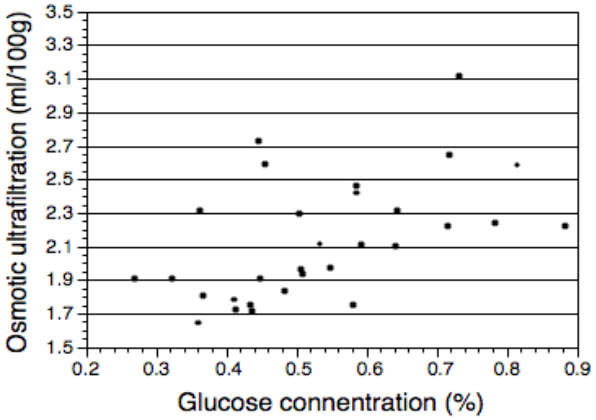

Figure 5. Intraperitoneal glucose concentration significantly correlated with osmotic ultrafiltration (ml/100 g body weight) at two and four hours (data corresponding two hours is shown), implying that increased retention of glucose resulted in the improved ultrafiltration induced by citrate PD fluid. The plot includes all rats, and citrate concentrations (0, 5, 10, and 15 mM).

A significant correlation between glucose concentration and osmotic ultrafiltration was found in the whole material (Fig. 5), indicating that changes in ultrafiltration were due to decreased glucose out-diffusion. In the control group, reabsorption of PD fluid, was 25.7 ± 3.6 µL/min, and in the 10 mM citrate substitution group it was 32.9 ± 1.6 µL/min. The difference between groups was not significant (p = 0.11), however there was a trend

in the same direction as the measured difference in net ultrafiltration (Fig. 3). Transport of glucose and urea was calculated as D/D_0 (dialysate concentration/initial dialysate concentration), and as the ratio D/P (dialysate concentration/plasma concentration) respectively. At 30 minutes dwell time, and 10 mM citrate substitution, urea D/P was significantly decreased (0.63 ± 0.022 compared to 0.72 ± 0.028).

In separate short dwell experiments the citrate, and calcium kinetics were evaluated. Ionised calcium concentration rose as citrate was eliminated. After roughly 30 minutes, citrate concentrations were half (5.5 mM) of those initially measured (10 mM), followed by an equally rapid increase in free calcium levels (0.6 mM and 1.2 mM at 30 and 60 minutes respectively) (Fig. 6).

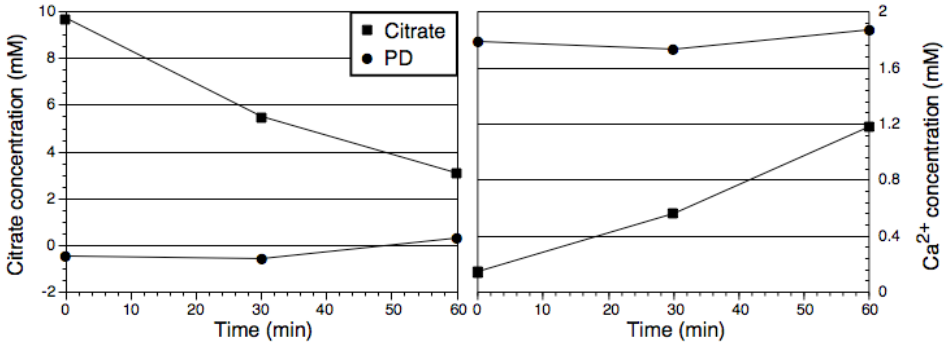

Figure 6. The kinetics of intraperitoneal citrate (left panel) and ionized calcium (right panel) studied in 1-hour dwells. Data show a rapid elimination of citrate and a simultaneous normalization of free calcium Citrate = 10 mM citrate; PD = 0 mM citrate.

Coagulation was not inhibited by citrate. However there was a tendency (71.4 ± 23.3 compared with 131.0 ± 25.0; p = 0.07) towards lower TAT-levels in citrate treated rats at time 0 compared to lactate treatment. Sampling time 0 corresponds to approximately 2 minutes of dwell time due to sampling procedure.

Paper II - Neuropeptide release in response to peritoneal dialysis exaggerates serum albumin loss and reduces ultrafiltration

A control, 20 ml, 2-hour dwell produced a rapid, and transient histamine release (ca 18 ng/ml), which peaked around 20 minutes (Fig. 7) of dwell time and corresponded to roughly 8 % of the total histamine release caused by the degranulating compound 48/80 (250 ng/ml) (Fig. 7). Blocking mast cell degranulation by the mast cell stabilizer doxantrazole significantly reduced histamine release, confirming that mast cells as the source of histamine.

