ACUTE AND CHRONIC EFFECTS OF PERITONEAL DIALYSATE EXPOSURE ON THE FUNCTION AND STRUCTURE OF THE PERITONEAL MEMBRANE

Siska Mortier

Promotoren:
Prof. Dr. Norbert H. Lameire
Prof. Dr. An S. De Vriese

Thesis submitted in fulfillment of the requirements for the degree of ‘Doctor in de medische wetenschappen’.

Opgedragen aan mijn ouders.
The road goes ever on and on
Down from the door where it began
Now far ahead the road has gone
And I must follow, if I can,
Persuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet,
And whither than? I cannot say.

*The studies were supported by:*
Grant from Fresenius Medical Care, Bad Homburg, Germany (2000-2003)
Grant from Baxter R&D Europe, Nivelles, Belgium (2000-2003)
# Table of Contents

LIST OF ABBREVIATIONS 8

CHAPTER 1 GENERAL INTRODUCTION AND OBJECTIVES 11

1.1 Peritoneal dialysis as a renal replacement therapy 13

1.2 The peritoneal membrane: more than a physical barrier 14

1.3 Complications during long-term PD 17

   Ultrafiltration failure (UFF) 17

   Peritonitis 18

1.4 Peritoneal membrane biology during long-term PD 18

1.5 Conventional and new PDF 19

1.6 Animal models in peritoneal dialysis research

   Mortier S, Lameire NH, De Vriese AS

   Submitted 25

1.7 Study aim 40

CHAPTER 2 ACUTE PERITONEAL EXPOSURE USING THE INTRAVITAL MICROSCOPY TECHNIQUE 51

2.1 Hemodynamic effects of peritoneal dialysis solutions on the rat peritoneal membrane: role of acidity, buffer choice, glucose concentration and glucose degradation products

   Mortier S, De Vriese AS, Van De Voorde J, Schaub TP, Passlick-Deetjen J, Lameire NH


2.2 Effects of conventional and new peritoneal dialysis fluids on leukocyte recruitment in the rat peritoneal membrane

   Mortier S, De Vriese AS, McLoughlin RM, Topley N, Schaub TP, Passlick-Deetjen J, Lameire NH

   *J Am Soc Nephrol* 2003;14:1296-1306 79
2.3  The effects of peritoneal dialysis solutions on peritoneal host defense  
Mortier S, Lameire NH, De Vriese AS  
Perit Dial Int 2004; 24:123-138

CHAPTER 3  A STANDARD RAT MODEL OF CHRONIC PERITONEAL EXPOSURE

3.1  The effects of heparin administration in an animal model of chronic peritoneal dialysate exposure  
Perit Dial Int 2002;22: 566-572

3.2  Antibiotic administration in an animal model of chronic peritoneal dialysate exposure  
Perit Dial Int 2003;23:331-338

CHAPTER 4  MEMBRANE ALTERATIONS AFTER CHRONIC EXPOSURE TO CONVENTIONAL AND NEW PDF

4.1  Long-term exposure to new peritoneal dialysis solutions: Effects on the peritoneal membrane  
Mortier S, Faict D, Schalkwijk CG, Lameire N, De Vriese AS  
Kidney Int 2004; 66:1257-1265

4.2  Benefits of switching from a conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model  
Mortier S, Faict D, Lameire NH, De Vriese AS  
Kidney Int, accepted
4.3 Inhibition of the interaction of AGE-RAGE prevents hyperglycemia-induced fibrosis of the peritoneal membrane
De Vriese AS, Flyvbjerg A, Mortier S, Tilton RG, Lameire NH
J Am Soc Nephrol 2003;14:2109-2118

4.4 Glucotoxicity of the peritoneal membrane: the case for VEGF
De Vriese AS, Mortier S, Lameire NH
Nephrol Dial Transplant 2001;16:2299-2302

CHAPTER 5 ESTABLISHED RAT MODELS OF CHRONIC RENAL FAILURE

5.1 Myofibroblast transdifferentiation of mesothelial cells is mediated by RAGE and contributes to peritoneal fibrosis in uremia
Mortier S, Tilton RG, Lameire NH, De Vriese AS
Submitted

CHAPTER 6 SUMMARY AND FUTURE PERSPECTIVES

DANKWOORD
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_w )</td>
<td>Wall shear rate</td>
</tr>
<tr>
<td>( \alpha )-SMA</td>
<td>( \alpha )-smooth muscle actin</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation endproduct</td>
</tr>
<tr>
<td>ALE</td>
<td>Advanced lipoxidation endproduct</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous ambulatory peritoneal dialysis</td>
</tr>
<tr>
<td>CEL</td>
<td>Carboxyethyllysine</td>
</tr>
<tr>
<td>CFS</td>
<td>Cell free supernatans</td>
</tr>
<tr>
<td>CML</td>
<td>Carboxymethyllysine</td>
</tr>
<tr>
<td>CNS</td>
<td>Coagulase-negative staphylococci</td>
</tr>
<tr>
<td>CrCl</td>
<td>Creatinine clearance</td>
</tr>
<tr>
<td>C Sp</td>
<td>Corynebacterium species</td>
</tr>
<tr>
<td>CTC</td>
<td>Catheter tip culture</td>
</tr>
<tr>
<td>D</td>
<td>Luminal diameter</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DC</td>
<td>Dialysate culture</td>
</tr>
<tr>
<td>2,4-DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>D/P</td>
<td>Dialysate to plasma ratio</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's balanced salt solution</td>
</tr>
<tr>
<td>EC</td>
<td>Enterococcus</td>
</tr>
<tr>
<td>ECi</td>
<td>Enterobacter Cloacae</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial NO synthase</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FB</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GDP</td>
<td>Glucose degradation products</td>
</tr>
<tr>
<td>5-HMF</td>
<td>5-hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPMC</td>
<td>Human peritoneal mesothelial cell</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>MC</td>
<td>Mesothelial cell</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MGO</td>
<td>Methylglyoxal</td>
</tr>
<tr>
<td>MN</td>
<td>Monocyte</td>
</tr>
<tr>
<td>MTAC</td>
<td>Mass transfer area coefficient</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>PAP</td>
<td>Peroxidase-antiperoxidase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td>PDF</td>
<td>Peritoneal dialysis fluid</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostaglandin I₂</td>
</tr>
<tr>
<td>PM</td>
<td>Peritoneal membrane</td>
</tr>
<tr>
<td>PM∅</td>
<td>Peritoneal macrophage</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>P Sp</td>
<td>Propionibacterium species</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for AGE</td>
</tr>
<tr>
<td>RCO</td>
<td>Reactive carbonyl compound</td>
</tr>
</tbody>
</table>
RRT  Renal replacement therapy  
SA   Staphylococcus aureus  
SEM  Standard error of the mean  
SFM  Serum free medium  
SV   Streptococcus viridans  
TBS  Tris buffer  
TGF-β Transforming growth factor β  
TNF-α Tumor necrosis factor α  
TXB₂ Thromboxane B₂  
UFF  Ultrafiltration failure  
V_{RBC} Red blood cell velocity  
VCAM Vascular cell adhesion molecule  
VEGF Vascular endothelial growth factor  
WBC  White blood cell
Chapter 1

General introduction and objectives
1.1 Peritoneal dialysis as a renal replacement therapy

End-stage renal disease (ESRD) presents a major medical challenge as the number of people developing ESRD increases annually with 6 to 8%. In 2003, the prevalent ESRD population is estimated at 1.681,000 worldwide, of which 22.7% is alive with a renal transplant. Although renal transplantation is the most optimal treatment, approaching most closely the function of the native kidneys, the majority of ESRD patients are, due to shortage of donor kidneys, subjected to alternative management forms of chronic renal failure, such as haemodialysis or peritoneal dialysis (PD).

The introduction of the concept 'continuous ambulatory peritoneal dialysis' (CAPD) by Popovich et al. was initially met with a lot of criticism, but has finally evolved into a successful primary form of dialysis therapy. CAPD involves 4 to 5 daily exchanges of 1.5 to 3 litres of a peritoneal dialysis fluid (PDF) into the peritoneal cavity through a permanently implanted Tenckhoff catheter in the abdominal wall. During dwell periods of 4 to 8 h, the peritoneum functions as a natural filter resulting in elimination of waste products and excessive fluid. The PDF induces a fluid shift from the blood capillaries to the dialysis solution by a process termed osmotic ultrafiltration. Simultaneously, uremic solute movement occurs by diffusion or convective transport, respectively driven along an chemical gradient and by ultrafiltration across the membrane. After the appropriate dwell period, the waste-containing dialysate is drained out of the peritoneal cavity and fresh PDF is instilled (Figure 1).

Today, 15% of the world dialysis population uses PD. This technique is ideal for patients willing to undertake home dialysis, which imposes less restrictions on the patients’ freedom and mobility. In addition, PD turns out to be an advisable renal replacement therapy (RRT) for the use in children (in whom circulation access and immobility are often problematic), patients with diabetes (better blood sugar control) and cardiovascular instability (lower haemodynamic stress, thanks to gradual fluid shifts).
1.2 The peritoneal membrane: more than a physical barrier

For efficient PD, the peritoneum must function as a dialyzing membrane, although this is not its primary purpose. The visceral and parietal peritoneum, respectively lining the visceral organs and the abdominal wall, both constitute the large serous peritoneal membrane (PM), which covers the abdominal cavity. Anatomically, this membrane consists of different cellular and extracellular components, each serving a specific set of physiological functions (Figure 2).

The mesothelium consists of a continuous monolayer of mesenchymal cells, joined by various intercellular junctions. The mesothelial cells are covered with microvilli and characterized by the presence of intracellular pinocytotic vesicles, rough endoplasmatic reticulum and lamellar bodies. The numerous microvilli and the secretions of the lamellar bodies (such as phosphatidylcholine) support the mesothelium in its function as protective barrier and smooth surface, which avoids friction between organs and opposing tissues. Furthermore, mesothelial cells have proven to act as a biosynthetic active layer, secreting cytokines and vasoactive agents, allowing them respectively to participate in the recruitment of inflammatory cells and modulate the peritoneal microcirculation.

The basal lamina, underlying the mesothelial cells, is a thin laminar network containing collagen, proteoglycans and glycoproteins. This functions as a support for the mesothelium and as selective cellular barrier, allowing macrophages and lymphocytes to pass through it, but preventing fibroblasts from the underlying connective tissue to contact the mesothelial cells.

The submesothelial interstitial tissue is composed of a complex network of collagen fibres, elastin fibres, proteoglycans, adhesive proteins and a salt solution. This extracellular matrix (ECM) provides mechanical strength to the tissue and acts as a framework for cell attachment and migration. Fibroblasts produce macromolecules that constitute the ECM and, like mesothelial cells, generate cytokines and chemokines, which contribute to the recruitment of leukocytes. Resident macrophages, underneath the basement membrane and around the blood vessels, are activated by invading microorganisms or their secreted products and subsequently ensure a broad variety of pro-inflammatory cytokines. In addition, interaction of macrophages with microorganisms also
Figure 1: (A) Schematic presentation of the peritoneal cavity with an implanted catheter. (B) The abdomen is filled with dialysis fluid via the catheter, (C) the fluid then removes toxins and water from the blood through the peritoneum, (D) the dirty fluid is drained out and replaced with new, clean fluid for further dialysis. (E) Detail of the peritoneal membrane which forms a semi-permeable barrier between the blood and abdominal compartment, (F-G) the uremic toxins are absorbed through the membrane along the chemical gradient by a process called diffusion. Additionally, the sugar in the dialysis fluid is 'sucking' the water across the membrane out of the blood by a process called ultrafiltration, (H) the 'dirty' fluid is discarded and replaced by fresh dialysis solution.
causes an increased secretion of chemoattractantia, resulting in emigration of leukocytes from the bloodstream to the site of inflammation. Next to macrophages, mastcells reinforce the peritoneal response to injury by releasing a large amount of inflammatory mediators and chemoattractantia.

Other important structures perfusing the interstitium at variable distance from the mesothelium are blood and lymph vessels. Peritoneal blood vessels, especially capillaries and post-capillary venules, take care of nutrient transport to and waste removal from tissues and organs within the peritoneal cavity. Lymphatic vessels, however, represent a drainage system, which helps to maintain the normal level of the intraperitoneal fluid (< 50 ml in non-PD patients), by returning excessive fluid and proteins to the systemic circulation. Furthermore, they can also participate in host defence by removing foreign bodies from the peritoneal cavity.

1.3 Complications during long-term PD

The success of PD as a long-term RRT completely depends on the preservation of the peritoneal membrane function responsible for the removal of waste products, salts and excessive body fluid. While a technique survival of 50% was observed for patients on PD for 3-5 years, the number of patients surviving on PD longer than 7 years falls below 10%.

The two main complications of PD resulting in technique failure are ultrafiltration failure (UFF) and recurrent or severe peritonitis episodes.

Ultrafiltration failure (UFF)

Loss of ultrafiltration capacity increases with time spent on treatment. The risk of developing clinically relevant UFF is assumed to be approximately 35% after 6 years. UFF can be ascribed to four main mechanisms: an increase in peritoneal transport of small soluta resulting in a loss of the osmotic gradient (type I); an impaired aquaporin-mediated transcellular water transport (type 2); an extremely small peritoneal surface area resulting in a hypopermeable peritoneum (type 3) and high lymphatic absorption (type 4). Type I UFF is by far the most frequent cause of ultrafiltration dysfunction and several studies demonstrated that this was associated with the presence of a large vascular surface area in
long-term PD patients \(^1\). This increased effective peritoneal surface area must be due to either recruitment of previously unperfused capillaries or to formation of new capillaries, as a change in the total size in the peritoneum is very unlikely \(^1\).

**Peritonitis**

Until now, it is not known how frequently bacteria penetrate into the peritoneal cavity during PD, though different sources of infection have been identified. The two principal routes for the development of peritonitis are 1) transluminal, resulting from contamination during dialysate exchange and 2) periluminal, caused by microorganisms that spread into the peritoneum along the tract of the catheter \(^1\). The risk that bacterial entrance will eventually induce peritonitis depends on a delicate balance between the quantity of bacteria invading the peritoneal cavity and the status of the local host defence system. The causative pathogens isolated from PD peritonitis are predominantly gram-positive microorganisms, with *coagulase-negative staphylococci* being responsible for 30-40\% of the peritonitis episodes. In the mid 1940s when PD was introduced, peritonitis rates could mount to 6.28 episodes per year \(^1\). Today, thanks to significant improvements in connection technology (Y-connection set and ‘flush and fill’ method), which reduced touch contamination considerably, the frequency of peritonitis has decreased to 0.5 episodes per year or even less \(^1\). In addition, the pattern of causative pathogens has changed, with a reduction in the proportion of gram-positive peritonitis and a relative increase in gram-negative infections. Nevertheless, peritonitis remains one of the principal causes of hospitalization, transfer to haemodialysis or even death in PD patients.

**1.4 Peritoneal membrane biology during long-term PD**

Long-term PD is associated with the development of functional and structural alterations of the peritoneal membrane. The most common change in the peritoneal function is a rise in small solute transport, resulting in a rapid dissipation of the transperitoneal osmotic gradient and a decrease of ultrafiltration capacity \(^1\). The pathogenesis of these functional changes still remains poorly understood, but there is increasing evidence that they reflect underlying structural changes of the membrane. Peritoneal biopsies indeed
demonstrated that the peritoneal membrane of long-term PD patients is featured morphologically by mesothelial, interstitial and vascular changes, such as loss or degeneration of the mesothelium, submesothelial thickening (fibrosis), neoangiogenesis, hyalinization of blood vessel media and reduplication of the vascular as well as the mesothelial basement membrane \(^{21-24}\).

The Biopsy Registry Study Group were the first to establish that these interstitial and vascular changes are also present in predialysis and hemodialysis patients, implicating the uremic syndrome as causative. Furthermore, the extent of the alterations appeared to increase with longer time spent on PD, suggesting an additional effect of PDF. Finally, the most pronounced alterations occurred in patients who experienced clinical problems, including recurrent episodes of peritonitis, indicating the importance of infection. Conversely, as patients with UFF were featured by these pronounced changes, this underlines the clinical importance of peritoneal membrane alterations \(^{25}\).

1.5 Conventional and new PDF

Conventional PDF does by no means resemble the physiologic peritoneal fluid and contains several bioincompatible components with the potential to induce peritoneal injury (table1). The routinely used PDF is hyperosmolar (385-511 mOsmol/L) due to the presence of high glucose concentrations (83-236 mmol/L), creating a transperitoneal osmotic gradient, necessary to obtain efficient ultrafiltration. Heat sterilization of the PDF causes the glucose to degrade and induces the formation of toxic glucose degradation products (GDPs). Lowering the pH of the solution to 5.2 partially prevents, though does not completely exclude GDP formation in conventional PDF. In addition, due to the chemical instability of bicarbonates during heat sterilization and the risk of calcium carbonate precipitation, lactate (35-40 mmol/L) is used as the buffer system instead of its physiological counterpart (Table 1) \(^{26}\).

PDF bioincompatibility is a major concern and has given impetus to the development of alternative, more biocompatible solutions. Whereas conventional PDF were primarily designed to offer an adequate elimination of metabolic waste products, to maintain fluid and electrolyte balance and to correct metabolic acidosis, the development of
new PD solutions focuses more on improving outcomes and long-term viability of the peritoneal membrane. The ideal solution, minimizing PDF-related complications, would have a neutral pH, a glucose-free osmotic agent, a reduced GDP content and bicarbonate instead of lactate as the buffer system\(^27\). The glucose-free alternative osmotic agent must still have the potential to produce a predictable ultrafiltration profile, while exerting neither local nor systemic toxicity. Of the different approaches tested to identify the ideal osmotic agent, two reached clinical practice: amino acids and polyglucose (Table 1)\(^28\). Besides their near neutral pH (6.7) and the absence of glucose and associated GDPs, amino acid-based solutions have the advantage to correct the negative nitrogen balance of PD patients and to manage malnutrition\(^29\). Their use on a regular basis, however, remains limited as generation of nitrogenous waste occurs and might aggravate metabolic acidosis. Polyglucose-based solutions with decreased GDP formation provide a sustained ultrafiltration during longer dwell times because of the relative slow absorption rate of polyglucose compared to dextrose. However, their use is also limited to one exchange per day owing to systemic accumulation of dissacharides and other polyglucose metabolites\(^30\).

<table>
<thead>
<tr>
<th>pH</th>
<th>Osmolality (mOsmol/L)</th>
<th>Osmotic agent (mmol/L)</th>
<th>Buffer (mmol/L)</th>
<th>GDP content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial fluid</td>
<td>7.4</td>
<td>280-295</td>
<td>glucose (5.6)</td>
<td>bicarbonate (24)</td>
</tr>
<tr>
<td>Conventional PDF</td>
<td>5.2</td>
<td>358-511</td>
<td>glucose (83-236)</td>
<td>lactate (35-40)</td>
</tr>
<tr>
<td>New PDF: Icodextrin-based</td>
<td>7.4</td>
<td>284</td>
<td>glucose polymer</td>
<td>lactate (40)</td>
</tr>
<tr>
<td>Amino acid-based</td>
<td>6.7</td>
<td>367</td>
<td>amino acids (87)</td>
<td>lactate (40)</td>
</tr>
<tr>
<td>Double Chamber bag</td>
<td>7.4</td>
<td>358-511</td>
<td>glucose (83-236)</td>
<td>lactate (35), bicarbonate (40) or lactate/bicarbonate (15/25)</td>
</tr>
</tbody>
</table>

**Table 1:** Composition of the interstitial fluid and commercially available PDF solutions

Recently, the design of the double chamber dialysate bag opened up new perspectives towards the development of more biocompatible dialysis solutions (Figure 3). Keeping glucose in a separate compartment of the bag allows heat-sterilization at a very
Chapter 1: Introduction

Figure 2: Peritoneal structure, functioning as a semi-permeable membrane between the blood and the dialysate. PMΦ = peritoneal macrophages.

Figure 3: Comparison of single chamber and double chamber bag design.
low pH (2.8 to 3.1), thus limiting GDP formation substantially. The sealed seam also separates electrolytes from the buffer components, allowing the use of bicarbonate-alone or in combination with lactate- without the risk of calcium carbonate precipitation. Next to a low GDP content and a physiologic buffer, the two chamber system -after mixing both compartments immediately before use- also provides a pH neutral dialysis solution (Table 1) \textsuperscript{31}.

The biocompatibility of these new PDF, either non-glucose based or with low GDP content, has been demonstrated extensively in \textit{in vitro} test systems. In addition, a number of whole animal experiments have been carried out, with promising preliminary results. Although these solutions can be used safely and effectively in humans, their superiority versus standard PDF still remains to be shown in clinical trials.
Chapter 1: Introduction

1.6

Animal Models in Peritoneal Dialysis Research

Mortier S, Lameire NH, De Vriese AS

Submitted

25
Chapter 1: Introduction

ABSTRACT

The development of an adequate animal model for peritoneal research remains an object of concern. In vivo peritoneal dialysis research is hampered by the large variety of available models, which makes the interpretation of results and comparison of studies very difficult. Species and strain of experimental animals, method of peritoneal access, study duration, measures of solute transport and ultrafiltration and sampling for histology differ substantially among the various research groups. A collective effort to discuss the shortcomings and merits of the different experimental models may lead to a consensus on a standardized animal model of peritoneal dialysis.

INTRODUCTION

In vivo animal models are designed to mimic the process of PD in patients: 1) study fluids are brought in the peritoneal cavity, 2) fluids undergo modification as dwell time proceeds, 3) interaction occurs between the different cell types in the membrane and 4) exchange of electrolytes and fluid occurs at the level of the peritoneal microcirculation. An in vivo model thus has the potential to provide important information on peritoneal transport pathophysiology, structural changes in the peritoneum and local defense mechanisms.

A large number of research groups works with animal models that differ substantially according to species and strain of experimental animals, method of peritoneal access, study duration and measures of solute transport and ultrafiltration and sampling for histology. The present paper provides an overview of the variety of animal models that are currently being used in the field of peritoneal dialysis research and highlights the differences in approach.
Chapter 1: Introduction

ACUTE PERITONEAL EXPOSURE

Intravital microscopy is a sophisticated research tool to evaluate function and structure of a living tissue (Figure 4). As the visceral peritoneum can easily be exteriorized and is highly translucent, it is extremely suitable for intravital microscopy. Intravital microscopy provides valuable information about diverse functional parameters, such as blood flow rate $^{32-35}$, vessel diameter $^{32,36}$, permeability to macromolecules $^{37}$, leukocyte-endothelial interaction $^{33-35,38}$, capillary recruitment $^{32}$ and lymph vessel kinetics. In addition, the architecture and density of the microvascular network can be studied $^{39}$.

The strength of intravital microscopy lies in its possibility to evaluate different parameters in a living experimental animal, allowing integration of function and structure $^{40}$. Functional parameters can be evaluated before and after an intervention, such as dialysate exposure or administration of a drug. The major drawback of the technique is that it does not permit long-term or repetitive observations. The duration of the experiments is limited to a few hours at best and afterwards the animals need to be sacrificed.