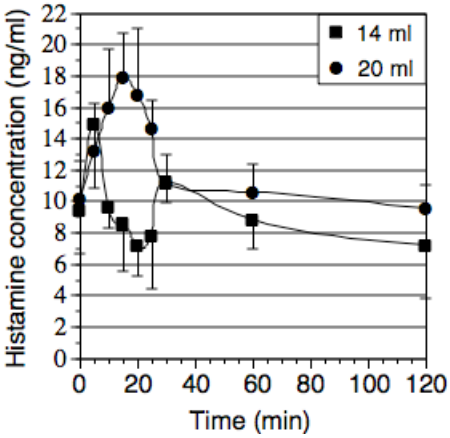

Figure 7. Histamine release pattern in two-hour dwells of 14 and 20 ml PD fluid respectively. The intraperitoneal histamine concentrations peaked within the first 30 minutes

Further assessment of histamine release showed that reducing the PD fluid volume from 20 ml to 14 ml, and inhibition of the NK1 receptor significantly reduced histamine release. However there was no visible effect on histamine secretion due to blocking the activation of the TRPV1 receptor or reducing the osmolarity to physiological levels (glucose concentration reduced from 4 % to 0. 5 %) (Fig. 8).

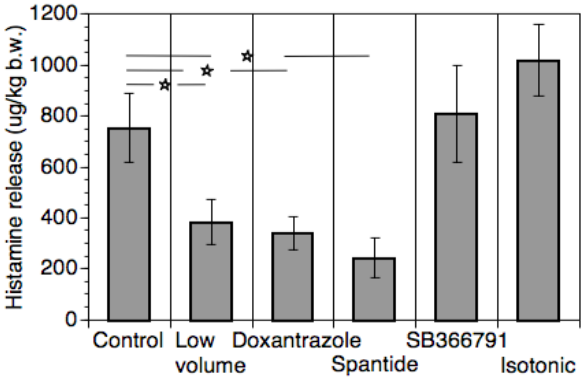

Figure 8. Low-volume PD dwells (14 ml), mast cell stabilizer Doxantrazole, and the SP receptor antagonist Spantide significantly reduced histamine release. The TRPV1 receptor blocker (SB366791) did not affect histamine release. Histamine release was averaged for the first 30 minutes of dwell time and calculated as histamine quantity/kg b.w. $p \leq 0.05$* (after correction for multiple comparisons). * = significant difference

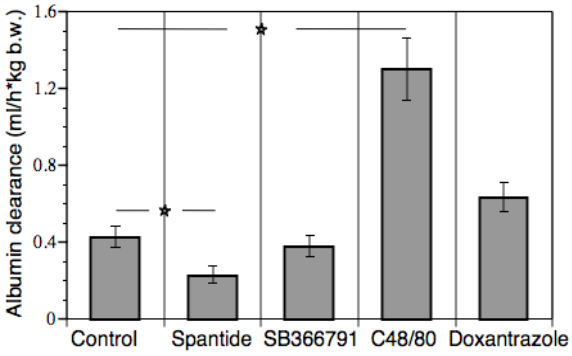

Figure 9. Plasma albumin leakage, calculated as average clearance of labelled bovine serum albumin from plasma to intraperitoneal space during two-hour dwells of 20 ml of lactate PD fluid. Mast cell degranulation by C48/80 significantly increased albumin leakage, and the substance P receptor antagonist Spantide significantly reduced albumin leakage compared with controls (a PD fluid dwell without additional treatment). * = significant difference

Different pharmacological interventions were performed at 20 ml PD fluid volume and affected transperitoneal transport in different ways. Receptor antagonist spantide (NK1 inhibitor) was the only treatment that significantly affected albumin clearance (Fig. 9). NK1 inhibition reduced clearance of albumin by approximately 50 %. The loss of albumin from plasma was measured as the clearance of ^{131}I bovine serum albumin into the PD fluid during the duration of the entire dwell, since an initial evaluation suggested that albumin was transported from plasma at a constant rate over the 120 minutes of dwell time. Lack of noticeable effect with mast cell stabiliser suggests histamine-independent mechanisms. However inducing a more complete mast cell degranulation by adding C48/80 to the PD fluid significantly increased albumin clearance. Additional investigation showed that ultrafiltration was affected by blocking activation of the CGRP receptor using the CGRP analogue, $CGRP_{8-37}$, thus inhibiting activation. Competitive inhibition of the CGRP receptor resulted in a significant increase in osmotic ultrafiltration and net ultrafiltration (Fig. 10). The increase observed during inhibition of the CGRP receptor was probable in part due to a significant increase in glucose retention at the end of 2-hour dwell time. (168.2 ± 22 compared to 112.3 ± 6 mg, P = 0.04). Usage of the TRPV receptor blocker, SB366791 resulted in a reduced reabsorption, and osmotic ultrafiltration (Fig. 10). Due to the assumed relation: net ultrafiltration = osmotic ultrafiltration – reabsorption, the effects of TRPV inhibition cancelled each other, resulting in no change to net ultrafiltration.