CHRONIC PERITONEAL EXPOSURE

A variety of chronic PD models has been developed, mainly to study long-term effects of peritoneal dialysate on peritoneal membrane function and structure. Rats are cheap, easy to handle and affordable, and therefore preferred by most investigators. Mice $^{41-43}$ and rabbit models $^{44-46}$ have also been used in PD research. Although rabbit models allow PD to be performed for long periods of time and mimic certain aspects of the human situation (ratio of peritoneal surface area and exchange volume of rabbits and human is similar), rabbits are very sensitive animals and difficult to house $^{47}$. The importance of genetically modified mice can not be underestimated as a tool to investigate the molecular bases of peritoneal changes during PD $^{43}$, but the miniature size of mice makes manipulations very difficult. This review focuses on rat models, as these are the most commonly used animal models for PD.
Figure 4: Intravital microscopy system. (1) Continuous monitoring of mean arterial blood pressure via cannulated carotid artery, (2) warm water bath, keeping the test solutions at 37 °C at the site of superfusion, (3) roller pump, bringing the solution to the exteriorized peritoneum, (4) intravital microscope with a motor driven microscopical stage, on which a rat with exteriorized peritoneum is placed in supine position, a regular camera is connected to the microscope, (5) continuous infusion of isotonic saline via a cannulated jugular vein, (6) high-speed video camera, allowing analysis of red blood cell velocities > 2 mm/s, (7) displayed image of visceral peritoneum, (8) videorecorder recording images for off-line analysis, (9) Computer containing the multifunctional image analysis software program.
Peritoneal access

Methods of fluid instillation are highly variable. Some investigators directly inject the test fluids into the peritoneal cavity, using a 22 gauge needle. The injected fluid is absorbed as no drainage is possible. To avoid trauma to muscle, bladder or intestine, some investigators perform the intraperitoneal injections under anesthesia. Although it is possible to maintain rats up to 20 weeks, major concerns have been raised about the repeated needle sticks as they can cause intraperitoneal bleeding or introduce infection, potentially interfering with the experimental results. In addition, anesthesia has been reported to change peritoneal permeability and to affect peritoneal transport kinetics, owing to effects on lymphatic drainage.

Alternatively, the development of custom-made miniature peritoneal catheters allows the use of an 'open' permanent system with easy instillation and removal of test fluids, thus imitating more closely the clinical situation. Two varieties of this 'open' PD system have been described. In both cases a tubing is tunneled from the peritoneal cavity subcutaneously to the neck. In one system, instillation of test fluids is achieved by passing a sterile catheter down the indwelling tubing. Subsequently, the catheter is withdrawn and the tubing is sealed from the external environment with a firm fitting plug. After a specified dwell period a catheter with multiple perforations at the tip is inserted in the tubing to drain spent fluid, usually passively by gravity or occasionally by gentle abdominal massage. In the other system, the subcutaneous tubing itself operates as peritoneal catheter through which test fluids are injected directly and which is secured from the surroundings by a cap. Identical to the former system, fluid is allowed to flow out passively. An advantage of these 'open' PD systems is that they allow chronic PD (ranging from 48 h to 24 weeks) to be performed in unrestrained awake animals with free access to food and water. Thus, potential artefacts induced by anesthesia are excluded. Nevertheless, the investigators using this type of PD model are confronted with varying dropout of experimental animals, ranging from 10 % to 30-40 % and even 60 %. The principal cause of dropout was catheter malfunctioning owing to omental wrapping, adhesions and fibrosis, primarily as a reaction to peritonitis. Using this system, the risk of infection is high as open contact with the environment is established every time the test fluid is instilled or drained.
Recently, a third type of an experimental animal model of chronic PD has been designed, the so-called 'closed' system. A permanent catheter is tunneled from the peritoneal cavity to the neck and connected to a subcutaneous port (Figure 5) \cite{34,78,86}. As draining dialysate through the catheter is not possible, it is left in the peritoneal cavity to absorb. The port device aims to minimize the risk of infection, because there is no need to make connections during fluid instillation. Still, there is 30-40\% of dropout due to catheter blockage, when no additional measures to prevent catheter malfunction or infection are undertaken \cite{78,85}.

**Catheter malfunction**

The most important technical problems in animal models of chronic PD remain frequent obstruction of peritoneal access and high incidence of infection, which are often interrelated. To avoid mechanical catheter obstruction, several investigators perform omentectomy before the catheter is implanted \cite{53,71,72,77}. The implications of omentectomy on the immune status of the animal, however, may not be underestimated as the omentum is an important source of mesothelial cells and macrophages and its absence impairs antibacterial defense \cite{87,88}. Furthermore, omentectomy does not always prevent catheter obstruction \cite{53}.

Alternatively, heparin may be added to the test solutions to reduce the formation of peritoneal adhesions. Heparin is a glycosaminoglycan that prevents fibrin formation by virtue of interference in the clotting cascade \cite{71,89}. However, heparin exerts effects beyond its well-known action as an anticoagulant, such as modulation of the activity of inflammatory cells, synthesis of the extracellular matrix, proliferation of cells and neoangiogenesis \cite{89}. Intraperitoneal administered heparin is known to accumulate in the peritoneum \cite{90} and substantial effects on peritoneal morphology and function may thus be expected. In addition, intraperitoneal administration of heparin did not completely prevent catheter obstruction \cite{90}. Coating the catheter with heparin, however, largely prevented obstruction and was not associated with heparin incorporation in the peritoneum \cite{90}. The use of a heparin-coated catheter thus appears to be the preferable method of peritoneal access.
Figure 5: (A) A heparin-coated catheter with port attached and beads at 2 and 2.3 cm, (B-C) a small incision was made in the skin and subsequently the skin was made free from the underlying muscle tissue, (D-E) the port with attached catheter was inserted into the incision and then tunneled over the left flank to the neck area, (F) a small incision was made in the abdominal layer.
Figure 5: (G-H) The catheter tip was inserted and secured into the abdominal cavity, (I) infusion of peritoneal dialysis solution via subcutaneous port in unrestrained awake animal
Chapter 1: Introduction

**Peritonitis**

Peritoneal infection often results in catheter blockage and subsequent drop-out. In addition, peritonitis *per se* is associated with long-term functional and structural peritoneal membrane changes, such as increased solute transport, decreased ultrafiltration, increased vascular density and fibrosis \(^ {91-96}\). Peritonitis may thus severely compromise the interpretation of the experimental results.

Peritonitis is diagnosed by dialysate culture or WBC counts. Suzuki *et al.* \(^ {97}\) reported a mean baseline WBC count in a rat model of PD of about 1000-1700 cells/mm\(^3\). In previous work \(^ {98}\), the mean WBC count in animals with negative effluent cultures was 853 cells/mm\(^3\). We therefore arbitrarily defined peritonitis as a combination of a positive dialysate culture and a dialysate WBC count higher than 1000 cells/mm\(^3\). Although this definition of peritonitis is arbitrary, we have found it workable in subsequent studies \(^ {86;99;100}\).

In the studies using an ‘open’ catheter system, peritonitis incidence varied from 0.23-0.5 episodes/animal/month \(^ {65;67}\). Comparison with peritonitis rate in the ‘closed’ system is difficult as some research groups do not systematically check for peritonitis \(^ {78;79;85}\). Using a closed system, we found that peritonitis occurs frequently (0.42-2.3 episodes/month/rat) when no prophylactic antibiotic treatment is given \(^ {98;99}\).

Several investigators have administered antibiotics either post-operatively for a few days \(^ {53;67;68}\) or only when peritonitis was diagnosed \(^ {57;67}\). Recent experiments demonstrated that prophylactic administration of antibiotics during the entire study period adequately prevented intraperitoneal infection and the associated alterations of peritoneal structure and function \(^ {99}\). No evidence was found of isolated effects of the antibiotics *per se* on the peritoneal membrane \(^ {99}\).

**Instillation volume**

The instillation volume has also been a matter of debate. Rats with a body weight of 350 g have a peritoneal surface area of about 600 cm\(^2\) \(^ {45}\). As humans have a peritoneal surface area of 10,000 -17,000 cm\(^2\), an instillation volume of 70 to100 ml in the rat would be proportional to the quantity used clinically. Rat respiratory compliance, however, only permits 30 to 40 ml to be instilled. Instillation volumes used in rat models of PD vary from
10 ml\textsuperscript{74,82,86,100} to 20 ml\textsuperscript{48,57,97,80,101} or even 25 ml\textsuperscript{50,53,67,68,102,103}. Infusion of too high volumes causes distress and peritoneal leakage. In our laboratory, the instilled volume is determined empirically in preliminary experiments as the highest volume that causes the least discomfort to the animals.

Frequency of instillation

The frequency of instillation varies strongly among the different research groups, ranging from once\textsuperscript{49,53,78,80} to twice\textsuperscript{61,68,86,101,102} or even three times daily\textsuperscript{67,104}. Comparison of different instillation frequencies (once vs twice daily) showed that the levels of the observed parameters in rats exposed twice daily to the dialysate at week 8 were equivalent to the levels observed in rats that were injected once daily at week 16\textsuperscript{52}. The repeated exposure during one day more closely resembles the multiple exchange program of PD patients and appears to be preferable.

Exposure period

No consensus exists on the optimal exposure period. It has been suggested that a period of adaptation of about one month after catheter implantation should be taken in account. Implantation of biomaterials has been reported to induce mesothelial changes that take 15 days to subside\textsuperscript{105} and a non-specific inflammatory reaction in the peritoneal cavity stabilizes after 3 weeks\textsuperscript{66}. In addition, a sufficiently long exposure period is required to allow clinically relevant alterations to develop. In our experience, treatment of 12 weeks or longer is needed to obtain significant differences between the tested solutions\textsuperscript{86,100}. Growth or aging did not influence membrane morphology\textsuperscript{79} or function\textsuperscript{103}.

Tissue sampling

Most research groups mainly use the visceral peritoneum\textsuperscript{79,86,96,99,106-108}, while others exclusively use the parietal peritoneum\textsuperscript{80}. Analysis of paired biopsies of parietal and visceral peritoneum in humans suggests that alterations in the visceral membrane are less pronounced than in the parietal membrane\textsuperscript{24}. The reverse was observed in some animal studies\textsuperscript{98,106}. Another research group demonstrated that different peritoneal tissues (visceral peritoneum, parietal peritoneum, omentum) may respond differently to PDF exposure\textsuperscript{82,85}. 
The density of vascular network in the visceral peritoneum increased progressively towards the distal loop of the ileum (Mortier and De Vriese, unpublished observations). Errors may thus be introduced when samples are taken in different sections of the peritoneum. Although there is presently no consensus on where representative peritoneal samples should be ideally obtained, it is clear that systematic sampling is essential.

CONCLUSION

At present, no standardized model has been agreed upon by the different groups in peritoneal dialysis research, hampering comparisons of experimental results. A consensus conference with a critical appraisal of the advantages and disadvantages of each experimental model in order to obtain an agreement on the superiority of a specific approach is urgently required. A valid animal model should allow an unbiased interpretation of the evaluated parameters. To this end, drop-out must be minimal and factors that may confound the experimental data, such as intercurrent peritonitis and heparin administration, should be avoided. In our hands, the use of a closed system, a heparin-coated catheter and prophylactic antibiotic administration is associated with very little dropout even during long-term exposure (12-20 weeks). Such long exposure periods are required to allow membrane alterations to develop.
1.7 Study aim

In the present thesis, acute and chronic rat models of peritoneal PDF exposure were designed and used to evaluate the influence of PDF and their components \textit{in vivo} on different parameters of the peritoneal membrane. The aim of the thesis was to examine if and how conventional PDF could contribute to the major reasons of technique failure: loss of ultrafiltration capacity and recurrent peritonitis. Additionally, it was evaluated whether newly developed PDF has the potential to preserve the longevity of peritoneal membrane.

\textbf{Part 2} examines the acute effects of conventional and new PDF on the peritoneal circulation, using the intravital microscopy technique. Conventional PDF is known to possess vasoactive properties\textsuperscript{109-112}, but the responsible factors and the pathophysiological consequences are unknown. By measuring blood flow rate, blood vessel diameter and capillary recruitment, the vasoactive potential of the standard and new PDF is assessed. Further, the effects of standard and new PDF on the leukocyte-endothelial interactions in response to an inflammatory stimulus are evaluated. Finally, a comprehensive review of previous studies on the effects of PDF on host defence is made and the results of the present thesis are situated critically within the large body of literature on this subject.

\textbf{Part 3} addresses two major areas of concern in models of chronic peritoneal exposure to overcome the frequently occurring technical problems: obstruction of peritoneal access and high incidence of infection. Heparin administration has been recommended to limit the formation of fibrin deposits, resulting in catheter obstruction and peritoneal adhesions. As various properties of heparin beyond its traditional anticoagulant role are known, it was reasonable to hypothesize that intraperitoneal infused heparin accumulates in the peritoneal membrane and affects peritoneal function and structure. On the other hand, the incidence of peritoneal infection was reduced by prophylactic antibiotics therapy. Therefore, the isolated effects of both heparin and antibiotic administration are explored in order to conclude whether administration of heparin and/or antibiotics may interfere with PDF-induced alterations.
Part 4 explores the pathogenic role of standard PDF in the development of functional and structural changes of the peritoneal membrane using a standardized rat model of PDF exposure. These results were compared with the effects of new, low-GDP or non-glucose based PDF and PDF with alternative buffers in order to draw conclusions about their potential superiority towards peritoneal integrity. In addition, it was examined whether the observed alterations in peritoneal structure and function observed after exposure to standard PDF could be reversed or reduced by a subsequent exposure to a low-GDP based PDF. Further, the effects of high glucose exposure and associated advanced glycation endproducts (AGE) accumulation on peritoneal structure and function are examined. The potential pathogenic effects of the interaction of AGE with their cell surface receptor RAGE is examined by inhibiting this interaction with a neutralizing monoclonal anti-RAGE antibody. Finally, the extant evidence that chronic high glucose exposure contributes to peritoneal changes is discussed in a review, highlighting the role of vascular endothelial growth factor (VEGF) as downstream mediator.

Part 5 investigates the functional and structural alterations of the peritoneum in an established animal model of chronic renal failure, in order to establish the effect of uraemia in the absence of PDF exposure on the peritoneal membrane.
Chapter 1: Introduction

REFERENCES

1. Fresenius Medical Care. ESRD Patients in 2003. A Global Perspective: 4


42


41. Gotloib L, Waisbrut V, Shostak A, Kushner R. Acute and long-term changes observed in imprints of mouse mesothelium exposed to glucose-enriched, lactated, buffered dialysis solutions. *Nephron* 1995; 70: 466-477


Chapter 1: Introduction


100. Mortier S, Faict D, Lameire NH, De Vriese AS. Benefits of switching from a conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model. *In press* 2004;


48


Chapter 2

Acute peritoneal exposure using the intravital microscopy technique
2.1

Hemodynamic Effects of Peritoneal Dialysis Solutions on the Rat Peritoneal Membrane: Role of Acidity, Buffer Choice, Glucose Concentration, and Glucose Degradation Products

Mortier S, De Vriese AS, Van De Voorde J, Schaub TP, Passlick-Deetjen J, Lameire NH

ABSTRACT

Conventional peritoneal dialysis fluids (PDFs) are unphysiologic due to their hypertonicity, high glucose and lactate concentrations, acidic pH and presence of glucose degradation products (GDP). Long-term exposure to conventional PDFs may cause functional and structural alterations of the peritoneal membrane. New PDFs have a neutral pH, a low GDP content and contain bicarbonate or lactate as the buffer. We examined by intravital microscopy the vasoactive effects of conventional and new PDFs on the rat peritoneal membrane.

A conventional, acidic pH, lactate-buffered 4.25 % glucose PDF induced maximal vasodilation of mesenteric arteries, resulting in doubling of the arteriolar flow and a 20 % increase of the perfused capillary length per area. The hemodynamic effects of conventional PDF were similar after pH-adjustment with NaOH, indicating that acidity \textit{per se} is not essential for the changes. Superfusion by a pH-neutral, lactate-buffered PDF with low GDP content caused only a transient arterial vasodilation despite continuous exposure, with a commensurate effect on arteriolar flow and capillary recruitment. Application of a pH-neutral, bicarbonate-buffered PDF with low GDP content did not affect the hemodynamic parameters at all. Resterilization of the bicarbonate solution increased GDP levels and completely restored the vasodilatory capacity. The corresponding 1.5 % glucose PDFs induced similar, yet less pronounced changes.

In conclusion, conventional PDFs have important vasoactive effects on the peritoneal circulation, mainly due to the presence of GDPs and transiently due to high lactate concentrations. Capillary recruitment may increase effective peritoneal vascular surface area. In addition, chronic vasodilation may induce structural adaptations in the blood vessel wall, contributing to vascular sclerosis. PDF with reduced GDP content induce no major hemodynamic effects and may thus have the potential to better preserve peritoneal vascular integrity.
INTRODUCTION

Long-term peritoneal dialysis is associated with the development of functional and structural alterations of the peritoneal membrane reviewed in 1-2. Ultrafiltration capacity tends to decrease with time spent on dialysis, along with a progressive rise in small solute transport 3-4. Diverse morphological changes have been observed in the peritoneum of PD patients, including reduplication of the basal lamina of the mesothelium and stromal blood vessels, interstitial fibrosis, presence of a hyalinizing vasculopathy and neoangiogenesis 5-9. The prevalence of these alterations increases with time on PD, suggesting that chronic exposure of the peritoneum to the unphysiological peritoneal dialysis fluids (PDFs) is an important causative factor.

Conventional PDFs contain high glucose concentrations to create a transperitoneal osmotic gradient and high lactate concentrations as the buffer system. To curtail caramelization of glucose with formation of a variety of toxic glucose degradation products (GDPs), conventional PDFs are heat-sterilized at a pH in the realm of 5.5, but even at this low pH considerable GDP formation occurs 10-11. The recognition that dialysate bioincompatibility has adverse effects on peritoneal structure and function, has given impetus to the development of new PDFs. A substantial reduction in GDP formation can be achieved by sterilizing glucose separately at a pH of approximately 3. The electrolytes and buffer are kept in another bag compartment at a pH of about 8. The contents of both chambers are mixed immediately prior to use, yielding a solution with neutral pH 12. Until recently, routine use of bicarbonate as the buffer anion has not been possible because of technical difficulties, including the precipitation of calcium and magnesium carbonate 13. Application of a similar double-chamber system allows the mixing of bicarbonate and divalent ions immediately before use. As glucose is also sterilized separately at a low pH, formation of GDPs is markedly reduced 12.

Conventional PDFs have been reported to possess vasoactive properties 14-18, but the causative mechanisms are still unclear. Also, the pathophysiological consequences of this PDF-induced vasodilation, in particular potential acute effects on fluid and solute
transport as well as long-term changes of peritoneal function and structure, are incompletely understood. The aim of the present study was to evaluate the vasoactive effects of conventional and new PDFs on the circulation of the rat peritoneal membrane using a well-standardized intravital microscopy model. Attempts were made to separate the potential contribution of low pH, hyperosmolality, buffer system, glucose concentration and presence of GDPs in mediating the hemodynamic changes. Finally, the impact on effective peritoneal surface area was studied by directly measuring capillary recruitment after acute dialysate exposure.

MATERIALS AND METHODS

Laboratory animals and dialysate solutions

The studies were performed in 102 female Wistar rats (Iffa Credo, Brussels, Belgium), that received care in accordance with the national guidelines for animal protection. The following PDFs (Fresenius Medical Care, Bad Homburg, Germany) were evaluated:
1) a conventional, single-chamber bag, acidic pH, lactate-buffered PDF, containing 1.5 % glucose (CAPD 2) or 4.25 % glucose (CAPD 3);
2) a conventional, single-chamber bag, acidic pH, lactate-buffered PDF, adjusted to pH 7.4 with NaOH, containing 1.5 % glucose (CAPD 2 - NaOH) or 4.25 % glucose (CAPD 3 - NaOH);
3) a new, double-chamber bag, pH-neutral, lactate-buffered PDF with low GDP content, containing 1.5 % glucose (CAPD 2 Balance) or 4.25 % glucose (CAPD 3 Balance);
4) a new, double-chamber bag, pH-neutral, bicarbonate-buffered PDF, containing 1.5 % glucose (CAPD 20 Bicarbonate) or 4.25 % glucose (CAPD 30 Bicarbonate);
5) a new, double-chamber bag, pH-neutral, bicarbonate-buffered PDF, resterilized (second steam sterilization process) (22) to increase GDP content, containing 1.5 % glucose (CAPD 20 Bicarbonate - R) or 4.25 % glucose (CAPD 30 Bicarbonate - R).

As control solution we used Earle’s Balanced Salt Solution (EBSS, Life Technologies Ltd., Paisley, Scotland), containing 5.6 mmol/l glucose, 26 mmol/l NaHCO₃, 117 mmol/l NaCl, 1.8 mmol/l CaCl₂, 5.3 mmol/l KCl, 0.8 mmol/l MgSO₄ and 1 mmol/l NaH₂PO₄.
Monofunctional aldehydes (formaldehyde, acetaldehyde, 5-hydroxymethylfurfural (5-HMF), 2-furaldehyde) were determined as 2,4-dinitrophenylhydrazine (DNPH) derivatives, as previously described 11. 0.05 g DNPH was dissolved in 0.2 ml concentrated sulfuric acid and 0.3 ml water, then diluted with 10 ml acetonitrile. 0.1 ml DNPH solution was added to 0.5 ml PDF, mixed and allowed to stand at room-temperature for 1 h. The determination was performed with a sample volume of 20 µl by reversed phase HPLC at 40 °C with gradient elution (flow 1 ml/min) and UV detection at a wavelength of 365 nm. The used HPLC equipment was: autosampler type GINA 50, gradient pump type M480, column oven type STH 585, detector type UVD 160 S, software chromeleon, all Dionex, former Gynkotek, Munich, Germany; column type Luna C18(2), 250 mm x 4 mm, 5 µ, Phenomenex, Aschaffenburg, Germany; gradient elution (A = acetonitrile, B = phosphate buffer, pH 7.5; gradient: given is % A, addition to 100 % is B: 30 %/ 5min -> 35 %/ 17min -> 40 %/ 25min-> 43 %/ 42min -> 60 %/ 48min -> 75 %/ 50min -> 75 %/ 60min -> 30 %/ 60, 5 min -> 30 %/ 65min).

Bifunctional aldehydes (glyoxal, methylglyoxal, 3-deoxyglucosone) were analyzed as o-phenylenediamine derivatives. 0.04 g o-phenylenediamine was dissolved in 10 ml water. 0.3 ml diamine solution was added to 0.5 ml PDF, mixed and allowed to stand at room-temperature for 2 h in the dark. HPLC was performed with the same chromatographic equipment (detection wavelength 235 nm) and isocratic elution (18 % A, 82 % B).

Intravital microscopy

Rats were anesthetized with thiobutabarbitral (Inactin, RBI, Natick, USA, 100 mg/kg s.c.). The trachea was intubated to facilitate breathing, a jugular vein was cannulated for continuous infusion of isotonic saline, and a carotid artery was cannulated for continuous monitoring of arterial blood pressure. Cromoglycate (cromolyn sodium salt, 10 mg/kg i.v., Sigma) was administered 15 min before surgery, to block degranulation of mast cells induced by the surgical manipulation. A small midline abdominal incision was made and a short segment of the small bowel was exteriorized, carefully avoiding stretching, spread over a plexiglass plate and superfused continuously with EBSS maintained at 37° C. The
preparation was allowed to stabilize for 30 min after completion of surgery. Observations were made with an Axiotech Vario 100 HD microscope (Zeiss, Jena, Germany) using water immersion objectives (Achromat 10x, 40x) and a non-immersion objective (Plan-Neofluar 5x). The microscopical stage was driven by a stepping motor control MCL-2 (Lang, Hüttenberg, Germany), operated by a joystick or a software program (Wincommander, Märzhäuser-Wetzlar, Wetzlar, Germany) via a RS-232 interface. The tissue was transilluminated via a fiberoptic using a light source (KL 1500, Schott, Wiesbaden, Germany) equipped with a 150 W halogen lamp. The resulting image was displayed on a television monitor by a TK-1281 camera (Victor Company of Japan LTD-JVC, Tokyo, Japan) or a high-speed video camera (Kodak Motioncorder Analyser, Eastman Kodak Company, San Diego, CA, USA) and recorded by a videorecorder (S-VHS Panasonic AG-7355, Matsushita, Japan) for off-line analysis. The video images were digitized with an IP-8/AT Matrox image processing board and analyzed with image analysis software (Cap-Image, Ingenieurbüro Zeintl, Heidelberg, Germany), as previously described 19-20.

**Experimental protocols**

A) Mesenteric arteries

In each experimental animal, one mesenteric artery with a diameter of 250-350 μm was dissected from the surrounding tissue (Figure 1) and the luminal diameter was measured at different experimental conditions 19-20. A concentration-response curve was performed for acetylcholine (10^{-7} M to 10^{-5} M), nitroglycerin (10^{-6} M to 10^{-4} M), verapamil (10^{-6} M to 10^{-4} M) and papaverine (10^{-8} M to 10^{-4} M), dissolved in EBSS and applied in randomized order (n=6).

For each of the 4.25 % glucose PDF, mesenteric arterial reactivity was tested after superfusion with PDF, nitroglycerin 10^{-4} M dissolved in EBSS and nitroglycerin 10^{-4} M dissolved in PDF (n=6). The conditions were applied in randomized order to exclude time-dependent effects. Solutions containing bicarbonate were bubbled continuously with CO₂ in order to maintain pH neutral and pCO₂ and HCO₃⁻ concentration stable throughout the entire experiment.
Figure 1: Schematic drawing of the peritoneal microcirculation. Mesenteric arteries with a diameter of 250 to 350 µm were dissected from the surrounding tissue, and changes of luminal diameters in response to local application of vasodilators and dialysate were measured. In a separate series of experiments, arterioles with a diameter of 15 to 25 µm were selected, and flow changes in response to dialysate exposure were analyzed. In addition, the length of the perfused capillaries per area measured before and after dialysate exposure to evaluate capillary recruitment.

B) The peritoneal microcirculation

In a separate group of experimental animals, the peritoneal microcirculation was studied. In each experimental animal, two arterioles with a diameter of 15-25 µm were selected for measurement of luminal diameter and red blood cell velocity (Figure 1). Blood flow rate (BFR) was calculated from the equation: 

\[ \text{BFR} = \text{VRBC} \times \pi D^2/4 \]

with \( \text{VRBC} \) = red blood cell velocity and \( D \) = luminal diameter. In order to evaluate perfused capillary length per area, the microscopical stage was driven through a meander consisting of 2 steps of 0.9 mm in the X-direction and 3 steps of 0.55 mm in the Y-direction. The microscopical image was recorded at each of these 12 positions. Vessel length per area was determined for each
Chapter 2: Hemodynamic effects of PDF

Microscopical image and the average was calculated. Only vessels with active flow were included in the analysis. For each of the 1.5% and 4.25% glucose PDF, luminal diameter and red blood cell velocity in the arterioles, as well as capillary recruitment were measured after exposure to EBSS for 10 min, PDF for 20 min and re-exposure to EBSS for 20 min (n=6). Solutions containing bicarbonate were bubbled continuously with CO₂ in order to maintain pH neutral and pCO₂ and HCO₃⁻ concentration stable throughout the entire experiment.

Statistical analysis

Dialysate-induced alterations were expressed as percentage changes versus the mean of two baseline observations. In univariate analysis, subgroups characterized by different PDF components were compared with respect to percent changes in vasoactive outcomes according to Student's t-test. In order to evaluate the independent contributions of PDF components on these vasoactive outcomes after simultaneous adjustment in a multivariate framework, multiple regression analyses were performed. Model assumptions were checked by visualisation of Pearson residuals. An a priori level of alpha=0.05 was used to indicate statistical significance. All analyses were done using SAS (version 6.12) software.

RESULTS

GDP Concentrations

GDP levels were higher in CAPD 2/3 than in CAPD 2/3 Balance and CAPD 20/30 Bicarbonate, except for 5-HMF (Table 1). Resterilization of CAPD 20/30 Bicarbonate increased GDP concentrations, although not to the same levels as in CAPD 2/3 (Table 1).

Mesenteric arteries

Local application of acetylcholine, nitroglycerin, verapamil and papaverine caused significant vasodilation of mesenteric arteries (Table 2), in the absence of any effects on systemic blood pressure. Nitroglycerin 10⁻⁴ M was used for subsequent studies, as it
induced a maximal and rapidly reversible vasodilation and acts independently of the endothelium.

Superfusion with CAPD 3 caused a similar arterial vasodilation as nitroglycerin $10^{-4}$ M during the entire exposure period. No additive effects were seen when nitroglycerin $10^{-4}$ M and CAPD 3 were applied concomitantly (Figure 2A). Luminal diameters rapidly returned to baseline values after withdrawal of dialysate and/or nitroglycerin.

After adjustment of the pH to 7.4 with NaOH, CAPD 3 still induced a maximal vasodilation in the mesenteric arteries (Figure 2B). In contrast, application of CAPD 3 Balance induced a transient vasodilation that was maximal 2 min after exposure and decreased to values not significantly different from control after 20 min of ongoing superfusion (Figure 2C). Exposure to CAPD 30 Bicarbonate had no major effects on the diameters of the mesenteric arteries (Figure 2D). After resterilization, the vasodilatory capacity of CAPD 30 Bicarbonate was similar to that of CAPD 3 (Figure 2E). There was no apparent effect of the order in which the interventions were applied. None of the PDF caused significant changes in systemic blood pressure (data not shown).

The peritoneal microcirculation

Arteriolar flow increased up to 94.3±12.2 % after superfusion with CAPD 3 and recovered to baseline values after withdrawal of PDF and superfusion with EBSS. Application of CAPD 2 induced a less pronounced but still significant increase of the arteriolar flow up to 53.3±5.5 % (Figure 3A). The flow changes could entirely be attributed to a rise of the red blood cell velocity, as the luminal diameter of the arterioles did not change significantly (data not shown). The elevated flow induced by CAPD 3 resulted in an increase of the perfused capillary length per area of up to 21.9±4.4%. No capillary recruitment was observed after application of CAPD 2 (1.7±2.5%) (Figure 3B). Adjustment of the pH to 7.4 with NaOH did not alter the hemodynamic effects of the CAPD 2 and 3 solutions: similar increases in arteriolar flow (up to 129.2±10.1 %) and perfused capillary length per area (up to 16.9±2.2 %) were observed as with the acidic solutions (Figure 4A and 4B).
Figure 2: Percentage change of luminal diameters of mesenteric arteries after local application of continuous ambulatory peritoneal dialysis (CAPD) 3 (A, n=6), CAPD3 neutralized with NaOH (B, n=6), CAPD3 Balance (C, n=6), CAPD30 Bicarbonate (D, n=6) and resterilized CAPD30 Bicarbonate to increase glucose degradation product levels (E, n=6). The vasodilatory capacities of nitroglycerin 10^{-4}M dissolved in Earle's balanced salt solution (EBSS) (NG), dialysate (D), and nitroglycerin 10^{-4}M dissolved in dialysate (D+NG) were compared. The three interventions were applied in random order. *P<0.001 versus EBSS, #P<0.05 versus EBSS, §P <0.01 versus NG and D+NG, $P<0.01 versus D+NG, &P<0.01 versus D2min.
Table 1: Concentrations of glucose degradation products in the different peritoneal dialysis fluids (µM).
CAPD = continuous ambulatory peritoneal dialysis

<table>
<thead>
<tr>
<th>Dialysate</th>
<th>5-HMF</th>
<th>Formaldehyde</th>
<th>Acetaldehyde</th>
<th>2-Furaldehyde</th>
<th>Glyoxal</th>
<th>Methylglyoxal</th>
<th>3-deoxyglucosone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPD 2</td>
<td>2.6</td>
<td>&lt;3.0</td>
<td>94.9</td>
<td>&lt;1.0</td>
<td>4.6</td>
<td>5.3</td>
<td>167.4</td>
</tr>
<tr>
<td>CAPD 3</td>
<td>7.1</td>
<td>5.0</td>
<td>149.8</td>
<td>&lt;1.0</td>
<td>7.0</td>
<td>8.9</td>
<td>399.8</td>
</tr>
<tr>
<td>CAPD 2 Balance</td>
<td>7.6</td>
<td>&lt;3.0</td>
<td>2.4</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>17.3</td>
</tr>
<tr>
<td>CAPD 3 Balance</td>
<td>27.2</td>
<td>&lt;3.0</td>
<td>2.5</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>38.7</td>
</tr>
<tr>
<td>CAPD 20 Bicarbonate</td>
<td>8.5</td>
<td>&lt;3.0</td>
<td>4.7</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>17.8</td>
</tr>
<tr>
<td>CAPD 30 Bicarbonate</td>
<td>28.9</td>
<td>&lt;3.0</td>
<td>3.7</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>43.1</td>
</tr>
<tr>
<td>CAPD 20 Bicarbonate-R</td>
<td>23.8</td>
<td>&lt;3.0</td>
<td>3.9</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>28.9</td>
</tr>
<tr>
<td>CAPD 30 Bicarbonate-R</td>
<td>63.4</td>
<td>3.3</td>
<td>4.1</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;1.4</td>
<td>73.3</td>
</tr>
</tbody>
</table>

Table 2: Percentage changes of luminal diameters of mesenteric arteries after local application of vasodilators

<table>
<thead>
<tr>
<th>Vasodilator</th>
<th>10^-7 M</th>
<th>10^-6 M</th>
<th>10^-5 M</th>
<th>10^-4 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>7.88 ± 3.3</td>
<td>16.67 ± 4.2(^a)</td>
<td>21.20 ± 3.6(^a)</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>9.69 ± 3.0(^a)</td>
<td>20.40 ± 2.7(^a)</td>
<td>26.60 ± 2.0(^b)</td>
<td></td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>8.33 ± 2.8(^a)</td>
<td>16.45 ± 3.3(^a)</td>
<td>25.35 ± 1.7(^b)</td>
<td></td>
</tr>
<tr>
<td>Papaverine</td>
<td>7.95 ± 2.9(^a)</td>
<td>9.95 ± 2.2(^a)</td>
<td>14.09 ± 1.8(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) P < 0.05 versus Earle's balanced salt solution (EBSS).
\(^b\) P < 0.001 versus EBSS.
Chapter 2: Hemodynamic effects of PDF

Figure 3: Percentage change of (A) arteriolar flow or (B) perfused capillary length per area after superfusion with Earle’s balanced salt solution (EBSS) (open bars, n=6), continuous ambulatory peritoneal dialysis (CAPD) 2 (hatched bars, n=6), or CAPD3 (solid bars, n=6). Measurements were made before exposure to dialysate (EBSS0), 2 min after dialysate (D2), 10 min after dialysate (D10), 20 min after dialysate (D20), 10 min after withdrawal of dialysate (EBSS10), and 20 min after withdrawal of dialysate (EBSS20). (A) *P<0.0001 versus EBSS0, #P<0.0001 versus D20. (B) *P<0.05 versus EBSS0, #P<0.05 versus D20.

Figure 4: Percentage change (A) of arteriolar flow or (B) perfused capillary length per area after superfusion with Earle’s balanced salt solution (EBSS) (open bars, n=6), continuous ambulatory peritoneal dialysis (CAPD) 2 neutralized with NaOH (hatched bars, n=6), or CAPD3 neutralized with NaOH (solid bars, n=6). Measurements were made before exposure to dialysate (EBSS0), 2 min after dialysate (D2), 10 min after dialysate (D10), 20 min after dialysate (D20), 10 min after withdrawal of dialysate (EBSS10), and 20 min after withdrawal of dialysate (EBSS20). (A) *P<0.0001 versus EBSS0, #P<0.0001 versus D20. (B) *P<0.05 versus EBSS0, #P<0.05 versus D20.
Chapter 2: Hemodynamic effects of PDF

Figure 5: Percentage change of (A) arteriolar flow or (B) capillary length per area after superfusion with Earle’s balanced salt solution (EBSS) (open bars, n=6), continuous ambulatory peritoneal dialysis (CAPD) 2 balance (hatched bars, n=6), or CAPD3 balance (solid bars, n=6). Measurements were made before exposure to dialysate (EBSS0), 2 min after dialysate (D2), 10 min after dialysate (D10), 20 min after dialysate (D20), 10 min after withdrawal of dialysate (EBSS10), and 20 min after withdrawal of dialysate (EBSS20). (A) *P<0.001 versus EBSS0, #P<0.01 versus D2. (B) *P<0.05 versus EBSS0.

Superfusion of CAPD 3 Balance induced a transient rise in arteriolar flow (71.7±13.6 %), with a commensurate effect on capillary recruitment (8.8±1.8 %). Arteriolar flow and
perfused capillary length per area returned to baseline values, despite ongoing exposure to PDF (Figure 5A and 5B). No changes in arteriolar flow or capillary recruitment were observed after exposure to CAPD 20 and 30 Bicarbonate (Figure 6A and 6B). In contrast, resterilized CAPD 20 and 30 Bicarbonate caused a similar increase in arteriolar flow (up to 63.9±7.0 % and 109.0±9.3 %, respectively) and perfused capillary length per area (up to 11.1 ±2.5 % and 28.5±5.0 %, respectively) as compared to CAPD 2 and 3 (Figure 7A and 7B). None of the PDFs had significant effects on mean arterial blood pressure (data not shown).

**Figure 7:** Percentage change of (A) arteriolar flow or (B) perfused capillary length per area after superfusion with Earle's balanced salt solution (EBSS) (open bars, n=6), resterilized continuous ambulatory peritoneal dialysis (CAPD) 20 bicarbonate (hatched bars, n=6), or resterilized CAPD 30 bicarbonate (solid bars, n=6). Measurements were performed before exposure to dialysate (EBSS0), 2 min after dialysate (D2), 10 min after dialysate (D10), 20 min after dialysate (D20), 10 min after withdrawal of dialysate (EBSS10) and 20 min after withdrawal of dialysate (EBSS20).

**Uni- and multivariate analysis**

In both uni- and multivariate analysis, no significant effect of pH on hemodynamic variables was found (Table 3-4). The use of lactate as the buffer anion was associated with a greater vasodilation and increase in flow only after 2 min of PDF exposure, but the effect on capillary recruitment was not significant (Table 3-4). The presence of high glucose concentrations and GDPs was consistently associated with more pronounced hemodynamic effects (Table 3-4).
Table 3: Results of univariate analysis

<table>
<thead>
<tr>
<th>PDF Component</th>
<th>Mesenteric arteries (% Vasodilatation)</th>
<th>Peritoneal microcirculation (% Increase in flow)</th>
<th>Peritoneal microcirculation (% Increase in capillary recruitment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>2 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Acidic pH</td>
<td>6</td>
<td>122.9 (3.2)</td>
<td>123.3 (3.9)</td>
</tr>
<tr>
<td>Lactate buffer</td>
<td>18</td>
<td>121.0 (5.0)</td>
<td>119.2 (6.0)</td>
</tr>
<tr>
<td>Bicarbonate buffer</td>
<td>12</td>
<td>113.2 (12.0)</td>
<td>114.6 (13.4)</td>
</tr>
<tr>
<td>High glucose</td>
<td>30</td>
<td>117.9 (9.2)</td>
<td>117.3 (9.7)</td>
</tr>
<tr>
<td>Low glucose</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>High GDP</td>
<td>18</td>
<td>122.9 (6.3)</td>
<td>123.4 (6.2)</td>
</tr>
<tr>
<td>Low GDP</td>
<td>12</td>
<td>110.3 (7.5)</td>
<td>108.2 (6.3)</td>
</tr>
</tbody>
</table>

\(^a\)Significance (p) according to unpaired t-test. PDF, peritoneal dialysis fluid and GDP, glucose degradation products.
Table 4: Results of multivariate analysis

<table>
<thead>
<tr>
<th>PDF Component</th>
<th>Mesenteric Arteries (% Vasodilation)</th>
<th>Peritoneal Microcirculation (% Increase in flow)</th>
<th>Peritoneal microcirculation (% Increase in capillary recruitment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ (SE)$^a$</td>
<td>Statistic$^b$</td>
<td>Significance$^c$</td>
</tr>
<tr>
<td>After 2 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>constant</td>
<td>122.9 (2.6)</td>
<td>47.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH (neutral versus acidic)</td>
<td>3.4 (3.4)</td>
<td>1.00</td>
<td>0.32</td>
</tr>
<tr>
<td>buffer (bicarbonate versus lactate)</td>
<td>-6.8 (2.6)</td>
<td>-2.64</td>
<td>0.01</td>
</tr>
<tr>
<td>glucose (low versus high)</td>
<td>-12.3 (2.6)</td>
<td>-4.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GDP (low versus high)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>constant</td>
<td>123.3 (2.6)</td>
<td>47.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH (neutral versus acidic)</td>
<td>1.4 (3.4)</td>
<td>0.42</td>
<td>0.68</td>
</tr>
<tr>
<td>buffer (bicarbonate versus lactate)</td>
<td>-2.5 (2.6)</td>
<td>-0.97</td>
<td>0.34</td>
</tr>
<tr>
<td>glucose (low versus high)</td>
<td>-15.3 (2.6)</td>
<td>-5.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GDP (low versus high)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 20 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>constant</td>
<td>123.5 (2.1)</td>
<td>59.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH (neutral versus acidic)</td>
<td>0.1 (2.7)</td>
<td>0.03</td>
<td>0.98</td>
</tr>
<tr>
<td>buffer (bicarbonate versus lactate)</td>
<td>4.3 (2.1)</td>
<td>2.08</td>
<td>0.04</td>
</tr>
<tr>
<td>glucose (low versus high)</td>
<td>-21.9 (2.1)</td>
<td>-10.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GDP (low versus high)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Estimated difference between levels (SE), $^b$ t Statistic for evaluating null hypothesis that difference between levels is zero, $^c$ P value associated with testing this null hypothesis.
Chapter 2: Hemodynamic effects of PDF

DISCUSSION

The results of the present study demonstrate that exposure to conventional PDFs exerts important vasoactive effects in the circulation of the rat peritoneal membrane. An acidic pH, lactate-buffered 4.25% glucose PDF reversibly dilated mesenteric arteries by more than 20%. The extent of the PDF-induced vasodilation was similar to that of nitroglycerin $10^{-4}$ M and no additive effects were observed when dialysate and nitroglycerin were applied simultaneously. In pilot experiments, nitroglycerin $10^{-4}$ M was found to have a maximal vasodilatory capacity. It thus follows that conventional PDFs produce maximal vasodilation of mesenteric arteries. In contrast, the arterioles in the peritoneal membrane did not appear to respond directly to the vasodilatory stimuli, as their luminal diameters remained unchanged during PDF exposure. However, the blood flow in these arterioles nearly doubled, indicating that they are passively conducting the rise in flow caused by the upstream vasodilation. The PDF-induced vasodilation resulted in capillary recruitment, increasing the number of perfused peritoneal capillaries by more than 20%.

The present results are in line with and extend previous observations of PDF-induced vasodilation in cremaster muscle arterioles and cecal arteries and PDF-induced increments of coeliac blood flow. The nature of the PDF components that are responsible for these hemodynamic effects is, however, incompletely understood. Conventional PDF is highly unphysiologic due to its acidic pH, high glucose and lactate concentrations, hyperosmolarity and the more recently described presence of GDPs. The vasodilatory effects of low pH, lactate and hyperosmolality are well-recognized. As a consequence, PDF-induced vasodilation has generally been attributed to these factors, although little direct evidence supports this contention. In addition, there is a dearth of information on the potential hemodynamic effects of GDPs in PDF. Exposure to heat-sterilized PDF was found to decrease the concentration of rolling leukocytes and to increase venular flow velocity as compared to filter-sterilized PDF, but no systematic assessment of the effects of GDPs on peritoneal hemodynamics has been performed.
Further experiments were therefore conducted to attain a better understanding of the factors responsible for dialysate-induced vasoreactivity. Adjustment of the pH to 7.4 with NaOH did not affect the vasoreactivity, indicating that, although low pH per se may cause vasodilation, it is not essential for the observed dialysate-induced hemodynamic effects. The results are in accordance with previous findings that pH adjustment does not alter the vasodilatory capacity and small solute clearances of conventional PDF. These observations are important, as acidity is rapidly corrected following infusion of standard PDF in the abdominal cavity. Conventional PDF may thus maintain its vasodilatory potential during the entire dwell period.

PDF with low GDP content and high lactate concentrations induced only transient vasodilation and capillary recruitment despite ongoing exposure, whereas PDF with low GDP content and bicarbonate as the buffer anion was found to be entirely neutral with respect to hemodynamic parameters. The results thus suggest that lactate may only in part be responsible for the PDF-induced vasoreactivity, while GDPs appear to exert major hemodynamic effects. As demonstrated previously, the concentrations of GDPs in CAPD 2/3 Balance and CAPD 20/30 Bicarbonate were lower than in CAPD 2/3, except for 5-HMF. Although 5-HMF has been used as a parameter to assess PDF biocompatibility, several investigators have been unable to correlate its concentrations with toxicity, which is underlined by the present results. Resterilization of CAPD 20/30 Bicarbonate induced similar vasoreactivity as caused by CAPD 2/3. Since resterilization is expected to increase GDP levels without otherwise altering the chemical composition of the PDF, the results further support the causative role of GDPs in peritoneal arterial vasodilation. It is of note that, while the levels of 5-HMF in the resterilized bicarbonate solutions exceeded those found in conventional dialysate, the concentrations of acetaldehyde, glyoxal, methylglyoxal and 3-deoxyglucosone were actually lower. Nevertheless, the hemodynamic effects caused by resterilized CAPD 20/30 Bicarbonate were of the same magnitude as those of CAPD 2/3. These findings are in line with previous observations that application of individual GDPs do not affect fibroblast and mesothelial cell viability and function to the same extent as heat-
Chapter 2: Hemodynamic effects of PDF

sterilized PDF. Taken together, the data suggest that other -as yet unknown- compounds are formed during heat-sterilization and may exert toxic effects.

The present results do not support a role for hyperosmolality in PDF-induced vasoreactivity, as the bicarbonate solutions were hemodynamically inert, even though their osmolality is identical to that of conventional PDF. In addition, the neutral effect of the bicarbonate solutions demonstrates that high glucose concentrations per se do not have hemodynamic effects. The more pronounced vasodilation induced by the 4.25 % glucose than by the 1.5 % glucose acidic lactate PDF most likely results from higher GDP levels. Collectively, the data indicate that the vasoactive properties of conventional PDF result mainly from the presence of GDPs and to a minor extent from the high lactate concentrations.

The number of perfused peritoneal capillaries is not a static property. Under basal circumstances only 25-50 % of peritoneal capillaries are perfused. Additional capillaries can be recruited by a rise in splanchnic blood flow or during local inflammatory reactions. In the present study, local application of conventional acidic pH, lactate-buffered PDF induced a capillary recruitment of about 20 %. Expansion of the effective peritoneal vascular surface area gives rise to an increased diffusive transport of small solutes. A rapid loss of glucose from the peritoneal cavity results in an early dissipation of the osmotic gradient and, consequently, in a decreased transcapillary ultrafiltration rate. It can thus be inferred that hemodynamically neutral PDF may improve ultrafiltration capacity in PD patients.

Several experimental studies have examined the effects of pH, different buffer anions and the presence of GDPs on solute transport and ultrafiltration capacity, yielding disparate results. PH adjustment of a conventional dialysate did not affect small solute clearances. No differences in ultrafiltration rate were observed in rabbits treated with a pH-neutral bicarbonate-buffered PDF (without separate sterilization of glucose) or with a conventional dialysate. In contrast, higher ultrafiltration rates and lower glucose absorption were reported with a pH-neutral lactate-buffered PDF and with a filter-sterilized bicarbonate/ glycylglycine-buffered PDF, compared to standard dialysate. Another group found less glucose absorption and a better ultrafiltration profile with a pH-neutral filter-
sterilized PDF than with an acidic heat-sterilized solution. Additional experiments showed that fluid and solute transport were not affected by either acidity or presence of GDPs, only by the combination of the two factors. Clinical studies with regard to the influence of GDPs and buffer anion on solute and water transport have been similarly conflicting. Dialysis with a bicarbonate-buffered PDF significantly improved ultrafiltration rate. In contrast, two randomized multicenter trials found no difference in ultrafiltration between patients treated with a double-chamber bicarbonate solution or conventional PDF. Computer simulations assuming a 40% difference in peritoneal surface area during the initial 70 min of a dwell with a conventional PDF or a pH-neutral, low-GDP solution, predicted a net ultrafiltration gain of 100 ml after 4 h in favor of the pH-neutral, low-GDP solution. In the present study, conventional PDF induced a capillary recruitment of 20% during the entire exposure time, whereas CAPD Balance caused only a transient recruitment of about 10% and the bicarbonate PDFs had no effect on capillary perfusion at all. Taking into account rather large intra-individual and interindividual variations in drained volume, PDF-related differences in ultrafiltration rate may require large clinical trials to be studied validly.