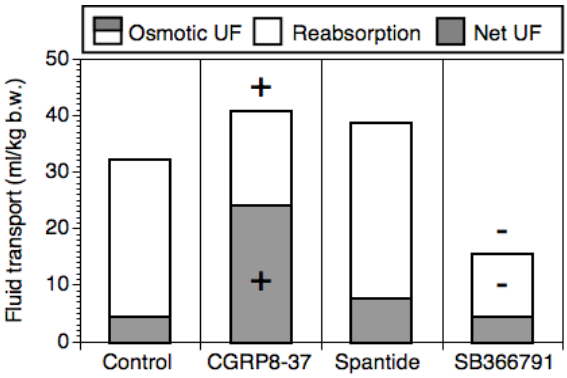

Figure 10. Total bar height corresponding to osmotic UF, is the sum of Net UF (grey bar) and Reabsorption (white bar). + and - represent significant increases and decreases respectively of the three transport components. Pharmacological treatments are compared with a standard PD dwell (20 ml lactate PD fluid). The CGRP receptor antagonist CGRP8-37 significantly increased Osmotic UF and Net UF whereas blockage of the TRPV1 receptor by SB366791 significantly reduced Reabsorption and Osmotic UF.

Paper III - Citrate-substitued PD fluid: effects on fibrosis, angiogenesis and catheter patency during 5 weeks of experimental PD in rats

The study in paper III was divided into two parts; an experimental evaluation of catheter patency and a second part which studied transperitoneal transport, fibrosis, and angiogenesis following long-term exposure to PD fluid with or without 10 mM citrate substitution.

The control group suffered no reduction in patency after 36 days of exposure to PD fluid; the citrate group (10 mM substitution) had 98.8 ± 1.2 % of average patency time, whilst exposure to regular PD fluid (lactate based) resulted in significantly decreased patency (54.7 ± 9.5 %) compared to the control and citrate groups. A decrease in patency was paralleled by an increase in fibrotic adhesions, estimated according to a scoring system (0 - 2). The control group scored 1.00 ± 0.45; the citrate group scored 1.12 ± 035; lactate group scored 2. However, only two animals were able to finish the study in the lactate group. The usage of heparinised catheters in the second experiment successfully reduced dropout and no significant difference in patency was observed between treatments.

Transperitoneal transport was measured from a single dwell at the onset of the study. The evaluation was performed using lactate PD fluid in the lactate and control groups, and citrate PD fluid in the citrate group. Net ultrafiltration was improved in the citrate group, but no differences were detected in osmotic ultrafiltration, and reabsorption (Table 2, Fig. 11). At this point of the study, none of the rats had been previously exposed to PD fluid and thus the control and the lactate group could be merged into one group (both were evaluated using lactate PD fluid). This combined group produced a significantly lower net ultrafiltration than the citrate group (Table 2, Fig. 11). A similar transport evaluation was performed at the end of the observation period (day 36) and a paired comparison with the initial measurements was performed. This evaluation showed that PD fluid exposure, both lactate and citrate, over time lead to a significant reduction in net ultrafiltration. Net ultrafiltration in the citrate group was still significantly higher than that of the lactate group. Ultrafiltration, and reabsorption in the control group did not change significantly.

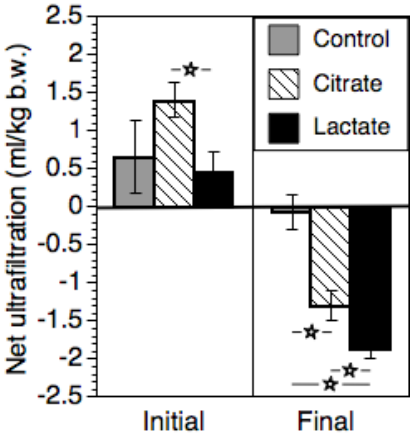

Fig. 11 Net ultrafiltration measured at the beginning and at the end of the study.
A paired test showed that PD fluid exposure (Citrate and Lactate groups) lead to a highly significant reduction in Net ultrafiltration over time.
* = significant difference

Exposure	Osmotic UF (ml/g b.w.)		Reabsorption (ml/g b.wt.)	
	Initial	Final	Initial	Final
Control	2.21±0.35	2.05±0.46	1.55±0.29	2.11±0.60
Citrate	3.25±0.34	1.38±0.45	1.84±0.38	2.67±0.47
Lactate	2.75±0.29	1.23±0.36	2.28±0.52	3.09±0.47
Expsore	Net UF (ml/g b.wt.)		Body weight (g)	
	Initial	Final	Initial	Final
Control	0.66±0.48	-0.07±0.23	346±10	459±13
Citrate	1.41±0.22^	-1.30±0.19*^	360±13	445±20^
Lactate	0.47±0.26	-1.86±0.13*	340±12	380±13*

Table 2: Initial and final body weights. Initial trans-peritoneal water transport (first PD fluid exposure), and final measurements after 5 weeks exposure to Citrate PD or Lactate PD, and control group (without continuous fluid infusions). Water transport was calculated as volume in ml/kg b.w. Data presented as mean±SEM. Osmotic UF – Reabsorption = Net UF.
The Initial evaluation was based on 7, 6 and 8 animals and the Final evaluation on 6, 6 and 5 animals in the Control, Citrate and Lactate groups respectively.
* = significantly different from Control, ^ = significantly different from Lactate.