Besides the potential acute effects on solute transport and ultrafiltration capacity, the PDF-induced vasoreactivity may have long-term effects on peritoneal vascular function and structure. Several authors have reported that the peritoneal vasculature of long-term PD patients is characterized by fibrosis and hyalinization of the media. The prevalence of this vasculopathy significantly increased with time spent on PD, suggesting that the continuous contact with the unphysiological PDF is an important pathogenetic element. Chronic exposure to the high glucose concentrations in PDF with resultant AGE formation and accumulation has been incriminated as a causative factor. The present results may provide an alternative explanation for the PD-related vascular changes. Long-term elevations in arteriolar blood flow and perfusion pressure may cause structural adaptations in the vascular wall, similar to those found in hypertensive vasculopathy, and ultimately lead to vascular sclerosis. The possible pathophysiologic link of the chronic vascular alterations with the dialysate-induced vasoreactivity needs further exploration.
In conclusion, conventional PDF induces maximal vasodilation of the larger mesenteric arteries, resulting in an increased flow in the peritoneal microcirculation and an expansion of the effective peritoneal vascular surface area. Presence of GDPs and to a minor extent high lactate concentrations may be responsible for these hemodynamic effects, while low pH, hyperosmolality and high glucose concentrations do not appear to be essential. Although the pathophysiological consequences of PDF-induced vasoreactivity remain to be fully determined, it can be speculated that chronic capillary recruitment may decrease ultrafiltration capacity. More importantly, continuous elevations of vascular flow may induce vascular remodelling and, ultimately, result in vascular sclerosis. New dialysates exert no major hemodynamic effects on the peritoneal circulation and thus may have the potential to better preserve vascular integrity.

ACKNOWLEDGEMENTS

The authors thank Julien Dupont and Marc Gillis for their expert technical assistance and Dr. Michael Fünfrocken and Dr. Thomas Knerr for the measurement of glucose degradation products. SM is supported by a grant from Fresenius Medical Care-Germany and ADV is supported by a grant from the Fund for Scientific Research-Flanders (N20/0).
REFERENCES


2.2

Effects of Conventional and New Peritoneal Dialysis Fluids on Leukocyte Recruitment in the Rat Peritoneal Membrane

Mortier S, De Vriese AS, McLoughlin RM, Topley N, Schaub TP, Passlick-Deetjen J, Lameire NH

J Am Soc Nephrol 2003, 14: 1296-1306
Chapter 2: Effects of PDF on leukocyte recruitment

ABSTRACT

Peritonitis remains an important cause of morbidity and technique failure in PD. Conventional peritoneal dialysate fluids (PDFs) inhibit peritoneal leukocyte function in vitro and may thus adversely affect the immune response to peritonitis. New PDFs have been designed with neutral pH, low glucose degradation product (GDP) contents and bicarbonate as buffer. The present intravital microscopy study examined the effects of conventional and new PDFs on leukocyte behavior in the peritoneal microcirculation of Wistar rats. The visceral peritoneum was superfused by a control solution (EBSS), a conventional (CAPD) or a new bicarbonate-buffered PDF with neutral pH and low GDP content (CAPD BicaVera). In addition, spent conventional and new PDFs were tested. The number of rolling, adhering and extravasated leukocytes and leukocyte rolling velocity were assessed at different time intervals after exposure to lipopolysaccharide (LPS) or cell-free supernatants of coagulase-negative staphylococci (CNS-CFS).

Exposure to LPS or CNS-CFS dissolved in EBSS dramatically increased the number of rolling, adhering and extravasated leukocytes and decreased leukocyte rolling velocity. Superfusion by CAPD abolished the LPS- or CNS-CFS-induced leukocyte recruitment, whereas CAPD BicaVera had significantly less depressant effects. Spent PDFs affected the leukocyte response in a similar way as fresh PDF. High lactate concentrations, GDPs and hypertonicity appeared to be mainly responsible for the inhibition of leukocyte recruitment.

In conclusion, conventional PDFs abolish in vivo leukocyte recruitment in response to potent inflammatory stimuli. Bicarbonate-buffered pH-neutral PDFs with low GDP contents have less depressant effects and, therefore, may contribute to a better preservation of peritoneal host defence.

INTRODUCTION

In spite of substantial improvements in bag connection technology, peritonitis remains an important cause of morbidity and technique failure in patients undergoing
peritoneal dialysis (PD). Gram-positive organisms are the most common pathogens, with coagulase-negative staphylococci being responsible for 30 to 40 % of peritonitis episodes \(^1\). However, the relative contribution of gram-negative organisms to PD-related peritonitis has risen considerably in the past few years \(^1\).

The peritoneal immune system plays a central role in the prevention and clearance of peritonitis in PD. The chief components of this system are resident macrophages, neutrophils that are recruited from the systemic circulation, mesothelial cells and fibroblasts. Shortly after peritoneal infection, local macrophages and mesothelial cells secrete inflammatory cytokines and chemoattractants, resulting in emigration of neutrophils from the bloodstream to the site of inflammation \(^2\). Leukocyte recruitment is a multi-step process, directed by specific adhesive interactions between the leukocyte and the endothelium. Selectins and their carbohydrate-containing ligands mediate the initial and transient contact between the circulating leukocyte and the vascular endothelium, the so-called “rolling”. The leukocyte thus becomes exposed to tissue-derived chemokines and other activating stimuli. For a rolling cell to subsequently adhere, a reduction in leukocyte rolling velocity must occur. The molecular mechanisms for slow rolling are incompletely understood, but may include an increased expression of selectins at the surface of the leukocyte \(^3\). Finally, firm adherence and transendothelial migration takes place, mediated by interaction of integrins with their immunoglobulin-like receptors \(^4\).

A large body of evidence indicates that conventional peritoneal dialysate fluids (PDFs) cause a functional impairment of peritoneal host defence mechanisms. Viability, bactericidal activity and chemokine production of different leukocyte populations, mesothelial cells and fibroblasts are substantially inhibited after exposure to PDF \(^5\). Conventional PDFs are unphysiologic due to their acidic pH, hypertonicity, high lactate and high glucose concentrations and the formation of glucose degradation products (GDPs) during heat-sterilisation and storage of the dialysate. In an attempt to improve these biocompatibility aspects, new PDFs have been developed with pH-adjustment to physiologic values, use of bicarbonate as buffer system and markedly reduced GDP levels through the use of double-chamber bags \(^6\). Several authors have reported that the new PDFs

\(^1\) Chapter 2: Effects of PDF on leukocyte recruitment
may exert less suppressive effects on peritoneal leukocyte functions. The large majority of these studies have, however, been performed in vitro. As in vivo data on PDF biocompatibility are scarce, the potential clinical relevance of the reported perturbations of various cell functions remains unclear.

Against this background, we evaluated the effects of conventional and new PDFs on the recruitment of circulating leukocytes in response to different inflammatory stimuli in the rat peritoneal membrane, using a well-standardized intravital microscopy model. The consecutive leukocyte-endothelial interactions, i.e. leukocyte rolling, adhesion and emigration, were evaluated and quantified in venules of the rat peritoneal membrane. The potential contribution of different dialysate components in mediating changes in peritoneal leukocyte recruitment was assessed.

MATERIALS AND METHODS

Laboratory animals and dialysate solutions

The studies were performed in 102 female Wistar rats (Iffa Credo, Brussels, Belgium), that received care in accordance with the national guidelines for animal protection. The following PDFs (Fresenius Medical Care, Bad Homburg, Germany) and self-generated solutions were evaluated (Table 1):

1. Earle's Balanced Salt Solution (EBSS, Life Technologies Ltd., Paisley, Scotland), containing 5.6 mmol/l glucose, 26 mmol/l NaHCO₃, 117 mmol/l NaCl, 1.8 mmol/l CaCl₂, 5.3 mmol/l KCl, 0.8 mmol/l MgSO₄ and 1 mmol/l NaH₂PO₄ (n= 6);
2. A conventional, single-chamber bag, acidic pH, L-lactate-buffered PDF with 1.5 % (83 mmol/L) D-glucose and an osmolarity of 358 mOsm/L (CAPD 2) (n= 6) or with 4.25 % (236 mmol/L) D-glucose and an osmolarity of 511 mOsm/L (CAPD 3) (n= 6);
3. A conventional, single-chamber bag, acidic pH, lactate-buffered PDF, adjusted to pH 7.4 with NaOH, containing 4.25 % glucose (CAPD 3 - NaOH) (n= 6);
4. A new, double-chamber bag, pH-neutral, bicarbonate-buffered PDF with 1.5 % D-glucose (CAPD 20 BicaVera) (n= 6) or with 4.25 % D-glucose (CAPD 30 BicaVera) (n=...
5. A new, double-chamber bag, pH-neutral, bicarbonate-buffered PDF, resterilized (second steam sterilization process) (10) to increase GDP content, containing 4.25 % glucose (CAPD 30 BicaVera - R) (n= 6).
6. EBSS with addition of D-glucose to achieve a final glucose concentration of 236 mmol/L and a final osmolarity of 511 mOsm/L (n= 6);
7. EBSS with addition of D-mannitol to achieve a final osmolarity of 511 mOsm/L (n= 6);
8. Sterile water with addition of electrolytes (117 mmol/l NaCl, 1.8 mmol/l CaCl₂, 5.3 mmol/l KCl, 0.8 mmol/l MgSO₄), a D-glucose concentration of 5.6 mmol/l and a L-lactate concentration of 35 mmol/L (n= 6);
9. Sterile water with addition of electrolytes (117 mmol/l NaCl, 1.8 mmol/l CaCl₂, 5.3 mmol/l KCl, 0.8 mmol/l MgSO₄), a D-glucose concentration of 236 mmol/l and a L-lactate concentration of 35 mmol/L (n= 6);
10. Spent CAPD 2 (n= 6) and spent CAPD 20 BicaVera (n= 6) obtained after a 6-hour dwell of a single patient using 4 x 2 l dwells per day;

**Inflammatory stimuli**

Lipopolysaccharide (LPS) *Escherichia coli* serotype 0127:B8, Sigma, St. Louis, MO) was added to the different solutions at a concentration of 0.1 µg/ml. In pilot experiments, this concentration was found to induce a substantial leukocyte recruitment in the absence of systemic blood pressure effects.

Additional experiments were conducted utilizing lyophilized cell free supernatants (CFS) from a strain of *Coagulase-negative staphylococci* (CNS), which had previously been obtained from a PD patient with peritonitis 11. In brief, bacteria were isolated from a stationary phase culture by centrifugation (1800 g at 20 °C for 20 min) and resuspended in Tyrode’s salt solution without gelatin (Sigma) to an absorbance of 0.5 at 560 nm. Previous serial plate count analysis had established that an optical density of 0.5 at 560 nm is equivalent of 5 x 10⁸ colony forming units/ml 12. This solution was incubated at 37 °C for 24h. Suspensions were centrifuged (1800 g at 20 °C for 20 min) to remove the remaining bacteria.
particles. Thereafter, the supernatants were filter-sterilized through 0.2 µm filters (Millipore, Bedford, USA) and dialysed against distilled water at 4 °C through size 5 dialysis tubings (Medicell International Ltd., London, UK). Fractions were freeze dried and aliquots stored at -70 °C until use. In pilot experiments, a dose of 30 x 10⁹ colony forming units was found to induce a substantial leukocyte recruitment in the absence of systemic blood pressure effects.

**Table 1:** Differences in pH, osmolality, glucose concentration, buffer and GDP content of the PDFs and home-made solutions.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Osmolality (mOsmol/L)</th>
<th>Glucose (mmol/L)</th>
<th>Buffer</th>
<th>GDP content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>EBSS 7</td>
<td>7.4</td>
<td>290</td>
<td>5.6</td>
<td>Bicarbonate Absent</td>
</tr>
<tr>
<td>2.</td>
<td>CAPD 3</td>
<td>5.2</td>
<td>511</td>
<td>236</td>
<td>Lactate High</td>
</tr>
<tr>
<td>3.</td>
<td>CAPD 2</td>
<td>5.2</td>
<td>385</td>
<td>83</td>
<td>Lactate High</td>
</tr>
<tr>
<td>4.</td>
<td>CAPD 3 - NaOH</td>
<td>7.4</td>
<td>511</td>
<td>236</td>
<td>Lactate High</td>
</tr>
<tr>
<td>5.</td>
<td>BicaVera 30</td>
<td>7.4</td>
<td>511</td>
<td>236</td>
<td>Bicarbonate Low</td>
</tr>
<tr>
<td>6.</td>
<td>BicaVera 20</td>
<td>7.4</td>
<td>385</td>
<td>83</td>
<td>Bicarbonate Low</td>
</tr>
<tr>
<td>7.</td>
<td>BicaVera 30 - R</td>
<td>7.4</td>
<td>511</td>
<td>236</td>
<td>Bicarbonate High</td>
</tr>
<tr>
<td>8.</td>
<td>EBSS + D-glucose</td>
<td>7.4</td>
<td>511</td>
<td>236</td>
<td>Bicarbonate Absent</td>
</tr>
<tr>
<td>9.</td>
<td>EBSS + D-mannitol</td>
<td>7.4</td>
<td>511</td>
<td>5.6</td>
<td>Bicarbonate Absent</td>
</tr>
<tr>
<td>10.</td>
<td>Water + L-lactate</td>
<td>7.4</td>
<td>290</td>
<td>5.6</td>
<td>Lactate Absent</td>
</tr>
<tr>
<td>11.</td>
<td>Water + L-lactate/D-glucose</td>
<td>7.4</td>
<td>511</td>
<td>236</td>
<td>Lactate Absent</td>
</tr>
</tbody>
</table>

*a EBSS, Earle's Balanced Salt Solution*

**Intravital microscopy**

Rats were anaesthetised with thiobutabarbitral (Inactin, RBI, Natick, USA, 100 mg/kg s.c.). The trachea was intubated to facilitate breathing, a jugular vein was cannulated for continuous infusion of isotonic saline, and a carotid artery was cannulated for continuous monitoring of arterial blood pressure. Cromoglycate (cromolyn sodium salt, 10 mg/kg i.v., Sigma) was administered 15 min before surgery, to block degranulation of mast cells induced by the surgical manipulation. A small midline abdominal incision was made
Chapter 2: Effects of PDF on leukocyte recruitment

and a short segment of the small bowel was exteriorized, carefully avoiding stretching, spread over a plexiglass plate and superfused continuously with EBSS maintained at 37° C (Figure 1). The preparation was allowed to stabilise for 30 min after completion of surgery. Observations were made with an Axiotech Vario 100 HD microscope (Zeiss, Jena, Germany) using a water immersion objective (Achroplan 40x). The tissue was transilluminated via a fiberoptic using a light source (KL 1500, Schott, Wiesbaden, Germany) equipped with a 150 W halogen lamp. The resulting image was displayed on a television monitor by a TK-1281 camera (Victor Company of Japan LTD-JVC, Tokyo, Japan) or a high-speed video camera (Kodak Motioncorder Analyser, Eastman Kodak Company, San Diego, CA, USA) and recorded by a videorecorder (S-VHS Panasonic AG-7355, Matsushita, Japan) for off-line analysis. The video images were digitised with an IP-8/AT Matrox image processing board and analysed with image analysis software (Cap-Image, Ingenieurbüro Zeintl, Heidelberg, Germany), as previously described 7-9.

Study of leukocyte recruitment

In each experimental animal, a single unbranched venule with a diameter of 20-30 µm and a length of 150-200 µm was selected. Rolling leukocytes were defined as those that moved at a velocity lower than that of the red blood cells and were in contact with the endothelial surface. The flux of rolling leukocytes was determined by counting the number of rollers crossing an imaginary line perpendicular to the axis of the venule per minute. The number of leukocytes adhering to the venular endothelial lining and not moving during a 30 second period was counted and expressed as the number per 100 µm length of venule. The number of extravasated leukocytes was determined as the number counted within a predefined area of perivenular tissue. To minimize the influence of preactivation of the tissue, only vessels in which baseline leukocyte rolling was <30 cells/min and baseline adhesion was <3 cells/100 µm of vascular endothelium were considered for further analysis.

After stabilisation, the peritoneal membrane was superfused with one of the above mentioned solutions. The number of rolling, adhering and extravasated leukocytes, leukocyte rolling velocity, red blood cell velocity (v_RBC) and vessel diameter (D) were
measured twice with an interval of 10 min. Venular wall shear rate ($\gamma_w$) was calculated as $\gamma_w = 8 \times v_{RBC}/D$. The measurements were repeated at 30, 60, 90, 120 and 150 min after exposure to LPS or CNS-CFS. Solutions containing bicarbonate were bubbled continuously with CO$_2$ in order to maintain the pH neutral and the pCO$_2$ and HCO$_3^-$ concentrations stable throughout the entire experiment.

**Circulating leukocytes**

A 25 µl sample of arterial blood was added to 475 µl of 2 % orthophosphoric acid (VWR International, Leuven, Belgium) in order to lyse the red blood cells. Thereafter, the total number of peripheral leukocytes was counted in a Bürker chamber and expressed as number/mm$^3$.

**Statistical analysis**

The results are expressed as mean +/- SEM. Statistical analysis was performed using ANOVA, and where appropriate the Tukey test was used as post hoc test. An a-priori level of alpha=0.05 was used to indicate statistical significance.

**RESULTS**

**Leukocyte recruitment in response to LPS and CNS-CFS**

Exposure to both LPS and CNS-CFS caused a dramatic rise in the number of rolling, adhering and extravasated leukocytes in the peritoneal microcirculation, as compared to superfusion with EBSS alone (Figure 2, Figure 3A-C). In addition, the velocity of the rolling leukocytes decreased after exposure to the infectious stimulus (Figure 3D). Leukocyte parameters were similar after LPS and CNS-CFS stimulation (Figure 3A-D).

**The effect of conventional and new PDFs on LPS-induced leukocyte recruitment**

Superfusion of the peritoneal membrane with CAPD 3 abolished leukocyte recruitment in response to LPS (Figure 2, Figure 4A-D). The number of rolling leukocytes decreased over
time, indicating that also baseline rolling was impaired (Figure 4A). The number of adhering and extravasated leukocytes did not increase after stimulation with LPS (Figure 4B-C). Leukocyte rolling velocity remained stable throughout the experiment (Figure 4D).

In contrast, CAPD 30 BicaVera had much milder effects on LPS-induced leukocyte recruitment. The number of rolling leukocytes increased initially to the same extent as in the EBSS-exposed peritoneal venules but decreased thereafter (Figure 4A). The number of adhering and extravasated leukocytes increased during the first 60 min after LPS exposure and remained stable thereafter (Figure 4B-C). Leukocyte rolling velocity fell initially, but returned to baseline values thereafter (Figure 4D).

CAPD 2 and CAPD 20 BicaVera had similar effects on LPS-induced leukocyte recruitment as CAPD 3 and CAPD 30 BicaVera, respectively (data not shown).

The effect of conventional and new PDFs on CNS-CFS-induced leukocyte recruitment

Exposure to CAPD 3 abolished the leukocyte response to CNS-CFS. The number of rolling, adhering and extravasated leukocytes, as well as leukocyte rolling velocity did not change over time (Figure 5A-D). CAPD 30 BicaVera partially inhibited leukocyte recruitment after CNS-CFS stimulation, in a similar way as during LPS stimulation (Figure 5A-D).

The effect of spent dialysate on LPS-induced leukocyte recruitment

Spent CAPD 2 inhibited the rise in rolling, adhering and extravasated leukocytes and the fall in leukocyte rolling velocity after LPS exposure, although to a somewhat lower extent than fresh dialysate (Figure 6A-D). Spent CAPD 20 BicaVera had similar effects on LPS-induced leukocyte recruitment as fresh CAPD BicaVera (Figure 6A-D).
Chapter 2: Effects of PDF on leukocyte recruitment

Figure 1: Intravital microscopy. A segment of the small bowel is exteriorized, spread over a plexiglass plate, and superfused with Earle's Balanced Salt Solution (EBSS) or peritoneal dialysate fluids (PDF). Observations are made with an Axiotech Vario 100 HD microscope, using a water immersion objective and transillumination.

Figure 2: Leukocyte rolling (open arrow), adhesion (closed arrow), and extravasation in response to LPS dissolved in EBSS after t=0 min (A) and t=150 min (B) and to LPS dissolved in CAPD3 after t=0 min (C) and t=150 min (D).
Figure 3: The number of rolling (A), adhering (B), and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points during superfusion by EBSS ($\bullet$, n=6), EBSS with LPS ($\blacktriangle$, n=6) and EBSS with cell free supernatants (CFS; $\blacklozenge$, n=6). (A) *$P<0.005$ versus EBSS, (B) *$P<0.05$ versus EBSS, (C) *$P<0.001$ versus EBSS, (D) *$P<0.01$ versus EBSS.

Figure 4: The number of rolling (A), adhering (B) and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points before and after addition of LPS during superfusion by EBSS ($\bullet$, n=6), CAPD 3 ($\bullet$, n=6), CAPD 30 BicaVera ($\blacklozenge$, n=6). (A) *$P<0.005$ versus EBSS, (B) #* $P<0.01$ versus CAPD3; (C) #* $P<0.05$ versus EBSS; (D) *$P<0.05$ versus EBSS.
Chapter 2: Effects of PDF on leukocyte recruitment

Figure 5: The number of rolling (A), adhering (B) and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points before and after addition of CFS during super-fusion by EBSS (▲, n=6), CAPD 3 (●, n=6) and CAPD 30 BicaVera (■, n=6). (A) *P<0.005 versus EBSS, †P<0.01 versus CAPD3, (B) ‡P<0.01 versus EBSS, †‡P<0.05 versus CAPD3, (C) *P<0.01 versus EBSS, †‡P<0.005 versus CAPD3, (D) *P<0.005 versus EBSS, †P<0.005 versus CAPD3.

The effect of lactate and hyperosmolarity on LPS-induced leukocyte recruitment

Addition of D-glucose to EBSS to obtain the same concentration as present in CAPD 3 and CAPD 30 BicaVera did not impair LPS-induced leukocyte rolling during the initial phase of the experiment, but decreased the number of rolling leukocytes thereafter (Figure 7A). The number of adhering and extravasated leukocytes increased initially, but remained unaltered thereafter (Figure 7B-C). Leukocyte rolling velocity only decreased during the first 30 min (Figure 7D). D-Mannitol, added to EBSS to obtain the same osmolarity as present in CAPD 3 and CAPD 30 BicaVera, had virtually identical effects on LPS-induced leukocyte recruitment as D-glucose. Addition of L-lactate in a concentration of 35 mmol/L substantially impaired leukocyte rolling, adhesion and extravasation and prevented the decrease of leukocyte rolling velocity. Finally, a combination of high L-lactate and high D-glucose concentrations abolished LPS-induced leukocyte recruitment (Figure 7A-D).
Chapter 2: Effects of PDF on leukocyte recruitment

Figure 6: The number of rolling (A), adhering (B) and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points before and after addition of LPS during superfusion by EBSS (▲, n=6), spent CAPD 2 (●, n=6) and spent CAPD 20 BicaVera (■, n=6). (A) *P<0.005 versus EBSS, #P<0.005 versus spent CAPD 2, (B) *P<0.05 versus EBSS, (C) *P<0.05 versus EBSS, #P<0.0005 versus spent CAPD 2, (D) *P<0.01 versus EBSS, #P<0.05 versus spent CAPD 2.