In order to assess effects of citrate substitution on the development of peritoneal angiogenesis and fibrosis, morphological analyses were performed on biopsies from diaphragm and peritoneal windows. Angiogenesis from immuno-histochemical imaging, and fibrosis from the average thickness of submesothelial tissue stained for collagen. Measurement of vascularised area fraction, and vascular density did not vary significantly between treatments (Table 3). Quantification of fibrosis showed no significant difference between groups, although the control group showed a tendency towards less fibrosis (Table 2). Analyses of correlations between transperitoneal transport and peritoneal morphology did not detect any significant results.

Exposure	N	Vascularized area fraction (%)	Mean vascular density (100/µm)	Submesothelial thickness (µ)
Control	6	75.5±7.5	1.99±0.14	15.7±1.9
Citrate	5	66.0±5.4	3.14±0.61	19.9±4.7
Lactate	4	66.6±9.6	3.00±0.94	20.3±3.6

Table 3: Peritoneal angiogenesis and fibrosis determined morphometrically from mesenteric window preparations and from biopsies of parietal peritoneum (diaphragm). Blood vessels were stained using Rat Endothelial Cell Antigen 1 (RECA-1) Ab and observed fluorescence microscopy. Vascularisation was characterized as mean vascularised area fraction (in %) of total window area. Vessel density within the vascularised areas was determined as average number of vessels/100 µm. There were no significant differences between groups. Peritoneal fibrosis was determined as average thickness of submesothelial tissue layer, stained for collagen by Masson Trichrome staining of paraffin sections. The groups did not differ significantly, and submesothelial thickness was generally high compared with normal rat peritoneal morphology

DISCUSSION

The clinical basis of the studies

PD is now a major form of replacement therapy for insufficient renal function. Acute peritonitis remains a common complication despite vast improvements in dialysis fluids, and delivery systems and the chronic changes of the peritoneal membrane that lead to ultrafiltration failure still limit the technique survival of PD. Therefore improvements to efficiency in PD would compensate for existing shortcomings in the technique and work towards a general increase in worth and usefulness. This study was initiated in order to further characterise the acute inflammatory response to peritoneal dialysis fluids and its effects on transperitoneal transport and to study the possible benefits of citrate as a pharmacological additive used to inhibit acute and chronic inflammation in PD (Zareie *et al.*, 2001), thereby improving PD efficiency.

Histamine release is part of the inflammatory response to PD

There was a release of histamine during the first 30 minutes of the PD dwell, blockable by mast cell stabilisation, indicating that mast cell activation may be an early step of the acute inflammatory response to PD fluid exposure. This is the first study to show histamin release and mast cell activation during an acute PD dwell. Previous studies in rat have shown a decrease in peritoneal mast cell numbers in PD fluid after long-term PD (Hekking *et al.*, 2001) (Zareie *et al.*, 2001). The same authors noted a significant increase, and simultaneous in the omental mast cell population. A decrease in the mast cell population can also be seen in patients treated with peritoneal dialysis (Jimenez-Heffernan *et al.*, 2006). An explanation to the observed decrease in the mast cell population could be total degranulation and destruction of cells. However, the observed increase of histamine in paper I suggest partial degranulation of the mast cell population due to PD fluid toxicity, as total degranulation by C48/80 caused a much greater histamine release. The increased degranulation by C48/80 indicates that mast cells are still present after exposure to PD fluid.

Samples from collected PD fluid showed a significant increase in histamine indicating activation of resident mast cells and possibly basophiles. In order to elucidate the mechanisms behind mast cell activation we used different volumes, and osmolarites of PDF. Histamine levels increased significantly when the PD fluid volume was increased from 14 ml to 20 ml, initially suggesting a direct link between peritoneal distension and mast cell activation. Isoosmotic PD fluid induced the same levels of histamine as treatment with 20 ml lactate buffered PD fluid. Morphological studies have visualised a close interaction between nociceptive fibres and mast cells, normally closely associated with capillaries, suggesting an important role during inflammation; leading us to further study this interaction. Unexpectedly, histamine release was shown to be dependent on SP release. Blocking SP binding to its receptor, NK1, inhibited histamine release. The histamine released in the beginning of the PD dwell did not, contrary to expectations, affect albumin leakage, ultrafiltration or reabsorption of fluid from the peritoneal cavity. However, exaggerated histamine release by C48/80 administration did, as expected increase albumin leakage into the peritoneal space during the PD dwell.

Evidence for a neurogenic inflammatory response to PD

In paper I we failed to show that coagulation explained the increased osmotic and net ultrafiltration in animals treated with citrate substituted buffered PD fluid; calcium was only temporary eliminated and effects on coagulation were not synchronised with the sustained effects on glucose transport and ultrafiltration. Still believing that the benefits of citrate were explained by its calcium chelating properties; the results suggested delayed effects, probably mediated by cell like mast cells (Wei *et al.*, 1994), and sensory nerve endings (Garland *et al.*, 1995).