The effect of pH and GDPs on LPS-induced leukocyte recruitment

Addition of NaOH to CAPD 3 in order to adjust the pH to 7.4 did not alter the effects on leukocyte recruitment. Leukocyte rolling, adhesion and extravasation, as well as leukocyte rolling velocity were affected to the same extent as by CAPD 3 (Figure 8A-D).

Resterilization of CAPD BicaVera increased GDP levels without otherwise altering the chemical composition of the PDF 10. CAPD 30 BicaVera – R inhibited leukocyte recruitment in a similar way as CAPD 3 (Figure 8A-D).

Blood pressure, circulating leukocytes, hematocrit and baseline leukocyte parameters

Blood pressure was not different between the experimental groups and did not change throughout the experiments (Table 2). The number of circulating leukocytes did not
change significantly during the experiments. There were no differences in the number of circulating leukocytes among the different experimental groups at any of the time points (Table 2). Hematocrit values were stable during the experiments and not different between the groups (Table 2).

The number of rolling, adhering and extravasated leukocytes, leukocyte rolling velocity and venular shear rate at baseline were not different between the experimental groups (Table 3). There was no correlation between the number of rolling leukocytes and venular shear rate at any of the time points (data not shown).

**Figure 7:** The number of rolling (A), adhering (B) and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points before and after addition of LPS during super-fusion by EBSS (∆, n=6), EBSS with 236 mmol/L glucose (○, n=6), EBSS with 511 mosm/L mannitol (●, n=6), sterile water with 35 mmol/L lactate (□, n=6) and sterile water with 35 mmol/L lactate and 236 mmol/L glucose (■, n=6). (A) *P<0.005 versus EBSS, 4P<0.05 versus sterile water with 35 mmol/L lactate and 236 mmol/L glucose, (B) *P<0.05 versus EBSS, 4P<0.05 versus sterile water with 35 mmol/L lactate and 236 mmol/L glucose, (C) *P<0.05 versus EBSS, 4P<0.05 versus sterile water with 35 mmol/L lactate and 236 mmol/L glucose, (D) *P<0.05 versus EBSS.
Chapter 2: Effects of PDF on leukocyte recruitment

Figure 8: The number of rolling (A), adhering (B) and extravasated (C) leukocytes and the velocity of the rolling leukocytes (D) at different time points before and after addition of LPS during superfusion by EBSS (▲, n=6), CAPD 3 (●, n=6), pH-neutralized CAPD 3-NaOH (○, n=6) and resterilized CAPD 3 BicaVera–R (■, n=6). (A) *P<0.005 versus EBSS, (B) *P<0.05 versus EBSS, (C) *P<0.001 versus EBSS, (D) *P<0.05 versus EBSS, 4P<0.05 versus CAPD 3.

DISCUSSION

Exposure of the rat peritoneal membrane to LPS derived from *Escherichia coli* causes an impressive rise in the number of rolling, adhering and extravasated leukocytes in the venules. In addition, leukocyte rolling velocity decreased substantially, allowing for intense signalling between the leukocyte and the vascular endothelium. These phenomena represent a physiological host response to infection. To determine whether the changes in leukocyte-endothelial interactions observed with LPS could be extrapolated to other inflammatory stimuli of relevance to PD-related peritonitis, additional experiments were conducted with supernatants of a strain of *Coagulase-negative staphylococci*, previously
Table 2: BP, the number of circulating leukocytes, and hematocrit in the experimental groups at different time points after the exposure to the inflammatory stimuli.

<table>
<thead>
<tr>
<th>Control</th>
<th>BP (mmHg)</th>
<th>Circulating leukocytes (x10^9/mm^3)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>90 min</td>
<td>150 min</td>
</tr>
<tr>
<td>EBSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>113 ± 7</td>
<td>114 ± 5</td>
<td>115 ± 6</td>
</tr>
<tr>
<td>LPS exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>122 ± 6</td>
<td>119 ± 10</td>
<td>116 ± 11</td>
</tr>
<tr>
<td>CAPD 3</td>
<td>103 ± 5</td>
<td>110 ± 3</td>
<td>101 ± 9</td>
</tr>
<tr>
<td>CAPD 2</td>
<td>108 ± 4</td>
<td>102 ± 4</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>CAPD 3-NaOH</td>
<td>103 ± 7</td>
<td>111 ± 7</td>
<td>110 ± 7</td>
</tr>
<tr>
<td>BicaVera 30</td>
<td>115 ± 6</td>
<td>123 ± 4</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>BicaVera 20</td>
<td>126 ± 6</td>
<td>130 ± 6</td>
<td>123 ± 6</td>
</tr>
<tr>
<td>BicaVera 30 - R</td>
<td>100 ± 3</td>
<td>116 ± 6</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>Sspent CAPD 2</td>
<td>107 ± 5</td>
<td>109 ± 4</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>Sspent BicaVera 20</td>
<td>109 ± 3</td>
<td>107 ± 7</td>
<td>109 ± 7</td>
</tr>
<tr>
<td>EBSS + D-glucose</td>
<td>111 ± 6</td>
<td>111 ± 7</td>
<td>110 ± 9</td>
</tr>
<tr>
<td>EBSS + D-mannitol</td>
<td>103 ± 5</td>
<td>112 ± 3</td>
<td>122 ± 5</td>
</tr>
<tr>
<td>Water + L-lactate</td>
<td>105 ± 3</td>
<td>100 ± 5</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Water + L-lactate/D-glucose</td>
<td>116 ± 5</td>
<td>112 ± 4</td>
<td>104 ± 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CNS-CFS exposure</th>
<th>BP (mmHg)</th>
<th>Circulating leukocytes (x10^9/mm^3)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBSS</td>
<td>110 ± 8</td>
<td>113 ± 7</td>
<td>116 ± 5</td>
</tr>
<tr>
<td>CAPD 3</td>
<td>104 ± 7</td>
<td>107 ± 7</td>
<td>116 ± 7</td>
</tr>
<tr>
<td>BicaVera 30</td>
<td>95 ± 7</td>
<td>108 ± 7</td>
<td>102 ± 2</td>
</tr>
</tbody>
</table>

a LPS, lipopolysaccharide; CNS-CFS, coagulase-negative staphylococci. No significant differences between groups and the different time points were found, except for b P<0.05 versus 0 min, c P<0.05 versus 0 min, d P<0.05 versus 0 min.
isolated from a PD patient with peritonitis. The resultant leukocyte recruitment appeared similar to that induced by LPS.

The leukocyte response to LPS and CNS-CFS was dramatically affected by concomitant exposure to conventional dialysate. In contrast, superfusion with a pH-neutral, bicarbonate-buffered PDF with a low GDP content had much less depressant effects on leukocyte recruitment. The differences could not be attributed to variability of systemic blood pressure, circulating leukocyte numbers or baseline levels of rolling, adhesion, extravasation, leukocyte rolling velocity or venular shear rate, as these parameters were not significantly different among the groups. In addition, no correlation was found between the number of rolling leukocytes and venular wall shear rate at any time point, indicating that potential dialysate-induced variations in blood flow were not responsible for the observed effects. Spent PDFs obtained from a patient after a 6-hour dwell affected leukocyte kinetics.

Table 3: Baseline leukocyte characteristics in the different experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Rolling leukocytes (number/min)</th>
<th>Hanging leukocytes (number/100µm)</th>
<th>Extravasated leukocytes (number/area)</th>
<th>Rolling velocity (mm/s)</th>
<th>Venular shear rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>15.6 ± 2.2</td>
<td>1.4 ± 0.4</td>
<td>100 ± 15</td>
<td>0.067 ± 0.008</td>
<td>886 ± 182</td>
</tr>
<tr>
<td>LPS exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>22.5 ± 1.4</td>
<td>1.5 ± 0.2</td>
<td>169 ± 34</td>
<td>0.076 ± 0.004</td>
<td>653 ± 122</td>
</tr>
<tr>
<td>CAPD 3</td>
<td>19.2 ± 3.7</td>
<td>1.6 ± 0.4</td>
<td>168 ± 40</td>
<td>0.072 ± 0.006</td>
<td>696 ± 109</td>
</tr>
<tr>
<td>CAPD 2</td>
<td>17.7 ± 4.8</td>
<td>1.3 ± 0.3</td>
<td>138 ± 29</td>
<td>0.081 ± 0.002</td>
<td>767 ± 102</td>
</tr>
<tr>
<td>CAPD 3-NaOH</td>
<td>17.9 ± 3.6</td>
<td>1.6 ± 0.3</td>
<td>183 ± 25</td>
<td>0.089 ± 0.004</td>
<td>510 ± 78</td>
</tr>
<tr>
<td>BicaVera 30</td>
<td>15.3 ± 3.6</td>
<td>0.9 ± 0.2</td>
<td>115 ± 28</td>
<td>0.080 ± 0.005</td>
<td>729 ± 145</td>
</tr>
<tr>
<td>BicaVera 20</td>
<td>17.1 ± 2.3</td>
<td>1.4 ± 0.4</td>
<td>207 ± 63</td>
<td>0.075 ± 0.002</td>
<td>642 ± 99</td>
</tr>
<tr>
<td>BicaVera 30-R</td>
<td>19.7 ± 3.6</td>
<td>1.2 ± 0.1</td>
<td>135 ± 26</td>
<td>0.082 ± 0.005</td>
<td>633 ± 73</td>
</tr>
<tr>
<td>Spent CAPD 2</td>
<td>21.7 ± 1.2</td>
<td>0.7 ± 0.1</td>
<td>121 ± 11</td>
<td>0.075 ± 0.004</td>
<td>677 ± 84</td>
</tr>
<tr>
<td>spent BicaVera 20</td>
<td>17.6 ± 2.4</td>
<td>1.5 ± 0.2</td>
<td>160 ± 18</td>
<td>0.080 ± 0.004</td>
<td>413 ± 65</td>
</tr>
<tr>
<td>EBSS + D-glucose</td>
<td>17.4 ± 1.8</td>
<td>1.5 ± 0.2</td>
<td>223 ± 41</td>
<td>0.082 ± 0.005</td>
<td>523 ± 70</td>
</tr>
<tr>
<td>EBSS + D-mannitol</td>
<td>19.9 ± 2.5</td>
<td>1.8 ± 0.3</td>
<td>149 ± 9</td>
<td>0.072 ± 0.008</td>
<td>794 ± 154</td>
</tr>
<tr>
<td>water + L-lactate</td>
<td>16.9 ± 3.8</td>
<td>1.4 ± 0.3</td>
<td>198 ± 48</td>
<td>0.067 ± 0.007</td>
<td>592 ± 64</td>
</tr>
<tr>
<td>Water+L-lactate/D-glucose</td>
<td>18.5 ± 2.9</td>
<td>1.8 ± 0.4</td>
<td>100 ± 8</td>
<td>0.064 ± 0.003</td>
<td>492 ± 60</td>
</tr>
<tr>
<td><strong>CNS-CFS exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>21.3 ± 3.1</td>
<td>1.6 ± 0.1</td>
<td>217 ± 34</td>
<td>0.081 ± 0.004</td>
<td>745 ± 131</td>
</tr>
<tr>
<td>CAPD 3</td>
<td>16.8 ± 1.8</td>
<td>1.2 ± 0.3</td>
<td>188 ± 22</td>
<td>0.090 ± 0.004</td>
<td>705 ± 65</td>
</tr>
<tr>
<td>BicaVera 30</td>
<td>21.3 ± 1.9</td>
<td>1.8 ± 0.3</td>
<td>143 ± 16</td>
<td>0.089 ± 0.003</td>
<td>751 ± 164</td>
</tr>
</tbody>
</table>

Parameters did not differ significantly between the groups.
Chapter 2: Effects of PDF on leukocyte recruitment

to a similar extent as fresh PDFs. Taken together, the results indicate that the presence of conventional PDF in the peritoneal cavity has important and persistent deleterious effects on the host response to peritonitis.

Additional experiments were conducted to identify the causative PDF-components in greater detail. Addition of D-glucose to EBSS in identical concentrations as found in conventional and new PDFs resulted in a partial inhibition of leukocyte-endothelial interaction that was similar to that caused by CAPD 30 BicaVera. Addition of D-mannitol, an osmotic agent that is not transported into the cell, yielded virtually identical results. These observations emphasise the importance of hyperosmolarity rather than glucose per se in mediating inhibitory effects on leukocyte recruitment. The results are in line with previous in vitro studies, showing that inhibition of phagocytosis and leukotriene generation by polymorphonuclear leukocytes (PMN) is related to the osmolarity but not to the glucose content of the fluid. An amino acid and glucose solution with similar osmolarity exerted comparable effects on monocyte cytokine release and cytotoxicity. In contrast, PMN cytokine release and cytotoxicity were found to be at least partly dependent on the glucose content of the solution. Finally, PMN respiratory burst activation remained unaffected by the hyperosmolarity and high glucose concentration of the solution. Taken together, the results indicate that the various leukocyte functions are differentially affected by glucose and hyperosmolarity. However, as efficient recruitment of leukocytes to the area of infection is a prerequisite for the effector functions to be meaningful, the effects of hyperosmolarity will be predominant.

While superfusion of the peritoneum with a pH-neutral solution containing high lactate concentrations and physiologic glucose levels caused a partial inhibition of leukocyte recruitment, a pH-neutral solution with both high lactate and high glucose concentrations abolished the leukocyte response similarly as conventional PDF, suggesting additive effects of lactate and hyperosmolarity on leukocyte kinetics. Impairment of leukocyte recruitment by conventional PDF persisted after pH-adjustment to 7.4, indicating that although low pH has well-documented inhibitory effects on various leukocyte effector functions in vitro, it does not appear to be essential for the observed inhibition in vivo.
After resterilization, CAPD BicaVera inhibited leukocyte recruitment to a similar extent as CAPD 3. Since resterilization is expected to increase GDP levels without otherwise altering the chemical composition of the PDF, the results support an inhibitory effect of GDPs on leukocyte recruitment, as suggested by *in vitro* experiments. However, as the combination of lactate and hyperosmolarity already caused a maximal suppression of leukocyte recruitment, lowering the GDP content of PDF alone may not be sufficient to improve peritoneal host defense. The subordinate effect of GDPs on leukocyte recruitment is supported by previous observations of a lower influx of neutrophils in the peritoneal cavity of rats infected with *Staphylococcus aureus*, after previous exposure to both a pH-neutral lactate-buffered PDF with low GDP content and a conventional dialysate.

Whereas lactate and GDPs caused an immediate suppression of leukocyte recruitment, the effects of hyperosmolarity were delayed. After 60 min exposure, leukocyte rolling and adhesion decreased, leukocyte rolling velocity increased and no further leukocytes extravasated. These results suggest that the underlying pathophysiological mechanisms of inhibition by lactate, GDPs and hyperosmolarity are different. Further work is required to clarify this issue.

The non-physiologic composition of PDF disappears progressively during the dwell time. Osmolarity decreases due to glucose absorption and water ultrafiltration, although it never reaches physiologic values. Lactate concentration also diminish rapidly during the dwell. We therefore determined the effect of spent dialysates on leukocyte recruitment. Results were very similar to those obtained with fresh dialysates, suggesting that osmolarity and lactate concentration remain sufficiently elevated to profoundly inhibit leukocyte recruitment. Alternatively, uremic toxins and reactive carbonyl compounds accumulating in the dialysate during the dwell may have affected peritoneal leukocyte behavior. Taken together, the results indicate that the inhibition of leukocyte recruitment by conventional dialysate will persist throughout the entire PD cycle.

The molecular mechanisms of the impaired leukocyte response were not investigated in the present study. Several possibilities may be advanced, including changes in the expression of adhesion molecules on the leukocyte membrane such as increased L-
selectin shedding \(^{20}\) or decreased CD11b/18 up-regulation \(^{21}\), competition by soluble adhesion molecules such as soluble P-selectin released from activated platelets \(^{22}\), defective generation of chemokines by mesothelial cells or resident macrophages or alterations of the adhesion molecules at the vascular endothelial cell surface. Additional experiments need to be conducted to elucidate this issue.

In conclusion, both fresh and spent conventional PDFs abolish leukocyte recruitment in response to LPS or CNS-CFS exposure, while a bicarbonate-buffered PDF exhibited less severe inhibitory effects. The depressant action largely results from a combination of high lactate concentrations, hyperosmolarity and GDPs. Glucose \textit{per se} and acidity do not appear to be essential for the inhibitory effects on leukocyte recruitment. Whether the use of bicarbonate-buffered PDF with low GDP content may portend an improvement in peritonitis rates in PD patients remains to be determined in long-term prospective clinical trials.

\textbf{ACKNOWLEDGEMENTS}

The authors thank Julien Dupont and Mieke Van Landschoot for their expert technical assistance and Wim Van Biesen for his kind cooperation. SM is supported by a grant from Fresenius Medical Care-Germany.
Chapter 2: Effects of PDF on leukocyte recruitment

REFERENCES


101


2.3

The Effects of Peritoneal Dialysis Solutions on Peritoneal Host Defense

Mortier S, Lameire NH, De Vriese AS

Chapter 2: Effects of PDF on peritoneal host defense

ABSTRACT

Conventional peritoneal dialysis fluid (PDF) is a bioincompatible solution owing to the acidic pH, the high glucose concentrations and the associated hyperosmolality, the high lactate concentrations and the presence of glucose degradation products (GDPs). This unphysiologic composition adversely affects peritoneal host defense and may thus contribute to the development of PD-related peritonitis. Viability of polymorphonuclear leukocytes (PMN), monocytes (MN), peritoneal macrophages (PM∅) and mesothelial cells (MC) is severely depressed in the presence of conventional PDF. In addition, the production of inflammatory cytokines and chemoattractants by these cells is markedly affected by conventional PDF. Further, conventional PDF hampers the recruitment of circulating leukocytes in response to an infectious stimulus. Finally, phagocytosis, respiratory burst and bacterial killing are markedly lower when PMN, MN or PM∅ are exposed to conventional PDF. Although there are a few discrepant results, all major PDF components have been implicated as causative factors. Generally, novel PDF with alternative osmotic agents or with alternative buffers, neutral pH and low GDP content have much milder inhibitory effects on peritoneal host defense. Clinical studies, however, still need to demonstrate their superiority with respect to the incidence of PD-related peritonitis.

INTRODUCTION

During past two decades, the success of peritoneal dialysis (PD) has grown considerably owing to improvements in both solution delivery systems and peritoneal dialysis fluids (PDF). Nevertheless, besides ultrafiltration loss and inadequate solute clearance, peritonitis remains a principal cause of dropout in PD, resulting in considerable morbidity and transfer to hemodialysis.

The role of PDF in the pathogenesis of PD-related peritonitis is still controversial. Their unphysiological composition may modulate peritoneal host defense negatively and thus increase the susceptibility to infection. The present communication provides an overview of
how PDF may influence a proper response of the peritoneal host defense and which PDF components play a significant role in the observed phenomena.

PERITONEAL HOST DEFENSE TO INFECTION

In normal circumstances, less than 50 ml of fluid is present in the peritoneal cavity and 3 to 15 ml of this fluid contains a range of 7 to 12 million cells. Determination of cell differentials has demonstrated that peritoneal macrophages (PM\(\Phi\)) constitute the predominant cell type (90 %), next to lymphocytes (5-10 %) and polymorphonuclear neutrophils (PMN) (<5 %) \(^1\). Furthermore, the peritoneal cavity is lined with a continuous monolayer of mesothelial cells (MC) with a density of about \(10^9\) cells/1-2 m\(^2\) \(^2\), which contribute to the peritoneal homeostasis through the expression and secretion of various mediators \(^3\). Finally, a low number of fibroblasts (FB) is present in the interstitium, surrounded by high molecular weight intercellular material. Following contamination, a coordinated set of events is initiated, in which both resident and infiltrating cells participate to eliminate the invading pathogen and restore normal tissue homeostasis (Figure 1).

Initiation of the host response

During the initial phase of the host response, resident peritoneal macrophages (PM\(\Phi\)) are activated by the invading micro-organisms or their secreted products. Before a phagocyte is able to ingest a bacterium recognition must take place. Opsonization is a process that promotes recognition of pathogens by coating them with serum factors, which in their turn interact with specific receptors present on the membrane of phagocytic cells. The opsonin concentrations in normal peritoneal fluid resemble those in normal serum \(^4\). IgG, C3 and to a minor extent fibronectin are the most important opsonins in the peritoneal cavity. In addition, microbial cell components itself (such as lipopolysaccharides) or interaction of IgG or IgM with microbial surface structures, are able to activate the complement cascade, in which cleavage of C3 results in C3b and its direct degradation product C3bi, both of which have opsonic capacity and are recognized by CR1 (C3b) and CR3 (C3bi) receptors, present on monocytes (MN), PM\(\Phi\) and PMN \(^5\).
Figure 1: Sequence of inflammatory events induced by microorganisms invading the peritoneal cavity. IL = interleukin; PGE₂ = prostaglandin E₂; TNF = tumor necrosis factor; PM = peritoneal macrophages; FB = fibroblasts; MCP-1 = monocyte chemotactic protein-1; PGI₂ = prostaglandin I₂; TXA₂ = thromboxane A₂; LTB₄ = leukotriene B₄; ICAM = intracellular adhesion molecule; VCAM = vascular cell adhesion molecule.
Opsonization, however, is not always a prerequisite for phagocytosis. Certain microorganisms, such as *Staphylococcus aureus*, can also be ingested as the result of a direct interaction between a microbial cell surface structure (e.g. protein A) and surface molecules present on PMφ membranes (IgG molecules).

**Amplification of the host response**

Activated PMφ release a broad variety of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-8. Furthermore, the interaction of the PMφ with the micro-organisms also causes an increased secretion of the chemoattractants leukotriene B₄ (LTB₄) and monocyte chemotactic protein -1 (MCP-1) as well as generation of prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂). IL-1 and TNF-α will activate MC that respond by synthesizing the prostaglandins PGE₂ and PGI₂, which cause vasodilatation and an increased vascular permeability to proteins. This allows more opsonins to pass from the blood into the peritoneal cavity. In addition, activated MC release IL-6, IL-8 and MCP-1. Whereas IL-8, MCP-1, and LTB₄ will reinforce the inflammatory response by attracting peripheral PMN and MN to the site of inflammation, IL-6 and PGE₂ additionally may downregulate the PMφ and control the extent of inflammation. Furthermore, IL-1 receptor antagonists (IL-1ra) and TNF soluble receptors, released by PMN and MN, could also restrain inflammatory activation. Until recently, the potential involvement of peritoneal FB in the sequence of inflammatory events received little or no attention. Peritoneal FB are capable of, at least in vitro, secreting IL6, IL-8 and MCP-1 upon stimulation with IL-1 and TNF-α and may thus contribute to leukocyte recruitment in the peritoneal cavity.

**Recruitment of leukocytes**

IL-8, MCP-1 and LTB₄ released by PMφ and MC contribute to the creation of a chemotactic gradient, resulting in emigration of leukocytes from the bloodstream to the site of inflammation. Leukocyte recruitment is a multi-step process, directed by specific adhesive interactions between the leukocyte and the endothelium. The initial cell contact between leukocytes and vascular endothelium is mediated by selectins and their carbohydrate ligands.
(e.g. Sialyl-LewisX) and is called "rolling": the selectin-mediated capture of the leukocyte in combination with the rapidly flowing bloodstream induces a rolling movement. L-selectin is found on most types of leukocytes, whereas E-selectin is specific for endothelial cells and P-selectin is present on both endothelial cells and platelets. Selectins bring the cells in close proximity of the endothelium, where they have the potential to sense signals from the endothelial cells which promotes them to adhere more firmly to the vascular wall. Activation of integrins (e.g. CD11b, CD18 and CD14) and binding to their immunoglobulin-like counterreceptors (e.g. ICAM and VCAM) are a prerequisite for firm adhesion and subsequent migration through the layer of endothelial cells. Extravasation of leukocytes over time is type-specific: initially (6h-24h) polymorphonuclear leukocytes (PMN) are recruited and then subsequently replaced by mononuclear cells. It has been suggested that this switch sets off the resolution of inflammation.

**Phagocytosis**

Following identification, the pathogen is engulfed and sequestered inside a vesicle lined with membrane derived from the cell’s own plasma-membrane (primary phagosome). The cytoskeleton plays an important role in this process: actin polymerization and depolymerization allows cytoskeletal reorganisation and results in a change of leukocyte shape, necessary for the engulfment of micro-organisms. The microbicidal activities of phagocytic cells may be divided in oxygen-independent and oxygen-dependent mechanisms. The former involves acidification of the phagosome by fusion between the phagosome and cytoplasmatic granules containing hydrolytic lysosomal enzymes. The latter is called respiratory burst and is associated with a coordinated series of metabolic events, characterised by an increased oxygen uptake upon stimulation of the phagocyte. Firstly, the consumed oxygen is reduced to superoxide (O$_2^-$), using NADPH as electron donor. H$_2$O$_2$ is then produced by the reaction of O$_2^-$ with itself, spontaneously or catalysed by superoxide dismutase. The hexose monophosphate shunt metabolizes glucose with the reduction of two molecules of NADP to NADPH, thus supplying the reducing agent necessary for the continued operation of the respiratory burst. O$_2^-$ and H$_2$O$_2$, which by themselves are relatively harmless, are converted through a complex series of secondary reactions into either oxidizing radicals, such as the hydroxyl ion (OH$^-$) or oxidized halogens (figure 2). Due to their
impressive reactivity, these oxidants have the potential to degenerate most biological proteins and may not only destroy the targeted micro-organisms, but also damage the surrounding tissues at the site of infection. A commonly used method to quantify respiratory burst activity in cells is taking advantage of light emitted during the oxygenation of a chemiluminogenic probe by oxygen radicals.⁴¹

**Figure 2:** Metabolic pathways during respiratory burst. Increased oxygen uptake results in the formation of reactive oxidants.

**THE EFFECT OF PDF ON HOST DEFENSE**

The PD procedure *per se* may adversely affect peritoneal host defense to infection through several mechanisms. The volume of the instilled PDF decreases the concentration of the fluid-phase leukocytes in the peritoneal cavity by 100- to 1000-fold,²² dramatically reducing the probability of phagocyte-bacterium interaction.²³ The instilled PDF volume also dilutes the opsonin concentration present in the peritoneal cavity, which can have substantial consequences for the recognition of pathogens by the phagocytic cells.³ In addition, about 3-4 x 10⁷ peritoneal MN/PMØ are lost in the discarded dialysate each day. This continuous removal of MN/PMØ recruits immature MN from the bone marrow into the circulation at an
Chapter 2: Effects of PDF on peritoneal host defense

excessive rate, as demonstrated by studies that evaluate chemotactic activity of the peritoneal PMO and expression of certain cell surface markers.23

Besides the PD procedure itself, the presence of PDF in the peritoneal cavity may also exert negative influences on peritoneal defense mechanisms, though its exact role in pathogenesis of peritonitis remains controversial. Conventional PDF are hyperosmolar (385-511 mOsmol/L) owing to the high glucose concentrations (83-236 mmol/L) that serve to create a transperitoneal osmotic gradient. A lactate concentration of 35-40 mmol/L is used as a the buffer system. To curtail caramelization of glucose with formation of a variety of toxic glucose degradation products (GDPs), conventional PDF are heat-sterilized at a pH of about 5.1-5.2, but even at this low pH considerable GDP formation occurs. Studies designed to unravel the effects of different PDF components on the various aspects of peritoneal host defense will be reviewed below.

**pH**

_In vitro_ exposure of immunocompetent cells to a conventional acidic, lactate-based PDF can induce the development of a prompt and pronounced intracellular acidosis, characterized by a decline of the intracellular pH to 5.7 within 2-3 min, which can impair various cell functions.24 25 This rapid intracellular acidification appears to be dependent on the presence of lactate. Lactic acid enters the cell through a "H" + lactate" cotransport system, after which it dissociates immediately into the conjugate anion and its proton.

Exposure of cultured MN and MC to conventional acidic lactate-based PDF adversely affected cell viability. Neutralization of the pH was sufficient to cancel out the detrimental effect, irrespective of osmolality, glucose concentration or presence of lactate.26-28 In contrast, variable results were obtained concerning the viability of cultured PMN. Several studies reported decreased viability of PMN after exposure to conventional acidic PDF, with complete reversal after neutralization of pH.26,27,29 In contrast, Liberek _et al._30 was unable to demonstrate an adverse effect of conventional PDF on PMN viability. This discrepancy may be related to differences in methodology used to evaluate cell viability (trypan blue extrusion assay vs. LDH assay). Comparison of both methods demonstrated that the trypan blue method overestimates cell death.30
Chapter 2: Effects of PDF on peritoneal host defense

Conventional PDF also has suppressive effects on cytokine production. Neutralization of pH only partially reversed the inhibition of TNF-α and LTB₄ release by MN ²⁶, PMN ²⁷,³¹ and PMØ ³²-³⁵ in vitro, indicating that other PDF components also play a contributing role. IL-6 release by cultured MC was significantly depressed after exposure to a conventional acidic low or high glucose PDF, but normalized after neutralizing the pH ²⁸,³⁶.

In vivo exposure of rat peritoneal microcirculation to an infectious stimulus (LPS or cell free supernatans of S. epidermidis) mimics a physiological host response to infection: a dramatic rise in the number of rolling, adhering and extravasated leukocytes is observed. This leukocyte response was abolished by concomittant exposure to a conventional acidic lactate-based PDF. Neutralization of the pH with NaOH did not reverse the supressing effects, indicating that low pH is not essential for the in vivo inhibition of leukocyte recruitment ³⁷.

Further, in vitro exposure to conventional acidic lactate-based PDF reduces the phagocytotic capacity of cells. Several studies have attributed the reduction in phagocytosis to the low initial pH of the PDF and subsequent development of intracellular acidosis in the cells ²⁷,²⁹,³⁸. Normal phagocytosis was observed when pH was increased above 6.0, independently of the glucose concentration of the PDF ³⁸,³⁹. In contrast, Van Bronswijk et al. failed to demonstrate in vitro a reduced phagocytosis of preopsonised Escherichia coli, when different pH values were evaluated within a range of 5-8 ⁴⁰. The observed reduction of phagocytosis may be a consequence of the effect of low pH on cytoskeleton reorganisation.

Actine polymerisation in cultured PMN was showed to be reduced in a pH- and time-dependent manner ¹⁸. Incubation of the cells at a pH of 5.2 abolished cytoskeletal reformation and pH adjustment after 10 minutes only partially reversed the inhibitory effects. Measurements of intracellular pH indicated that this was due to an incomplete recovery of intracellular acidosis ¹⁸,⁴¹. As the acidic pH is able to inhibit phagocytosis, a logical consequence would be that no subsequent killing could occur. Duwe et al. ⁴⁰, indeed, observed that in vitro bacterial killing by peripheral blood leukocytes, as measured by a bactericidal assay using E. Coli, was dramatically decreased when pH of the PDF was below 6.2.

MN and PMØ in culture, isolated from the peritoneal cavity, are capable of maintaining a normal resting and stimulated oxygen uptake over a wide range of external
oxygen concentrations. In contrast, when those cells are exposed to a conventional PDF, the
phagocytic oxygen uptake of the cells was severely depressed to about 15% of the normal
response. Similar results were found for their chemiluminescent (CL) response. Varying
pH of the dialysis solution induced a significant suppression of CL response within an
interval of 5.2 to 6.0, but a substantial increase was found once pH had mounted above 6.5. Further,
oxygen uptake as well as superoxide generation were depressed during in vitro
exposure to the conventional PDF, but restored when pH was neutralized. In
contrast, when PMN and PMΦ were incubated in an acidified control solution, no adverse
effects on respiratory burst were observed, suggesting pH is not the only factor participating
in the inhibition of respiratory function of these cells. Additional experiments showed that a
combination of both low pH and lactate was necessary to significantly reduce respiratory
burst. Involvement of other PDF components was also presumed when was demonstrated
in vitro that the suppressed superoxide production could not be recuperated when pH was
neutralized to 7.4 after preincubation to pH 5.2. An alternative explanation could be,
however, that once intracellular pH has been reduced permanent cellular damage occurs. Indeed, if the pH of conventional PDF was titrated to 7.4 before cell exposure, superoxide
production was preserved, no matter what the nature of the buffer was (lactate or a mixture of
lactate/bicarbonate).

The clinical importance of pH as bioincompatible component has been intensively
disputed, as the initially low pH (5.2) of the PDF rises rapidly to a more physiological level
of 6.4 within 30 min and equilibrates at about 7.2 after 1 h. Nevertheless, Dobos et al. have
found in vitro that the cytoplasmatic pH does not change correspondingly and remains
cytotoxic. In contrast, Ing et al. demonstrated that, after preincubation of cultured PMN in
pH 5.2 and subsequent neutralization to pH 7.4, pHi was instantly restored to baseline levels.
Even when pHi was restored to baseline levels, some effector functions, such as
phagocytosis and TNF-α release remained inhibited. Respiratory burst, however, did return
to control values after pretreatment with spent dialysate of a low glucose conventional PDF
when dwell time was longer than 15 to 30 min. These observations correlated directly with
the increase in pH of the spent fluid. Taken together, whether the pHi of the cells is
neutralized or not, during the initial period of each dwell cycle with a conventional PDF, the
Chapter 2: Effects of PDF on peritoneal host defense

Peritoneal cavity is confronted with an acidic solution and a subsequent drop in pH. This may be sufficient to-at least temporarily- impair the effector functions of immunocompetent cells, although it may not answer for all the derangements in the peritoneal cell populations induced by the PDF.

Osmotic agents
Glucose:

High glucose concentrations contributed to cytotoxicity, assessed by LDH release of cultured PMN. To differentiate whether these effects were caused by glucose *per se* or its associated hyperosmolality, osmolality was increased by adding NaCl to PDF. This action did not affect LDH release, suggesting that the cytotoxic effects of high glucose PDF are related to the glucose itself. In contrast, glucose, mannitol, glycerol and glycine had similar inhibitory effects on MC proliferation *in vitro*, pointing out a role for hyperosmolality rather than glucose *per se*.

*In vitro* exposure of LPS-stimulated PM∅ to conventional PDF suppressed TNF-α release, even though pH and osmolality were adjusted (to 7.4 and 300 mOsmol/L, respectively). In unstimulated cultured MN, however, both high glucose and high osmolality induced an increased production of TNF-α. Increasing the osmolality of PDF by addition of NaCl downregulated the expression of CD 14 receptor expression in cultured PMN. LTB₄ generation by PMN was also dramatically reduced after *in vitro* exposure to solutions with a high glucose content compared to low glucose counterparts, independently of the initial pH, which was be attributed to the high glucose *per se*, the high osmolality and/or the high GDP content. Liberek et al. demonstrated an osmolality-dependent inhibition of LTB₄ generation found in cultured zymosan-stimulated PMN. In addition, increased osmotic stress depressed IL-8 and IL-6 release by LPS-stimulated MN in culture. In contrast, an additional effect of glucose, next to hyperosmolality, on the inhibition IL-6 release by PMN was found when increasing glucose concentrations were evaluated in a moderate hyperosmolar environment (400 mOsmol/L).

Increasing glucose concentrations stimulated both MCP-1 mRNA expression and protein production in cultured human peritoneal MC (HPMC), independently of osmolality.
Chapter 2: Effects of PDF on peritoneal host defense

54. This increase in MCP-1 mRNA expression and protein secretion was confirmed by Wong et al. 55 when they incubated HPMC in a culture medium supplemented with increasing amount of D-glucose. Addition of D-mannitol or NaCl to achieve similar osmolality, resulted in a dose-dependent rise in MCP-1 expression and secretion, albeit at lower levels than induced by equivalent glucose concentrations, indicating that the stimulation of MCP-1 is at least partially osmolality-driven. Inhibition of the polyol pathway by the aldose reductase inhibitor sorbinil prevented the increase in MCP-1 synthesis in the presence of glucose, suggesting involvement of the polyol pathway 55. In contrast, an osmolality-dependent inhibition of LTB₄ generation was found in cultured zymosan-stimulated PMN 48.

Adherence capacity of PMN was completely disturbed after in vivo exposure to conventional PDF 51. The inhibition was independent of pH, but increased with rising glucose concentrations. Cell surface densities of CD11b, CD18 and CD14 on PMN decreased with increasing glucose concentrations. Additional experiments, in which PDF was supplemented with NaCl instead of glucose to reach identical osmolality, demonstrated that this was caused by hyperosmotic stress rather than high glucose per se 51. Long-term exposure of cultured endothelial cells to high glucose upregulated VCAM-1 expression, whereas ICAM-1 and E-selectin were unaffected. The concomitantly increased adherence of MN was related to this enhanced VCAM-1 density, as preincubation of the endothelial cells with anti-VCAM-1 abolished MN adherence 56. Adherence capacity of leukocytes to peritoneal venules was increased after 5-weeks in vivo exposure of the rat peritoneal membrane to a high glucose PDF, whereas such an increase did not occur after 5-weeks exposure to a control solution 57. This leukocyte response was analyzed with intravital microscopy during superfusion with a buffer solution. In contrast, acute in vivo exposure of the peritoneal microcirculation to conventional PDF abolished leukocyte rolling, adherence and extravasation. Addition of D-glucose to a buffer solution in identical concentrations as found in a conventional PDF partially inhibited leukocyte recruitment. Addition of D-mannitol, an osmotic agent that is not transported into the cell, yielded virtually identical results, emphasizing the importance of hyperosmolality rather than glucose per se in mediating the inhibitory effects on leukocyte recruitment 57.

Varying glucose concentration (osmolality) from 0.5 to 4.25 % (275 to 479 mOsmol/L), significantly depressed phagocytosis of PMN and PM∅ at glucose
concentrations (osmolality) higher than 2.5 % (375 mOsm/L). Adding NaCl resulted in similar effects on phagocytosis as increasing glucose concentration. In addition, when osmolality was maintained at serum level (275 mOsm/kg), increasing glucose concentrations (0.1-4.25 %) did not suppress phagocytosis. These in vitro data demonstrated that the inhibition of phagocytosis was due to hyperosmolality rather than the glucose concentrations per se\textsuperscript{39,48}. Similar inhibitory effects of hyperosmolarity on in vitro phagocytosis by PMN were reported by Cendoroglo et al.\textsuperscript{50}. However, increasing the glucose concentration while controlling osmolality by addition of NaCl or mannitol, also suppressed the phagocytotic capacity of cultured cells\textsuperscript{50}. Whether this observation was ascribable directly to glucose or to the effect of GDP remained unclear. In contrast with the previous studies, no effect of increased osmotic stress on phagocytosis in incubated MN was found when supplementing the test fluid with increasing glycerol concentrations\textsuperscript{52}.

High glucose concentrations and the associated hyperosmolality inhibited respiratory burst activation of cultured PM∅, measured by the CL or superoxide assay\textsuperscript{27,40,58}. Earlier, Duwe et al.\textsuperscript{39} showed that the depressed in vitro respiratory burst by PMN and PM∅ was due to the high osmolality rather than the high glucose concentration, respectively increasing the osmolality with NaCl, while maintaining glucose levels and increasing glucose concentrations, while keeping osmolality constant. Cendoroglo et al. found a lower oxidative burst by PMN, as evaluated in vitro by measurement of the superoxide radical, when glucose concentrations were increased while controlling for osmolality\textsuperscript{50}. It remained unclear whether these observations were due to glucose per se or to the presence of GDP. In contrast, Liberek et al. failed to observe a suppression of respiratory burst by cultured PMN, neither with hyperosmolar nor with iso-osmolar high glucose PDF at neutral pH\textsuperscript{48}. Kashem et al. even reported an increase in superoxide generation by PMN and MN, measured in vitro by CL response, exposed to a neutralized conventional 1.5 % glucose PDF\textsuperscript{59}. This stimulatory effect on respiratory burst was considered glucose-mediated as the increase in superoxide generation was not observed when PMN and MN were exposed to 1,1 % amino acid (AA)-PDF, which has an even slightly higher osmolality than the glucose-based PDF (365 mOsm/L vs 344 mOsm/L). A possible explanation for the observed increase in respiratory burst
activity is that the latter study has evaluated the cells during a longer incubation period (8h) than most other studies.

Finally, *in vitro* bacterial killing of *E. Coli* by peripheral blood leukocytes was adversely affected once glucose concentration of PDF was 2.5 % or higher. As the suppression of bactericidal activity was still present when similar osmolalities were obtained by NaCl instead of glucose, inhibition was considered to be caused by hyperosmolality and not by high glucose concentrations.\(^{39}\)

Taken together, most -if not all- aspects of peritoneal host defense are impaired by either glucose by itself or the associated hyperosmolality. As possible mechanism for osmolality-dependent suppression of cell functions, osmolality induced cell shrinkage has been speculated. The mechanism responsible for the direct glucotoxicity may be the Crabtree effect, which enholds a manifestation of respiratory inhibition and is characterized by contraction of mitochondria and a decreased glucose utilization occurring at the time of respiratory inhibition. This respiratory inhibition may result in an inadequate energy generation in the cells.\(^{60,61}\)

**Polyglucose:**

Several studies evaluated the effect of polyglucose on peritoneal host defense. While some used icodextrin, others have tested other –not further specified- polyglucose preparations. As their origin and molecular weight may be different, the type of polyglucose used may be a source of discrepancies between studies.

The *in vitro* cytotoxicity of polyglucose towards MN, as evaluated by LDH release, was as pronounced or even slightly higher than that of conventional lactate-based PDF.\(^{62}\)

LTB₄ generation was suppressed when cultured PMN were incubated with a neutralized polyglucose-based PDF\(^{30}\). The results could not be attributed to the electrolyte composition of the solution, as a conventional neutralized 1.36 % glucose PDF with a similar electrolyte composition did not adversely affect LTB₄ release. Further, it could not be ascribed to hyperosmolality, as polyglucose-based PDF has a more physiological osmolality than the 1.36 % glucose based PDF. These findings thus suggest that the glucose polymer *per se* suppresses LTB₄ release.\(^{30}\) The polyglucose-based PDF, at its initial low pH (5.5), also clearly reduced the *in vitro* release of IL-6 and TNF-α by LPS-stimulated MN. Neutralization
of pH improved the release of IL-6 and TNF-α, but could not restore it to normal levels ⁶³. The observation that cytokine gene transcription remained unaltered in cultured cells exposed to icodextrin-based PDF (irrespective of pH) indicates that the suppressive effect is probable caused by posttranscriptional events ⁶³. Similarly, another polyglucose-based PDF was as suppressive on LPS-stimulated IL-1β and IL-8 production by MN in culture as a conventional PDF⁶²,⁶⁴.

Polyglucose inhibited the phagocytotic capacity, measured by the number of cultured PMN that ingested 2 or more acridine orange stained *S. epidermidis* particles, as compared to a control solution ³⁰. However, the phagocytic capacity of PMN and MN, as evaluated by uptake of radiolabeled bacteria, was not different during *in vitro* exposure to either polyglucose-based PDF or 1.5 % glucose-based PDF ⁶⁵.

Glucose polymer-based PDF (pH 5.7) was less inhibitory than a 1.5 % glucose-based solution (pH 5.4) on the ability of both PMN and MN to mount a respiratory burst, as measured *in vitro* by the CL assay or superoxide generation. However, after pH adjustment the difference was markedly reduced ⁶⁵.

Exposure to a glucose polymer facilitated the bacterial growth of *S. epidermidis* and *P. aeruginosa*, assessed by total viable count in the inoculum, whereas bacterial survival was dramatically suppressed by conventional PDF ³⁰. This indicates that more biocompatible solutions may not only protect peritoneal cells, but could bring about a similar positive effect towards bacterial organisms.

**Amino acids (AA):**

PMN incubated in AA-containing PDF (pH 6.7, 365 mOsmol/L) showed an increased viability and phagocytic capacity compared to the cells incubated in conventional glucose-based PDF (pH 5.2, 486 mOsmol/L). At neutral pH (7.3), phagocytic capacity was still higher in 1.1 % AA-PDF than in a conventional 3.86 % glucose-based PDF, probably due to the lower osmolality of the AA-based solution ⁶⁶.

Incubation of endotoxin-stimulated blood MN with AA-PDF (365 mOsmol/L) or conventional 1.36 % glucose PDF (344 mOsmol/L) preserved TNF-α and IL-6 mRNA expression, as well as TNF-α and IL-6 release ⁶⁷. In contrast, prolonged *in vitro* exposure to
both high glucose lactate-based and bicarbonate-based PDF depressed the release of proinflammatory cytokines by MN, despite neutralization of pH. IL-1β and IL-6 release by cultured MN did not differ between AA bicarbonate-based PDF and low glucose bicarbonate-based PDF. In vivo exposure to AA-PDF, resulted in a lower ex vivo stimulated IL-1β and IL-8 release by PMZ, as compared to conventional glucose-based PDF.

Respiratory burst of PMN was suppressed during in vitro exposure to AA-PDF, compared to 1.36 % glucose-based PDF, but was still higher than with 3.86 % glucose-based PDF, suggesting an effect of hyperosmolality. Alternatively, this depression in respiratory burst could be due interference of one of the amino-acids in AA-PDF with the chemiluminogenic probe.

Taken together, the results suggest that the beneficial effects of AA-PDF towards viability, metabolic cell function and cytokine release over high glucose, lactate-based PDF in vitro appear to be mainly osmolality-dependent, as similar improvements were found with low glucose bicarbonate-based PDF.

Buffer

Viability of HPMC was suppressed by in vitro exposure to a conventional lactate-based PDF to about 65 % of that observed with a control solution. Replacement of lactate by pyruvate resulted in a significant improvement of HPMC viability and proliferation, measured in vitro by LDH release and 3H-methyl-thymidine incorporation respectively. Similarly, PMN and MC viability, evaluated by measurement of intracellular ATP levels, was better preserved when the cultured cells were exposed to double-chamber bicarbonate-buffered PDF compared to conventional lactate-based PDF. pH adjustment of the latter allowed partial recovery of intracellular ATP levels of both cell types, but they were still markedly lower than with the double-chamber bicarbonate-based PDF. Identical results were found when evaluating in vitro phagocytosis capacity of PMN: pH adjustment alone of the conventional lactate-based PDF could not restore zymosan phagocytosis to the level observed with double-chamber bicarbonate-based PDF. Mitochondrial dehydrogenase activity is a parameter of metabolic cell function that is evaluated by the cellular formazan production (MTT assay). MTT by MN was suppressed in vitro by a lactate-buffered PDF.
with our without pH neutralization, but it was better preserved in a bicarbonate-buffered PDF.