Although histamine was released during acute exposure to PD fluid, unexpectedly, blocking of histamine release from mast cells did not affect ultrafiltration or clearance of albumin from plasma to the intraperitoneal fluid. Closer examination showed that blocking of the substance P (SP) receptor (NK1) not only significantly decreased albumin clearance from plasma to PD fluid (Fig. 7) but also significantly reduced histamine release (Fig. 6), suggesting that actions of SP are histamine-independent, and that histamine release is greatly dependent on SP release. Capillary endothelial cells contain NK1 receptors; therefore it is possible that SP directly interacted with the endothelium in a mast cell-independent manner. It is therefore logical to expect that a reduced clearance independent of a parallel reduction in osmotic ultrafiltration (Fig. 8) suggests a permeability decrease for albumin. This is in line with documented direct effects of SP (Wick *et al.*, 2006) on blood vessels. Control experiments confirmed mast cells as the source of histamine (Fig. 6). Excessive mast cell degranulation with compound 48/80 (C48/80) showed that histamine produced responses in line with established information regarding mast cell and histamine involvement in inflammation. Although histamine did not affect peritoneal transport in the acute model, it is possible that increasing histamine levels, from recruited mast cells contribute to the chronic inflammation produced over time. It has recently been found that mast cells increase during long-term PD in rats, and are responsible for omental tissue remodelling (Zareie *et al.*, 2006).

Blocking of CGRP, increased osmotic ultrafiltration, accompanied by improved glucose retention at the end of the 2-hour dwell. According to Waniewski *et al* (1999), reducing CGRP-induced vasodilation should reduce glucose out-diffusion and increase osmotic ultrafiltration. The significant improvement of net ultrafiltration from blocking CGRP receptors was likely a combination due to a significant increase in osmotic ultrafiltration in combination with a trend towards reduced reabsorption (Fig. 8). In the present study, reabsorption was measured as the clearance of volume tracer from the peritoneal cavity. This is a common way to measure reabsorption in experimental PD but it implies a more restricted definition of reabsorption than that presented in the “INTRODUCTION” of this thesis. There, the term reabsorption was referring to all fluid transport from the peritoneal cavity to the intravascular space. Quantifying reabsorption as albumin clearance from the peritoneal cavity implicates that the reabsorbed fluid is lost through a pathway permeable to macromolecules like albumin. Others and we have shown that only roughly 10 % of the cleared volume tracer enters the circulation during the dwell, indicating that the remaining tracer is mostly lost into peritoneal tissue space as

oedema. CGRP is known to potentiate the permeability actions of SP and histamine is also likely that CGRP increases oedema, thus the effects seen on reabsorption are possibly due to a false positive feedback. Peritoneal oedema formation depends on intraperitoneal hydrostatic (Zakaria *et al.*, 2000, Flessner *et al.*, 1983) and colloid osmotic pressures (Wu *et al.*, 2009). There is also evidence that neurogenic inflammation can alter the contration state of the extracellular matrix, facilitating oedema formation (Reed *et al.*, 2001), possibly explaining why only 10 % of cleared volume tracer enters the circulation whilst remaining tracer is distributed in the peritoneal tissue (Rosengren *et al.*, 2004).

It is well established that the TRPV1 receptor mediates SP and CGRP release. Unexpectedly, blocking of TRPV1 did not produce results, which resembled those obtained from directly blocking the actions of SP and CGRP. Concerning fluid transport, TRPV1 receptor inhibition significantly reduced osmotic ultrafiltration, whereas CGRP receptor blockade significantly increased osmotic and net UF. Also, inhibiting SP binding to NK1 did not affect reabsorption in the manner blocking TRPV1 did. Thus the results suggest that there are other triggering mechanism, not involving TRPV1, involved in SP and CGRP release. Recent findings have shown that SP and CGRP not necessarily are expressed in the same neuron (Flessner *et al.*, 1983).

An evident question was whether mechanical and/or osmotic effects of the PD fluid triggered neurogenic inflammation, histamine release, and subsequent modifications on transperitoneal transport. Data from comparing different PD fluid volumes and osmolarites in the present study (Fig. 6) suggests that neuropeptide release was triggered by volume load rather than hyperosmolarity. TRPV2 and 4 are sensitive to sheer stress and membrane stretch (O'Neil and Heller, 2005). However, available data on pressure/volume relation indicates that the pressure only increases by about 2 mm Hg during the dwell at the PD volume (20 ml). Used in our study. There is, however evidence for a substantial oedema formation in the submesothelial connective tissue during the dwell. This is probably due to the low colloid osmotic pressure of the PD fluid (Wu *et al.*, 2009). Possibly, expansion of the tissue due to this oedema could activate mechanoreceptors of the TRPV family. Although results indicate a neurogenic component there some questions unanswered. We were not able to measure SP or CGRP; low quantities with localised actions, and degradation probably kept diffusion to a minimum. Thus, localised actions may result in a very low quantity reaching the peritoneal cavity, which then is further diluted by the PD fluid. Sampling volumes had to be kept to a minimum to reduce influence on transperitoneal transport measurements, therefore concentrating techniques were not used. Disparity in transperitoneal transport data from blockage of neurotransmitters directly vs. blockage of TRPV1 made it hard to confirm activation of nociceptive neuron, and receptors belonging to the TRPV family. Thus, in order to confirm neurotransmitter release as the result from neurogenic activation, a more thorough blocking of receptors belonging to the TRPV family should be attempted. SP, and CGRP are stored within granules; as a consequence it is hard to use quantitative methods from whole tissue. It is possible that detection of neurotransmitters with immuno-histochemical techniques could be done after improving our methodology. It is clear that further studies are needed.

PD catheter causes severe angiogenesis and fibrosis

Compared to an earlier study conducted by our group we received a surprisingly strong reaction in peritoneal tissues. The earlier long-term evaluation of lactate PD fluid exposure, in which fluid was injected by needle showed a low background of fibrosis and angiogenesis (Albrektsson *et al.*, 2006); lactate PD caused vascularisation of roughly a 1 mm wide zone of the mesenteric windows, and to thickening of the submesothelial tissue up to 20 µm. Control animals hardly showed any signs of vascularisation and submesothelial thickening (Albrektsson *et al.*, 2006) compared with the present study, which exhibited a 75 % vascularisation and 16 µm in the control group. PD fluid exposure showed a tendency towards a higher density in the vascularised area, suggesting that the catheter significantly contributes to the peritoneal inflammation. Flessner *et al* (2007) showed that catheters not only cause a strong inflammation but also potentiates the acute inflammatory reaction of standard PD fluid infused through the catheter. Although citrate could not block angiogenesis and fibrosis, the citrate group had almost 100 % functionality catheter patency in contrast to the lactate group having 55 %. Although catheters are rarely encapsulated in PD patients (Kazory *et al.*, 2007), there are other patency related problems (Santarelli *et al.*, 2006), e.g. omental wrapping, which cause considerable problems in clinical practice. However, in long-term patients (8 years) encapsulation can be serious problem resulting in the removal of the patient from PD. In fact, roughly 20 % of the patients on PD for eight years suffer from catheter occlusion by encapsulation (van Westrhenen *et al.*, 2007), suggesting that occlusion becomes increasingly problematic over time. Activation of fibrinolysis pathways have been shown to have positive effects on catheter occlusion (Zorzanello *et al.*, 2004), suggesting that fibrin clot formation is the governing mechanism of action (Holmdahl *et al.*, 2001). Formation of peritoneal adhesion is initiated by fibrin deposition, followed by a fibrin dissolution phase in which fibrin is gradually degraded and replaced with extracellular matrix components, e.g. collagen in the third stage called "the fibrous phase". Replacement of fibrin requires recruitment of various cells, and thus activation of various Ca^{2+} pathways. Another explanation could be partial coagulation inhibition. Earlier studies have shown that heparin improves technique survival (De Vriese *et al.*, 2002). Animals in the citrate group, paper I, displayed signs of coagulation inhibition during the first 30 minutes that could possibly explain the greatly improved results in paper III (Fig. 9).

Citrate substitution has acute and long-term beneficial effects on transperitoneal transport

Substituting 10mM lactate with citrate, a strong calcium chelator, we received a significant increase in osmotic and net ultrafiltration. Since effects on ultrafiltration persisted well after two hours of dwell time when calcium levels had normalised, they were not directly connected to the calcium concentration.

We initially speculated that the coagulation and complement cascades initiated the triggering mechanisms behind an acute peritoneal inflammation and the subsequent chronic damage. According to theories, the alternative pathway is activated by surfaces absent of complement inactivators. The catheter is one apparent structure present during PD, which does not have any inactivators. Previous data from our group has

shown that complement is also activated in animals lacking catheter, suggesting several triggering mechanisms. The mechanisms behind the intraperitoneal activation of coagulation and complement are still elusive, however, it is known that they are highly dependent on free Ca^{2+}.

Despite a lack of an obvious effect on coagulation, citrate has the possibility to interact with numerous inflammatory processes and mediators. Measurement from shorter, 1-hour dwells indicate that there might have been a short-lived, less than 30 minutes, anticoagulatory effect that might have contributed to the effects seen on ultrafiltration and glucose. It is therefore more likely that the gain in ultrafiltration is the result of indirect mechanisms, via cellular responses, initiated during the first 30 minutes of dwell time. Calcium is necessary in many biological processes besides complement and coagulation activation; also cell recruitment and nociceptive nerve activation depend on extracellular calcium. It is not unlikely that citrates chelating activity somehow is responsible for the ca 25 % gain in net ultrafiltration (Fig. 3) seen during single dwells and could thus be of clinical importance. A 36-day study to evaluate the persistent effects of citrate showed that the initial benefits of citrate remain during the study; however, citrate did not have any additional benefits on ultrafiltration. Both citrate and lactate dialysis fluid suffered equally from reduced net ultrafiltration over time compared with untreated control animals.