*In vitro* TNF-α release by MN was not affected by varying lactate concentrations between 0 and 35 mmol/L (tested doses: 0, 15, 25 and 35 mmol/L) at neutral pH, though an additive adverse effect of lactate was observed once the pH dropped below 6.0. Furthermore, MCP-1 secretion by cultured HPMC was increased when 40 mmol/L lactate was added to the culture medium. Lactate in combination with D-glucose had additive enhancing effects on the MCP-1 secretion. This activation of HPMC by lactate alone or with glucose was inhibited by sorbinil, suggesting the involvement of the polyol pathway. PM∅ exposed *in vitro* to pyruvate-buffered PDF generated a substantially higher amount of cytokines (IL-1β, IL-6 and TNFα) and superoxide anion than those exposed to lactate-PDF. In addition, *in vitro* exposure of MN to different solutions with varying pyruvate, lactate and glucose concentrations demonstrated that the presence of lactate reinforced the glucotoxicity on IL-1β release, whereas pyruvate neutralized it. *In vitro* incubation of MN with pH-neutral bicarbonate-buffered PDF induced a significantly higher release of cytokines, such as IL-1β and IL-6, than pH-neutral lactate-buffered PDF. Similarly, IL-1β-stimulated IL-6 release by MC in culture was better after pretreatment with double-chamber bicarbonate-buffered PDF than with lactate-buffered PDF, independently of glucose content. However, replacement of lactate by bicarbonate only allowed a normal cytokine response by cultured MN provided that hyperosmolality or glucose concentration were moderate. Similarly, no differences were found in TNF-α and IL-6 release by cells isolated from an 8h effluent of bicarbonate-buffered PDF or lactate-buffered PDF, suggesting that during long-term *in vivo* exposure the buffer has only a minor influence on cytokine generation. However, cytokine release was measured during incubation in culture medium, which may explain the discrepant results with the previous studies where it was evaluated during PDF exposure. Whereas acute *in vivo* exposure to a high-glucose PDF, buffered with either lactate or pure bicarbonate, did not affect *ex vivo* TNF-α release by stimulated PM∅, *in vivo* exposure to double chamber bicarbonate/lactate based PDF increased *ex vivo* TNF-α release by stimulated PM∅ significantly, compared to lactate-based PDF. Even after a 6 month exposure period, TNF-α release remained elevated, indicating
that beneficial short-term effects of bicarbonate/lactate buffered PDF on cytokine production are maintained during the long-term treatment. The differences between the effect of pure bicarbonate-and bicarbonate/lactate-buffered were unexpected, as both solutions have an identical composition except for the absence of lactate and a higher bicarbonate concentration and PCO₂ in the pure bicarbonate-buffered dialysate (resp. 38 mmol/L vs. 25 mmol/L and ± 77 vs 48 mmHg). IL-6 levels were markedly lower in dialysis effluent of uninfected patients treated long-term with double-chamber bicarbonate/lactate-based PDF as compared to a standard lactate-based PDF. It should be noted that this study evaluated constitutive rather than stimulated cytokine release. The decrease in IL-6 levels in the effluent suggests that a bicarbonate/lactate-based PDF may reduce chronic inflammation in the peritoneum.

Intracellular calcium ([Ca^{2+}]) mobilization changes after stimulation with chemoattractants and is one of the basic requirements for signal transduction. [Ca^{2+}]_{i} mobilization was better maintained in double-chamber bicarbonate-based PDF than in lactate-based PDF. pH adjustment of the latter improved, but did not restore [Ca^{2+}]_{i}. Addition of 35 mmol/L lactate to a control solution demonstrated an additional inhibitory effect of lactate itself on [Ca^{2+}]_{i}.

Varying the lactate concentration from 6.7 to 20.8 mmol/L (tested doses : 6.7, 12.5, 15, 16.7 and 20.8 mmol/L) showed that migration distance of cultured PMN into a micropore membrane placed upon a filter layer containing the chemoattractant casein, was adversely affected once lactate concentrations rose above 15 mmol/L. Furthermore, the chemotactic response of PMN, evaluated in vitro by effective migration distance towards the chemotactic N-formyl-methionyl-leucocyl-phenylalanine (fMLP), was markedly improved after incubation with pyruvate-based PDF compared to its lactate-based counterpart. Lactate-and bicarbonate-based effluents collected from patients at different time points both suppress PMN migration in vitro during the first hour of dialysis, though migration observed with bicarbonate effluent was slightly better. However, lactate-based effluent collected after a dwell of more than 60 min showed a rise in the bicarbonate concentration and an associated increase in PMN migration, which was even slightly higher than that observed with bicarbonate-based effluent obtained at the same time point. Altering the bicarbonate content of a solution from 10-27 mmol/L revealed that leukocyte migration was impaired.
once the bicarbonate concentration approached 27 mmol/L. Leukocyte migration was best preserved with a solution containing 12.5 mmol/L lactate and 20 mmol/L bicarbonate. *In vivo* leukocyte rolling, adherence and extravasation in the rat peritoneal microcirculation in response to LPS or cell free supernatans of *S. epidermidis* was abolished during exposure to a conventional lactate-based PDF, but partially preserved in a double-chamber bicarbonate-based PDF. Addition of 35 mmol/L lactate to a control solution moderately inhibited leukocyte recruitment, whereas addition of 35 mmol/L lactate and 236 mmol/L glucose impaired leukocyte response to the same extent as conventional PDF, suggesting additive effects of lactate and glucose on the observed parameters.

The *in vitro* CL response of peritoneal phagocytes was not affected by pretreatment with a pH-neutral solution containing 35 mmol/L lactate, but it was severely depressed when the solution was acidified to a pH of 5.2. Acidification of the solution in the absence of lactate did not influence the CL generation of the phagocytotic cells. Taken together, the results indicated that a combination of lactate and low pH is necessary to provoke these adverse effects by virtue of a rapid lowering of pH. In cultured PMN, however, lactate *per se* was found to have a pH-lowering effect at an extracellular pH of 7.0, suggesting that lactate in its undissociated state is capable of diffusing into the cytoplasm and directly cause intracellular acidosis. Oxygen consumption by PMN after *in vitro* exposure to conventional acidic lactate-based PDF was partially improved once pH was adjusted to 7.4, though it was even better when PMN were incubated in bicarbonate-based PDF. Similar results were observed when superoxide production of PMN was evaluated *in vitro*. Addition of 35 mmol/L lactate to a control solution had a partial suppressive effect on respiratory burst of PMN.

Furthermore, *in vivo* exposure of experimental animals to double-chamber bicarbonate/lactate PDF increased *ex vivo* bacterial killing of preopsonized bacteria by peritoneal cells, as compared to standard lactate-based PDF.

The inhibitory effect of lactate on different cellular functions has been ascribed to intracellular acidosis. However, lactate may also disturb cellular energy production through the inhibition of glycolysis. Instillation of lactate-based PDF may change the equilibrium between lactate and pyruvate and thus impair the reduction of pyruvate to lactate and the associated oxidation of NADH to NAD⁺. As NAD⁺ is essential as electron acceptor in
glycolysis, its exhaustion may paralyse the glycolysis pathway. Therefore, replacing lactate by pyruvate in PDF, may exert a beneficial effect by providing the cell with an energy source that is not affected by the blocking of lactate oxidation (the Crabtree effect) and thus reducing glucose-induced respiratory inhibition. H$_2$O$_2$-induced damage of rat MC, measured by the MTT assay, was prevented by the addition of pyruvate to the culture medium, confirming that pyruvate has a protective effect against oxidative injury.

The described inhibitory effect of high bicarbonate concentrations may be explained by the development of intracellular acidosis. Extracellular bicarbonate is in balance with CO$_2$, which diffuses more easily into cell than ionized bicarbonate, and reacts with water forming carbonic acid that reduces intracellular pH. However, in vitro viability, phagocytosis, CL response and cytokine release of HPMC and PMN were not different in solutions with a high CO$_2$ concentration, associated with high bicarbonate concentrations, and those with CO$_2$ values in a normal range. Ex vivo studies, however, demonstrated an unexpected difference between the effect of pure bicarbonate- and bicarbonate/lactate-buffered PDF on PMΦ function, suggesting a potential advantage of physiological bicarbonate concentrations and low P$_{CO_2}$. In general, the use of bicarbonate as a buffer system has been promoted as it allows an increase of pH and a decrease of lactate concentration of the solutions. In vitro, bicarbonate-based PDF are considerably more biocompatible towards peritoneal cells than their lactate-based counterparts, though several studies have suggested that this is mainly due to their more physiological initial pH. Unfortunately, most studies did not further differentiate contributing effects of bicarbonate per se, glucose and GDP content, so determination of the causative factors remains difficult.

**GDPs**

Growth of PMΦ was impaired during in vitro exposure to heat-sterilized as compared to filter-sterilized PDF. As the solutions only differed with respect to the presence of GDPs, the cytotoxic effects were attributed to these compounds. Similarly, in vitro proliferation of HPMC was substantially reduced after exposure to pH-neutralized heat-sterilized PDF, whereas the degree of inhibition exerted by its filter-sterilized counterpart was markedly lower. The inhibitory effect of individual GDPs (5-hydroxymethyl-furfural,
Chapter 2: Effects of PDF on peritoneal host defense

Furaldehyde, acetaldehyde, formaldehyde, glyoxal and methylglyoxal) was evaluated in vitro on HPMC proliferation, by measurement of $^3$H-thymidine incorporation assay, and HPMC viability, by measurement of LDH release and MTT assay. When applied in doses markedly exceeding those found in conventional PDF, GDPs suppressed HPMC proliferation and viability. However, none of the individual GDPs had the potential to impair cell proliferation and viability, if added at doses that approached those found in conventional PDF. Even when all the individual GDPs were added to filter-sterilized PDF, the toxicity level of the heat-sterilized PDF could not be achieved, suggesting a supplementary effect of not-yet identified GDPs, with or without an additive or synergistic effect of polymeric container leachables. However, in vitro exposure to GDPs at doses that appeared non-toxic on short-term (24 h), produced deleterious effects on HPMC viability when exposure time was sufficiently long (up to 36 days).

Incubation of peritoneal cells in conventional PDF may also result in cell death, either necrosis or apoptosis. The increased proportion of necrotic phagocytes observed after in vitro exposure to a conventional, heat-sterilized PDF was no longer present when phagocytic cells were exposed to its filter-sterilized counterpart. The acceleration of apoptotic cell death with conventional glucose-based PDF was also attributed to GDPs, as pH, osmolality, lactate and glucose were excluded as contributing factors in a series of additional experiments. Further, a double-chamber high glucose, bicarbonate-based PDF with low GDP content induced less apoptosis than a conventional high glucose PDF. Caspase-3 was identified as a possible effector of the observed apoptosis, as it was activated after exposure to glucose-containing PDF and its inhibition prevented cell death.

In addition, cytokine production appeared to be adversely affected by the presence of GDPs. HPMC released substantially less IL-6 after in vitro exposure to heat-sterilized PDF than after exposure to filter-sterilized PDF. Incubation of HPMC in the presence of individual GDPs revealed that acetaldehyde, formaldehyde, glyoxal and methyl-glyoxal exerted the most pronounced inhibitory effects. Comparison of a heat-sterilized conventional PDF with its filter-sterilized counterpart and a double-chambered bag PDF established that the latter solutions with low GDP content preserved release of IL-1β by cultured MN. TNF-α release by cultured PMØ was inhibited by heat-sterilized PDF, compared to a filter-sterilized
Chapter 2: Effects of PDF on peritoneal host defense

TNF-α production by MN in culture was similarly impaired by conventional heat-sterilized PDF compared with its filter-sterilized counterpart or a double-chamber bicarbonate-based PDF. Furthermore, in vitro exposure of HPMC to heat-sterilized PDF impaired the MCP-1 release to a greater extent than exposure to filter-sterilized PDF. Additionally, heat-sterilized PDF with high glucose concentration, associated with a higher GDP content exerted more pronounced inhibitory effects than its low glucose counterpart.

A contributory role of GDPs on inhibition of leukocyte recruitment was supported by the observations of a lower influx of PMN in the peritoneal cavity of rats infected with Staphylococcus aureus, after previous in vivo exposure to a conventional PDF compared to a pH-neutral bicarbonate/lactate-buffered PDF with low GDP content. In vivo leukocyte rolling in peritoneal venules was markedly decreased during exposure to heat-sterilized PDF, whereas filter-sterilized PDF preserved leukocyte rolling to an identical level as a reference solution. Similarly, in vivo leukocyte recruitment in the rat peritoneal microcirculation was markedly impaired by a conventional PDF, whereas a double-chamber PDF partially preserved the response. Resterilization of the double-chamber PDF, increasing GDP levels without otherwise altering the chemical composition of the solution, inhibited leukocyte recruitment to a similar extent as conventional PDF, supporting the suppressive role of GDPs on leukocyte recruitment.

PMN phagocytosis, measured in vitro by the uptake of 14C-labeled heat-killed Staphylococcus aureus, was better in a 4.25 % filter-sterilized PDF than in a 4.25 % heat-sterilized counterpart. In vitro PMN phagocytosis was also preserved by a 4.25 % double-chamber bicarbonate-based PDF.

Conventional heat-sterilized PDF, with pH adjusted to 7.3, did not inhibit the respiratory burst of cultured zymosan-stimulated PMN compared to its filter-sterilized counterpart. In agreement, ex vivo PMN respiratory burst did not differ after in vivo exposure of rats to either heat-sterilized or filter-sterilized PDF. In contrast, Cendoroglo et al. observed a significantly lower respiratory burst, measured by superoxide production, when PMN in culture were exposed to a pH-neutral, heat-sterilized conventional PDF as compared to a filter-sterilized solution. It should be noted that the latter study evaluated 4.25 % glucose PDF with much more extreme differences in GDP content between the heat- and filter-sterilized solutions. Nevertheless, even in low glucose heat-sterilized PDF, in vitro
superoxide generation of PMN and MN was impaired, as compared to a low glucose filter-sterilized PDF and a double-chamber PDF. Similarly, in vivo exposure of rats to heat-sterilized PDF substantially decreased PMØ respiratory burst, compared with its filter-sterilized counterpart. Comparing the in vitro effects of heat-and filter-sterilized PDF at low and normal pH and at different time points (1 h, 2 h and 4 h), allowed to characterize the kinetics of the GDP-induced respiratory burst inhibition of PMØ and PMN. Low pH inhibited respiratory burst rapidly, whereas the effect of heat-sterilization developed more slowly but progressively.

The molecular mechanism behind the inhibitory effects of GDPs is presently unknown, but several potential explanations have been advanced. As GDPs are electrophilic low molecular weight molecules, they can diffuse into the cell and inactivate the NADH-oxidase complex directly or through a second messenger. Alternatively, GDPs may adversely affect the cell in the same way as lipid peroxidation products, by alkylating SH-groups on the plasma membrane of cells. Yahyapour et al. have demonstrated that the inhibitory effects of GDPs on CL response can be dramatically reduced by the addition of glutathione to PDF. Three possible mechanisms were suggested: protection of the cell membrane by reaction of the SH-group of glutathione with oxidized membrane proteins, inactivation of GDPs by spontaneous reaction with aldehydes or protection of cell signaling molecules that are targets for redox modification. It was also speculated that the aldehydes among the GDPs could interfere with functionally important enzymes and proteins.

Spent dialysate

As described above, conventional PDF impairs peritoneal host defense by virtue of its acidic pH, high glucose concentrations with the associated hyperosmolality, high lactate concentrations and the presence of GDPs. However, in vivo PDF equilibrates during the dwell. The pH rises rapidly from 5.2 to about 6.8 within 30 min and stabilizes around 7.2 after one hour. Reduction of osmolality, due to glucose absorption and water ultrafiltration, occurs more slowly and does not reach physiological levels within 5 h. The lactate concentration declines from 35 mmol/L to 12.5 mmol/L within one hour, with a concomitant influx of bicarbonate to a concentration of 15.3 mmol/L. Recently, measurements of
methylglyoxal in the effluent demonstrated that this compound was totally absent after a 2 h dwell, indicating that GDPs may disappear from the dialysis solution during the dwell cycle. In the past it was therefore hypothesized that PDF bioincompatibility is only transient and improves with equilibration.

In fact, a recovery of cell function in spent dialysate of overnight dwell was found with respect to phagocytosis by PMΦ, measured in vitro by the uptake of opsonized sheep red blood cells and unopsonized latex beads, and respiratory burst, compared to fresh dialysis solution. In contrast, cytokine production by cultured MN (IL-6 and TNF-α) was not only significantly suppressed following exposure to fresh PDF, but was even further inhibited by PDF obtained from longer intra-peritoneal dwell periods. Similarly, acute in vivo exposure of the rat peritoneal microcirculation to spent PDF resulted in similar inhibitory effects on leukocyte recruitment as with fresh PDF.

As spent dialysate, despite equilibration, still impairs peritoneal host defense, it was hypothesized that immuno-inhibitory components were dialyzed from the systemic circulation. Tranperitoneal transported factors with inhibitory effects on host defense have been identified, including granulocyte inhibitory protein I (GIPI) and II (GIPII), degranulation inhibiting protein I (DIPI) and II (DIPII) and a chemotaxis inhibiting protein (CIPII). In addition, p-cresol, present in the same concentration in PD effluent as in uremic serum, impairs various aspects of phagocytic function. Furthermore, during peritoneal dialysis, the peritoneal cavity is exposed to increased carbonyl stress, not only originating from conventional heat-sterilized PDF, but also from reactive carbonyl compounds (RCOs) dialyzed from the uremic circulation. Although its direct role still needs to be proven, this carbonyl stress may also be an additional burden on the normal functioning of peritoneal immunocompetent cells.

**CONCLUSION**

A overwhelming body of evidence, largely consisting of in vitro, ex vivo and some whole animal experiments, indicates that conventional PDF profoundly affects peritoneal host defense (table 1). The above reviewed studies point with few inconsistencies to all major
PDF components - acidic pH, high glucose, hyperosmolality, lactate and GDPs - as the causative factors. Novel dialysis solutions with alternative osmotic agents or with alternative buffers, neutral pH and low GDP content generally have less suppressive effects of peritoneal host defense. The promising in vitro and experimental animal evidence leaves us now poised to examine whether these new solutions reduce peritonitis rates, curtail peritonitis severity or reduce the risk of relapsing peritonitis in PD patients. A retrospective survey demonstrated that patients exposed to low-GDP PDF had a lower peritonitis incidence and shorter peritonitis duration than those treated with conventional PDF. So far, no prospective randomised trials have been initiated. Against the background of declining peritonitis rates, a sufficiently powered study would need a large sample size and a follow-up for a reasonable length of time.
Table 1: Overview of studies evaluating the contribution of different PDF components to PDF biocompatibility. (↓) depressin effect, (↑) stimulating effect, (=) no effect. (MN) monocytes, (PM∅) peritoneal macrophages, (PMN) neutrophils, (MC) mesothelial cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell function</th>
<th>Low pH</th>
<th>Osmotic agent</th>
<th>Buffer</th>
<th>GDPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>Viability</td>
<td>↓ (24)</td>
<td>Icodextrin</td>
<td>Bicarbonate ↑ (60)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokine release</td>
<td>↓ (24)</td>
<td>Glucose ↑ (48), Hyperosmolality ↓ (23, 50), ↑ (48)</td>
<td>Lactate ↓ (24, 59), Pyruvate ↑ (59), Bicarbonate ↑ (60)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemotactant release (LTB4)</td>
<td>↓ (24)</td>
<td>Icodextrin ↓ (60, 61, 62)</td>
<td>Amino acids = (80, 65, 66)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recruitment</td>
<td></td>
<td>Glucose ↑ (54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phagocytosis</td>
<td>↓ (27, 37)</td>
<td>Hyperosmolality ↓ (37), = (50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respiratory burst</td>
<td>↓ (37, 38)</td>
<td>Glucose ↑ (57), Hyperosmolality ↓ (37)</td>
<td>Icodextrin ↑ (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacterial killing</td>
<td>↓ (37)</td>
<td>Glucose ↑ (37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM∅</td>
<td>Viability</td>
<td>↓ (30, 31, 33)</td>
<td>Glucose ↓ (24), Amino acids ↓ (64)</td>
<td>Pyruvate ↑ (59, 68), Bicarbonate = (72, 73), ↓ (75, 76, 77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokine release (TNF-α, IL-1β, IL-6, IL-8)</td>
<td>↓ (30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemotactant release (LTB4)</td>
<td>↓ (30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recruitment</td>
<td></td>
<td>Bicarbonate ↑ (77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phagocytosis</td>
<td>↓ (37)</td>
<td>Hyperosmolality ↓ (37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respiratory burst</td>
<td>↓ (37, 40)</td>
<td>Glucose ↓ (25, 38, 56), Hyperosmolality ↓ (37)</td>
<td>Amino acids ↓ (64), Glucose ↓ (37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacterial killing</td>
<td>↓ (37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMN</td>
<td>Viability</td>
<td>↓ (24, 25, 27) = (23)</td>
<td>Glucose ↓ (23), Amino acids ↑ (64), Glucose ↓ (23)</td>
<td>Bicarbonate ↑ (34), ↓ (88, 89)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokine release (IL-6)</td>
<td>↓ (24, 25, 29)</td>
<td>Glucose ↓ (25), Hyperosmolality ↓ (23)</td>
<td>Icodextrin ↓ (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemotactant release (LTB4)</td>
<td>↓ (24, 25, 29)</td>
<td>Hyperosmolality ↓ (35, 49)</td>
<td>Lactate ↓ (35, 76), Bicarbonate ↓ (34), ↓ (55, 93, 94)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recruitment</td>
<td></td>
<td>Bicarbonate ↓ (76), ↑ (55), Bicarbonate ↑ (34)</td>
<td>↓ (48, 91)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phagocytosis</td>
<td>↓ (25, 27, 36) = (38)</td>
<td>Glucose ↑ (48), Hyperosmolality ↓ (23, 48)</td>
<td>Icodextrin ↓ (28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoskeletal reorganization</td>
<td>↓ (16, 39)</td>
<td>Amino acids ↑ (64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respiratory burst</td>
<td>↓ (23, 38, 40, 41, 42, 43)</td>
<td>Glucose ↓ (48), = (23), ↑ (57)</td>
<td>Icodextrin ↑ (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Viability</td>
<td>↓ (25, 26)</td>
<td>Hyperosmolality ↓ (47)</td>
<td>Lactate ↓ (68, 69), Pyruvate ↑ (68, 69), Bicarbonate ↑ (70)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokine release (IL-6)</td>
<td>↓ (26, 34)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
|           | Chemotactant release (MCP-1) | Glucose ↑ (51, 52, 53), Hyperosmolality ↑ (53) | Lactate ↑ (53) | ↓ (92)
REFERENCES


Chapter 2: Effects of PDF on peritoneal host defense


133


44. Ing TS, Yu AW, Podila PV *et al.* Failure of neutrophils to recover their ability to produce superoxide after stunning by a conventional, acidic, lactate-based peritoneal dialysis solution. *Int. J. Artif. Organs* 1994; 17: 191-194


Chapter 2: Effects of PDF on peritoneal host defense


66. Brulez HF, Dekker HA, Oe PL *et al.* Biocompatibility of a 1.1% amino acid-containing peritoneal dialysis fluid compared to a 2.27% glucose-based peritoneal dialysis fluid. *Nephron* 1996; 74: 26-32


68. Plum J, Fussholler A, Schoenick G *et al.* In vivo and in vitro effects of amino-acid-based and bicarbonate-buffered peritoneal dialysis solutions with regard to peritoneal transport


Chapter 2: Effects of PDF on peritoneal host defense


Chapter 3

A standard rat model of chronic peritoneal exposure
3.1

The Effects of Heparin Administration in an Animal Model of Chronic Peritoneal Dialysate Exposure


Chapter 3: Heparin in animal models of PDF exposure

ABSTRACT

Diverse modes of heparin administration have been used in animal models of chronic peritoneal dialysate exposure to maintain catheter patency and prevent fibrinous adhesions. Heparin has biological actions independent of its well-known anticoagulant activity, including the ability to modulate extracellular matrix synthesis, cellular proliferation, angiogenesis and inflammation. These actions may interfere with peritoneal membrane homeostasis. The present study evaluated the influence of the mode of heparin administration on technique survival and infection rate in a rat model of chronic dialysate exposure. Further, the incorporation of heparin in the peritoneal membrane was examined.

A 3.86% glucose dialysate was injected twice daily in wistar rats, using a heparin-coated catheter (group A1), a standard catheter with heparin injections during the entire exposure time (group A2) or only during 1 wk (group A3). In a fourth group, sham manipulations were performed and a fifth group was left untreated. Technique survival was 80% in group A1, 60% in group A2 and 40% in group A3. The rate of infection was highest in group A1 and lowest in group A2. Intraperitoneally administered heparin accumulated in the peritoneal membrane, whereas a dextrane with a molecular weight similar to that of heparin was not incorporated in the peritoneum.