Although there was no statistically significant correlation between citrate dose and glucose concentration ($p = 0.08$ and 0.13 at 2 and 4 hours respectively) that directly explains increases in ultrafiltration, data implies that there is indeed a connection as glucose mass did significantly increase with 10 mM citrate substitution at 4 hours (Fig. 4). Also, glucose retention significantly correlated with osmotic ultrafiltration in the material as a whole (Fig. 5), suggesting that citrate increased ultrafiltration indirectly via a reduced glucose transport. While it is unclear by which pathways citrate increases glucose retention, results from the present study indicate that a change in the relation between filtration and diffusion is the cause. This is supported by our data on urea transport (paper I), which is reduced by citrate. Whether such a change is a direct or indirect effect remains unclear. Results from paper II suggest an indirect mechanism via neurogenic inflammation, as blocking activation of the CGRP receptor also significantly increased osmotic, net ultrafiltration, and increased glucose mass (retention).

Citrate substitution introduces direct osmotic effects via two mechanisms. Firstly, each citrate ion is accompanied by three sodium ions, two of which are dissociated and osmotically active. Compared with lactate, this adds one extra sodium ion for each citrate ion substituted. The extra sodium ions will diffuse together with chloride rather than with citrate according to the Gibbs-Donnan effect. Secondly, citrate is a larger molecule than lactate and will therefore be retained intraperitoneally for longer time. Thus, a difference in intraperitoneal concentration between lactate and citrate develops over dwell time, reaches a maximum and declines as both molecules diffuse out. Both types of osmotic effects may be simulated theoretically in order to estimate their contribution to the increase of osmotic ultrafiltration induced by citrate substitution.

Such a simulation was based on diffusion constants for citrate and lactate measured in humans. Here, the diffusion constant for citrate was 70 % of that measured for lactate. This ratio was applied to the rat model and combined with measurements of osmotic ultrafiltration and of sodium, citrate and glucose transport. Diffusion time histories for those solutes were calculated by solving a differential equation based on their diffusion constants and a polynomial best fit of the experimentally obtained osmotic ultrafiltration time history (Fig. 12). The contributions of osmotic ultrafiltration from the difference between lactate and citrate and from the extra sodium ions were calculated by numeric integration, applying the reflection coefficients of sodium, chloride, citrate and lactate. It was shown that, at 10 mM/L citrate substitution, both contributions together corresponded to 3.5 % of the total osmotic ultrafiltration created by 120 mM/L of glucose.

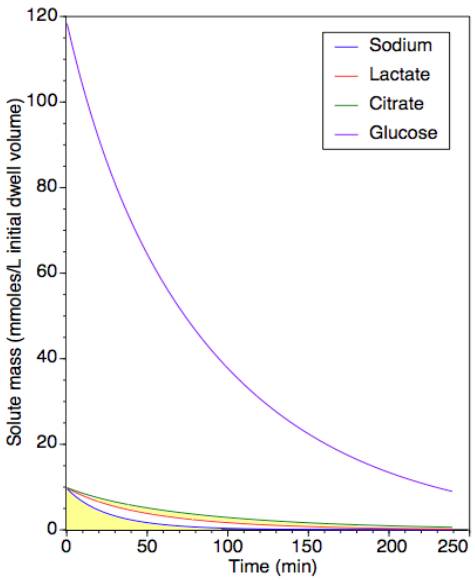

Figure 12. Plot of the theoretically simulated mass transfer of different solutes during a 4-hour PD dwell in the rat. The osmotic effect of 10 mM/L citrate for lactate substitution is created by 10mM/L added sodium ions (yellow area below the sodium curve) and the difference in particle number between 10 mM/L citrate and 10 mM/L lactate (yellow area between the citrate and lactate curves). The total osmotic effect of a lactate-based PD fluid is estimated as that of 120 mM/L glucose symbolized by the area below the glucose curve.

In order to compensate for the impact of the osmotic effect of citrate, 3.5% was added to the values for osmotic ultrafiltration in the lactate group (increasing net ultrafiltration by the same volume increment) and data was re-evaluated statistically. The increase of osmotic and net ultrafiltration induced by citrate was still statistically significant but reduced in magnitude by 0.07 ml/100 g BW (Fig.3).

The improved osmotic and net ultrafiltration shown in paper I was confirmed at the beginning of a 5-week evaluation of citrate on transperitoneal transport (Table 2). Although the significant gain in osmotic and net ultrafiltration was conserved over the study, osmotic and net ultrafiltration of both the citrate and lactate groups deteriorated compared to control animals (Fig. 9).

Ultrafiltration failure is often associated with angiogenesis in PD; therefore we tried to find any correlation between angiogenesis and ultrafiltration failure. However, no such correlation was found, angiogenesis did not differ significantly between the groups (Table 2). Neither did we find a significant difference between fluid treated groups and control animals. However, combining all treated animals into one group visualised a significant increase in vascularisation compared with control animals. A possible explanation as to why a lower vascular density in the control group was not discovered could be lack of statistical power. A recent study of PDGF-induced peritoneal angiogenesis (Cina *et al.*, 2009) verifies our findings and conclusions that angiogenesis may be necessary but insufficient for ultrafiltration failure to develop. It is more likely that new vessels exhibit different properties that could explain ultrafiltration failure.

Implications for the development of PD

An improvement in technique survival would make PD more competitive and close the gap in effectiveness between PD and haemodialysis as therapy for renal failure. Results from paper I show that substituting 10mM lactate by citrate improves net ultrafiltration in rat. A recent clinical study by our group shows similar results, although there are some differences. While our study in rat suggested that increase in net ultrafiltration was partly due to increased glucose retention (glucose mass), the study by Braide *et al*. (2009) showed no such correlation. Instead data indicated that improvements to ultrafiltration were at least in part due to a decreased reabsorption. The implications for the improvement of PD are interesting. Although heparin has showed promising results (Sjoland *et al.*, 2004, Braide *et al.*, 2009), both in acute and chronic patient trials there are some issues associated with the usage of heparin. The half-life is 3-4 hours and the elimination is up to 50 % renal, which could cause unnecessary stress on patients with partial kidney dysfunction. In clinical practice, concerns regarding low calcium levels created in the peritoneal cavity by citrate may potentially be harmful to cells, especially mesothelial cell and peritoneal resident cells. Severe depletion of calcium (below 4 μM) may result in disruption to the cytoskeleton and irreversible cell damage occurs in concentrations below 0.1 - 0.4 μM. However, measurement of calcium in paper I had almost returned to normal concentrations after 2 hours, and lowest concentration of calcium in our study was 150 μM. No negative effects were observed. Plasma calcium levels in patients during a single dwell of 5 mM citrate substitution dropped from 1,20 mM to 1.14 mM at 60 minutes, corresponding to a significant reduction compared with standard PD fluid (Braide *et al.*, 2009). However, calcium levels slowly normalised and at the end of the 4-hour dwell plasma calcium levels were completely normalised (Braide *et al.*, 2009). In the peritoneal cavity, the calcium content was gradually increasing during the dwell and some citrate-bound calcium was lost with the drain of PD fluid. This moderate calcium loss from stores mainly in the skeleton can probably be compensated for by dietary calcium supplementation. Therefore exposure to citrate at low concentrations seems to be safe. Long-term studies of citrate-substituted PD fluids are required to determine any effects on peritoneal membrane integrity.

In paper III we show that directly blocking the activities of substance P and CGRP has beneficial effects on albumin clearance, net ultrafiltration, and reabsorption. If the results translate to PD patients, intervention with neuropeptides mechanisms of action then has the potential to improve PD efficiency. Patients with renal dysfunction on PD often suffer from peritoneal fibrosis caused by the deposition of fibrin, which is up regulated by mast cells, fibroblasts, and macrophages. Peritoneal fibrosis and catheter occlusion is a major complication in PD (Santarelli *et al.*, 2006) and can result in the removal of the patient from PD. In a recent study, adhesion formation after peritoneal surgery was reduced or prevented entirely when rinsing the peritoneum after surgery with NK1 inhibitor (Reed *et al.*, 2008), suggesting additional benefits for peritoneal dialysis patients. Prevention of peritoneal fibrosis should enhance technique survival and patient comfort. Blocking activation of the NK1 receptor and a subsequent increase in fibrinolytic actions was explained by increases in plasminogen activator, matrix metalloproteinases, and reduction in oxidative stress, proposing that inhibition of a neurogenic inflammation not only would result in an acute improvement of net ultrafiltration but also in a reduction in angiogenesis and fibrosis during long term

treatment. Together, evidence hint at a number of different interventions related to neurogenic inflammation with potential to improve PD efficiency and technique survival.

FURTHER INVESTIGATIONS

Questions remain to be answered, and as such there are results that need to be further analysed. Inflammation, especially the importance of the neurogenic component, and how mechanisms are propelled down streams needs to be further studied in order to better understand the mechanisms behind peritoneal fibrosis, angiogenesis, and a subsequent degradation of transperitoneal transport. Primarily, the regulation of cytokine production should be evaluated. Thus, which cytokines have up regulated transcription? Real time PCR is a powerful technique able to quantify up-regulation of proteins with very high precision and could be a useful tool. Secondly, the involvement of specific neurogenic receptors should be studied. A better understanding of which receptors are activated, could explain how inflammation is trigged and how the signal is mediated. Understanding which TRP receptors are involved could have an impact on the improvement of PD. Another question needing answer is whether citrate acts through the neurogenic inflammation or if there are other mechanisms of action to be discovered.

CONCLUSIONS

The aim of this thesis was to attempt to explain the mechanism that trigger inflammation during instillation of PD fluid and to clarify the effects of sodium citrate as an additive to PD fluid.

The included studies have shown:

Citrate causes a significant increase in osmotic and net ultrafiltration accompanied by increased glucose retention

Citrate gains no obvious benefits over lactate over time, measured as angiogenesis, and fibrosis. However, the initial advantage in net ultrafiltration gained by citrate remain over time.

Mast cell activation with subsequent histamine release is an early step during the acute reaction to PD

Blockade of substance P leads to reduced clearance of albumin from plasma to blood, and inhibits activation of mast cell degranulation

Blockade of CGRP causes an increase in net ultrafiltration, resulting from increased osmotic ultrafiltration accompanied by a tendency to reduced glucose out-diffusion

There is reasonable evidence to suggest that neurogenic inflammation is an important early step in the acute inflammatory response to PD fluid exposure.

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