In conclusion, intraperitoneal heparin reduced the incidence of infection in an animal model of chronic dialysate exposure. The best technique survival was, however, obtained using a heparin-coated catheter. Heparin is incorporated in the peritoneal membrane, where it may exert diverse biological actions and thus bias study results. The use of a heparin-coated catheter in combination with antibiotics may be the optimal approach to obtain a peritoneal access in animal models of chronic dialysate exposure.

INTRODUCTION

Animal models of peritoneal dialysis remain an essential step between in vitro studies and clinical trials. In the past few years, substantial progress has been made in the development of experimental animal models of chronic dialysate exposure. The most important technical problem remains the high incidence of infection and the frequent
obstruction of the peritoneal access. In normal circumstances, high fibrin generation in the peritoneal cavity is balanced by active fibrinolysis. During peritonitis, the equilibrium between the procoagulant and fibrinolytic activity is disturbed in favor of increased fibrin formation. To curtail the formation of fibrin deposits resulting in catheter obstruction and peritoneal adhesions, intraperitoneal heparin administration has become a widely used practice.

The biological action of heparin extends, however, beyond its traditional role as an anticoagulant. Heparin profoundly affects the activity of diverse inflammatory cells. Further, heparin inhibits the proliferation of several different cell types, including vascular smooth muscle cells, fibroblasts and peritoneal mesothelial cells. Depending on the circumstances, both inhibitory and promoting effects on angiogenesis have been described. Finally, heparin modulates the synthesis of the extracellular matrix proteins by different cell types.

The effects of heparin on peritoneal membrane pathophysiology are at present largely unknown. It is however reasonable to assume that, if intraperitoneally administered heparin accumulates in the peritoneum, substantial effects on peritoneal function and structure may be expected.

Against this background, we examined the effects of different modes of heparin administration on technique survival and infection rate in an animal model of chronic peritoneal dialysate exposure. Further experiments were conducted to evaluate whether exogenous heparin is incorporated in the peritoneal membrane.

**MATERIALS AND METHODS**

*Laboratory animals*

The experiments were conducted in 64 female Wistar rats (Iffa Credo, Brussels, Belgium) with a mean body weight of 210±2.1 g. All animals received care in accordance with the national guidelines for the care and use of laboratory animals. A subcutaneous port (PMINA-CBAS-C30 Soloport, Instech Solomon, Plymouth Meeting, USA) was implanted in the neck under halothane (Fluothane, Zeneca, Destelbergen, Belgium) anesthesia in a
Chapter 3: Heparin in animal models of PDF exposure

sterile manner. The attached polyurethane catheter was tunneled over the left flank into the peritoneal cavity. The first week after implantation, catheters were flushed daily with 1 ml Earle’s Balanced Salt Solution (EBSS, Life Technologies Ltd., Paisley, Scotland). Thereafter, 10 ml conventional 3.86 % glucose dialysate (Dianeal, Baxter SA, Lessines, Belgium) was administered twice daily. Laboratory technicians wore masks and gloves during manipulations. The area of the port was desinfected with ethanol 97% 20 sec before puncture.

Study of the influence of the mode of heparin administration on catheter patency and infection rate

In a first set of experiments, the influence of the mode of heparin administration on catheter patency and infection rate was evaluated. Five groups of experimental animals were studied. In group A1 (n=10) a heparin-coated catheter was implanted and no heparin was administered intraperitoneally. The animals of group A2 (n=10) received a standard catheter and the infused solutions (EBSS or dialysate) were supplemented with heparin (5 IU/ml, Heparin Leo, Leo Pharmaceuticals, Zaventem, Belgium) during the entire treatment period. A standard catheter was also implanted in group A3 (n=10), but heparin (5 IU/ml) was only added to EBSS during the first week. Group A4 (n=10) received a standard catheter and the port was punctured twice daily without injecting dialysate, in order to mimic the stress and contamination associated with a transcutaneous needle passage. Group A5 (n=9) was left untreated.

The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice daily. In case of catheter obstruction, an attempt was made to infuse fluids under halothane anesthesia. In case of persistent catheter obstruction, skin lesions or severe weight loss, dialysate and catheter tip cultures as well as dialysate white blood cell (WBC) counts were obtained and the animal was sacrificed. At two week intervals, dialysate cultures and WBC counts were obtained through sterile abdominal puncture with a silicon catheter (Venflon, Becton Dickinson, Erembodegem-Aalst, Belgium) under halothane anesthesia. After 8 weeks of dialysate exposure, 10 ml of dialysate was infused in all experimental groups through sterile abdominal puncture with a silicon catheter. After 120 min, bulk dialysate was recovered
through the silicon catheter and samples were obtained for culture and WBC counts. The tunneled polyurethane catheter was removed in a sterile way and the tip was cultured. WBC counts were performed in a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a dialysate WBC count higher than 1000/mm³.

**Study of the incorporation of heparin in the peritoneal membrane**

In a second series of experiments, the incorporation of intraperitoneally administered heparin in the peritoneal membrane was evaluated. All experimental animals received a standard catheter. In group B1 (n=3) the infused solutions (EBSS and dialysate) were supplemented with biotinylated heparin (5 IU/ml, BH-0323, MW 12500 Da, Celsus Laboratories, Cincinnati, Ohio) during the entire study period. The animals of group B2 (n=3) received 5 IU/ml biotinylated heparin in EBSS during the first week. Thereafter, no further heparin was injected. To assure that the heparin molecule was incorporated rather than the biotin-label, a biotinylated macromolecule (5.6 µg/ml Biotin-dextran, MW 10000 Da, Sigma) with a molecular weight similar to that of biotinylated heparin was administered in group B3 (n=4). The dose was selected to match the number of biotin-labels with those of group B1. In group B4 (n=5), the solutions were supplemented with 5 IU/ml unlabelled heparin during the entire study period. After 2 weeks of dialysate exposure the rats were sacrificed for tissue sampling. Two samples of parietal and visceral peritoneum were obtained in each rat, fixed in 4% neutral buffered formalin and embedded in paraffin. Five µm sections were cut for immuno-histochemistry.

The incorporation of the biotinylated heparin was investigated using the indirect peroxidase antiperoxidase (PAP) method. Sections were deparaffinized, rehydrated and incubated in 3% H₂O₂ in PBS for 10 min to block endogenous peroxidase activity. The sections were subsequently washed in blocking serum (1% BSA, 0.2% Tween in PBS) for 30 min to block non-specific binding. To detect the biotin label, the sections were then incubated for 30 min with streptavidin-peroxidase, diluted 1/200 in PBS (Dako A/S, Glostrup, Denmark). 3,3’-diaminobenzidine (DAB) was used as the chromogenic substrate for the peroxidase, resulting in a brown precipitate. The chromogenic signal was enhanced using a TSA™-kit (Tyramide Signal Amplification, NEN™ Life Science Products, Zaventem, Belgium).
Morphometric measurements were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification x200. For each sample of parietal or visceral peritoneum, two sections were analyzed. Quantitative measurements of the incorporated heparin were made with a computerized image analysis system (LEICA Q500MC, Leica Cambridge Ltd, UK). A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the peritoneal tissue was analyzed with Qwin Software (Leica Cambridge Ltd., Cambridge, UK). Each pixel in a color image was divided into three color components (hue, saturation and intensity). The threshold for each color component of the brown staining was defined and kept constant throughout the analysis. In a predefined area covering the tissue within 500 µm of the mesothelium the staining was measured and expressed as percentage.

Statistical analyses

The results are expressed as mean +/- SEM. Statistical analysis was performed using chi-square tests, ANOVA and unpaired t-tests as appropriate. The significance level was set at P≤0.05.

RESULTS

Technique survival

No differences in body weight were observed between the experimental groups at the different time points (Figure 1). At the end of the study, the body weight of the rats with peritonitis (264±19 g) was not different from those without peritonitis (263±3 g). Technique survival in the animals treated with heparin during 1 week (group A3) and during the entire study period (group A2) was 40 % and 60 %, respectively. In the heparin-coated catheter group (group A1), technique survival was 80 % (Figure 2). All animals of the sham group (group A4) and the control (group A5) survived until the end of the study.
Figure 1: Body weight in the different experimental groups. No differences were observed between the animals in group A1 (closed squares, n=10; heparin-coated catheter, no intraperitoneal heparin), A2 (closed triangles, n=10; standard catheter, infused solutions supplemented with heparin during the entire treatment period), A3 (closed circles, n=10, standard catheter, heparin added to EBSS during the first week only), A4 (open squares, n=10; standard catheter, the port was punctured twice daily without injecting dialysate in order to mimic the stress and contamination associated with a transcutaneous needle passage, or A5 (open circles, n=9, untreated).

Figure 2: Technique survival in the different experimental groups. The best technique survival was obtained in the heparin-coated catheter group (closed squares, n=10), with lower survivals in the animals treated with continuous closed triangles, n=10) and temporal (closed circles, n=10) intraperitoneal heparin administration.
A total of 12 technique failures were observed. Less frequent causes of drop-out were severe weight loss (n=1), an open abdominal wound with catheter damage (n=1) and a severe skin lesion around the port (n=1). Problems related to catheter patency, including dialysate leakage around the port (n=3), dialysate leakage around the catheter (n=3) and frank catheter obstruction (n=3) were the major causes of technique failure.

Infection rate

In several animals of group A1, A2 and A3, one or more dialysate cultures and WBC counts were indicative of infection. In some cases infection was transient, as indicated by the presence of positive dialysate cultures and elevated WBC counts and subsequent negative cultures and WBC counts. Catheter tip cultures and dialysate cultures yielded the same microorganism in 5 experimental animals. In 7 animals, catheter tip cultures were positive, whereas the final dialysate culture was negative. Five of these had repeated positive cultures at earlier time points and elevated WBC counts, suggesting that the dialysate cultures were falsely negative. In group A4, 5 of 8 catheter tip cultures were positive, indicating that transcutaneous needle passage even without injecting fluid is sufficient to introduce bacteria. No infection was observed in group A5.

To compare the rate of infection among the experimental groups, the ratio of the number of infections and the number of observations (availability of both dialysate cultures and WBC counts) was calculated (Table 1). Overall, 43/113 episodes were considered positive for infection. The highest number of infective episodes (23/43) was recorded in the heparin-coated catheter group (group A1). In the animals that only received heparin during the first week (group A3) infection was diagnosed on 14/34 occasions. Continuous administration of heparin (group A2) yielded the lowest infection rate (6/36) (P=0.003 for the comparison between A1, A2 and A3). In 12/113 cases, WBC count was higher than 1000/µl in the absence of bacterial growth. In 5/113 cases, cultures yielded bacteria but WBC were lower than 1000/µl. The total number of animals with at least one episode of infection was 8/9 in group A1, 4/9 in group A2 and 7/10 in group A3.
# Table 1: Dialysate leukocyte counts and cultures in the experimental groups at different time points after catheter implantation. Episodes considered positive for infection are in bold face. The reasons for dropout are given.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>8 weeks</th>
<th>9 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>1</td>
<td>330</td>
<td>880</td>
<td>SA +</td>
<td>5460</td>
<td>SA ++</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>450</td>
<td>360</td>
<td>490</td>
<td>320</td>
<td>510</td>
</tr>
<tr>
<td>A2</td>
<td>1</td>
<td>3140</td>
<td>-</td>
<td>2600</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1060</td>
<td>-</td>
<td>1390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>1</td>
<td>1100</td>
<td>4400</td>
<td>SA +</td>
<td>1990</td>
<td>SA +</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>650</td>
<td>6930</td>
<td>SA +</td>
<td>6250</td>
<td>SA +</td>
</tr>
<tr>
<td>A4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>200</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A=dialysate cultures; B=dialysate leukocyte counts; C=catheter tip cultures; SA=Staphylococcus aureus; CoNS=Coagulase negative Staphylococcus; C sp=Corynebacterium species; SV=Streptococcus viridans; P sp=Propionibacterium species; Ec=Enterococcus; NA=data not available.

Chapter 3: Heparin in animal models of PDF exposure

Heparin incorporation

There were no technique failures in this series of experiments. Administration of biotinylated heparin during the entire treatment period resulted in a pronounced staining for biotin in the visceral peritoneum (Figure 3A, Table 2). The staining was also increased in the group that received minimal amounts of biotinylated heparin, dissolved in EBSS during the first week of treatment (Figure 3B, Table 2). In contrast, biotinylated dextran with a molecular weight similar to that of heparin did not accumulate in the peritoneal membrane (Figure 3C, Table 2). Only background staining was observed in the control group treated with unlabelled heparin (Figure 3D, Table 2).

For the parietal peritoneum, no significant differences in staining were found between the different experimental groups (Table 2).

Figure 3: Staining for biotin in the visceral peritoneum. Pronounced staining was observed in the animals treated with biotinylated heparin throughout the study (group B1, panel A). A short course of low-dose biotinylated heparin resulted in modest staining (group B2, panel B). No staining was observed in animals treated with biotinylated dextran (group B3, panel C) or with unlabelled heparin (group B4, panel D). Magnification 200x.
Table 2: Incorporation of the biotin-label.

<table>
<thead>
<tr>
<th>Group</th>
<th>Visceral peritoneum</th>
<th>Parietal peritoneum</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>5.213 ± 1.600 %</td>
<td>0.049 ± 0.045 %</td>
</tr>
<tr>
<td>B2</td>
<td>1.877 ± 0.271 %</td>
<td>0.050 ± 0.021 %</td>
</tr>
<tr>
<td>B3</td>
<td>0.700 ± 0.187 %</td>
<td>0.008 ± 0.007 %</td>
</tr>
<tr>
<td>B4</td>
<td>0.961 ± 0.138 %</td>
<td>0.078 ± 0.029 %</td>
</tr>
</tbody>
</table>

*aGroup B1 (n=3): infused solutions (EBSS and dialysate) supplemented with biotinylated heparin during the entire study period. Group B2 (n=3): biotinylated heparin in EBSS during first week only. Group B3 (n=4): biotinylated macromolecule with molecular weight similar to biotinylated heparin was administered to ensure the heparin molecule rather than the biotin label was incorporated in the peritoneal membrane. Group B4 (n=5): solutions were supplemented with unlabelled heparin during the entire study period. bP<0.005 vs. B3 & B4, cP<0.001 vs. B2.*

DISCUSSION

A high incidence of peritonitis was found in the present animal model of chronic dialysate exposure, although manipulations were performed as sterile as possible. Also in the sham group infections were frequent, indicating that transcutaneous needle passage even in the absence of fluid injection is sufficient to introduce bacteria and cause peritonitis. It is important to note that in some animals dialysate cultures were transiently positive. In addition, catheter tip cultures yielded more often bacteria than dialysate cultures in the same animal. It thus follows that the true incidence of peritonitis may be severely underestimated if screening is limited to dialysate cultures at the end of the study. It has been suggested that peritonitis may be diagnosed clinically on the basis of weight loss and decreased activity. In the present study, no difference was found in body weight between animals with and without peritonitis. Taken together, the data suggest that the incidence of peritoneal infection in animal models of chronic dialysate exposure should be evaluated by regular dialysate cultures throughout the study and by a catheter tip culture at the end of the study.

Intraperitoneally administered heparin reduced the rate of peritonitis in the present animal model of chronic dialysate exposure. These findings are in agreement with previously demonstrated beneficial effects of heparin on survival and bacterial clearance in
Chapter 3: Heparin in animal models of PDF exposure

experimental animals and humans with peritonitis \(^{17-19}\). These effects of heparin can most likely be attributed to decreased fibrin deposits within the peritoneal cavity, thus rendering micro-organisms more susceptible to clearance by the host defense mechanisms.

Although the rate of intraperitoneal infection was highest in the group with a heparin-coated catheter, technique survival was excellent in this group. In contrast, a poor technique survival was obtained in the animals with a standard catheter and intraperitoneal heparin administration. Intermittent injections of heparin apparently are not sufficient to completely impede the formation of fibrin deposits and the resultant catheter obstruction. Coating of the catheter with heparin, however, largely prevents obstruction.

Intraperitoneally administered heparin accumulates in the peritoneal membrane, as evidenced by the positive immunocytochemical staining. Even administration of low doses of heparin for a short period of time were sufficient to yield a positive staining. In contrast, a synthetic macromolecule of a similar molecular weight was not incorporated in the peritoneal membrane, indicating specificity of the findings. Heparin exerts its biological actions by binding to proteins in the extracellular fluid, on the cellular surface and in the extracellular matrix. It thus affects a variety of physiologic processes, including the immune response, cellular proliferation, extracellular matrix synthesis and angiogenesis. Very little is known about the effects of exogenous heparin on peritoneal function and structure. Intraperitoneal heparin administration augmented small solute transport rates in PD patients \(^{20,21}\). Sulodexide, which is a mixture of 80% low molecular weight heparin and 20% low molecular weight dermatan sulphate, increased D/P ratios of urea and creatinine and decreased peritoneal protein losses \(^{22}\). Against this background, it is reasonable to assume that heparin may have substantial acute and chronic effects on peritoneal membrane pathophysiology when administered intraperitoneally in an animal model of chronic dialysate exposure. As these effects may interfere with study results, the use of intraperitoneal heparin in these models should be avoided.

In conclusion, intraperitoneal heparin administration reduced the incidence of peritoneal infection in an animal model of chronic dialysate exposure, but did not prevent the frequent occurrence of catheter obstruction and resultant technique failure. In contrast, excellent technique survivals were found when heparin-coated catheters were used. In doses that are routinely used, heparin accumulates in the peritoneal membrane, where it
Chapter 3: Heparin in animal models of PDF exposure

may exert diverse effects on peritoneal function and structure. The preferred peritoneal access in animal models of dialysate exposure is a heparin-coated catheter, but this approach should probably be combined with the use of intraperitoneal antibiotics.

ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica and Mieke Van Landschoot for their expert technical assistance. The work was supported by a grant from Baxter Healthcare Co.
Chapter 3: Heparin in animal models of PDF exposure

REFERENCES


158


3.2

Antibiotic Administration in an Animal Model of Chronic Peritoneal Dialysate Exposure


Perit Dial Int 2003, 23: 331-338
Chapter 3: Antibiotics in animal models of PDF exposure

ABSTRACT

The high incidence of intraperitoneal infection remains an important problem in animal models of chronic dialysate exposure. Prophylactic antibiotic administration can be used to resolve this problem, but the isolated effects of antibiotics on peritoneal membrane function and structure are unknown. The present study examined the effects of prophylactic antibiotics on infection rate and peritoneal membrane function and structure in a rat model of chronic dialysate exposure.

A first group of rats (A, n=12) received twice daily 10 mL 3.86% glucose dialysate through a heparin-coated catheter. In a second group of animals (B, n=12), oxacillin 2.5 mg/day and gentamycin 0.04 mg/day were added to the dialysate. Group C (n=12) was injected twice daily with an identical dose of antibiotics dissolved in 1 mL of buffer solution. Group D (n=12) was left untreated. Dialysate cultures were obtained regularly. After 8 weeks of exposure, peritoneal transport studies were performed and samples for histology were obtained.

Technique survival was 92% in group A and 100% in the remaining groups. Five rats of group A, but none of the animals of the other groups developed peritonitis. The transport rates of small solutes were elevated and net ultrafiltration was decreased in group A, compared to the controls. Fibrosis, as evaluated by quantifying Picro Sirius Red staining with image analysis, was significantly elevated in group A (3.48 ± 1.06 % vs 0.72 ± 0.51 % in group D, P<0.05), but not in group B (0.29 ± 0.07 %) or in group C (0.52 ± 0.28 %). Vascular density, measured by counting the number of blood vessels that stained positive for eNOS, was increased in both groups that were exposed to dialysate (153.0 ± 12.9/µm² in group A and 131.6 ± 14.3/µm² in group B vs 76.76 ± 12.37/µm² in group C and 73.2 ± 10.4/µm² in group D, P<0.01).

Prophylactic administration of oxacillin and gentamicin adequately prevented intraperitoneal infection in an animal model of chronic dialysate exposure. In addition, fibrosis was absent suggesting that intraperitoneal infection rather than dialysate exposure is a causative factor.
INTRODUCTION

Animal models of peritoneal dialysis (PD) are essential to examine the effects of peritoneal dialysis fluids on the functional and structural properties of the peritoneal membrane. Although substantial progress has been made in the development of experimental models of chronic dialysate exposure, peritonitis and subsequent obstruction of the peritoneal access remain frequent technical problems. We have recently reported that the use of a heparin-coated catheter was associated with a low rate of catheter obstruction and an excellent technique survival. The incidence of intraperitoneal infection was, however, unacceptably high.

Intraperitoneal antibiotics are used by several investigators to prevent peritonitis in animal models of peritoneal dialysis. Very little data are available on the isolated effects of antibiotics on peritoneal membrane homeostasis. Intraperitoneal gentamicin decreased the transport of small solutes in PD patients. Several antibiotics, including cephalosporins and aminoglycosides, inhibit the growth of human peritoneal mesothelial cells in culture. In contrast, CA125 dialysate concentrations, a marker of mesothelial cell mass, did not change after intraperitoneal cefazolin administration in noninfected PD patients. It is unknown whether the prophylactic use of antibiotics in animal models affects the function and structure of the peritoneal membrane. This knowledge is, however, essential for the development of valid animal models of peritoneal dialysate exposure.

Against this background, we examined the effects of an intraperitoneal antibiotic regimen on infection rate in an animal model of chronic peritoneal dialysate exposure. Further experiments were conducted to evaluate whether the antibiotic regimen that adequately prevented the occurrence of intraperitoneal infection was associated with changes in peritoneal function and structure.

MATERIAL AND METHODS

Laboratory animals

The experiments were performed in 48 female Wistar rats (Iffa Credo, Brussels,
Chapter 3: Antibiotics in animal models of PDF exposure

Belgium) with a mean body weight of 215 ± 0.7 g, receiving care in accordance with the national guidelines for the care and use of laboratory animals. A subcutaneous port (PMINA-CBAS-C30 Soloport, Instech Solomon, Plymouth Meeting, USA) was implanted in the neck under halothane (Fluothane, Zeneca, Destelbergen, Belgium) anesthesia in a sterile manner. The attached polyurethane heparin-coated catheter (Instech Solomon, Plymouth Meeting, USA) was tunneled over the left flank into the peritoneal cavity. After surgery the animals received an intramuscular injection of buprenorphine (Temgesic, 0.1 mL/kg, Schering-Plough NV/SA, Brussels, Belgium). The first week after implantation, catheters were flushed daily with 1 mL Earle’s Balanced Salt Solution (EBSS, Life Technologies Ltd., Paisley, Scotland). Thereafter, 10 mL conventional 3.86 % glucose dialysate (Dianeal PD4, Baxter SA, Lessines, Belgium) was administered twice daily. Laboratory technicians wore masks and gloves during manipulations. The area of the port was disinfected with ethanol 97% 20 sec before puncture.

Experimental protocol

A first group of experimental animals (Group A, n=12) received twice daily 10 mL of Dianeal without further supplements. In a second group of rats (Group B, n=12), oxacillin (Penstapho, 2.5 mg/day, Bristol-Myers Squibb, Brussels, Belgium) and gentamycin (Geomycine, 0.04 mg/day, Schering-Plough, Brussels, Belgium) were added to the dialysate. The use of this regimen was supported by previous findings that intraperitoneal infections can be ascribed mainly to oxacillin-sensitive Staphylococcus aureus and epidermidis and to a lesser extent to gram-negative bacteria. In pilot studies, the presently used antibiotic doses were found to be the lowest that effectively prevented intraperitoneal infection. The animals of group C (n=12) were injected twice daily with an identical dose of antibiotics dissolved in 1 mL EBBS. Group D (n=12) was left untreated. Osmolality was measured in 9 dialysate samples with antibiotics (493.3±0.3 mOsm/l) and in 9 dialysate samples without antibiotics (492.1±0.3 mOsm/l), to exclude an osmotic activity of the antibiotics per se.

The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice daily. In case of catheter
obstruction, an attempt was made to infuse fluids under halothane anesthesia. In case of persistent catheter obstruction, skin lesions or severe weight loss, dialysate and catheter tip cultures as well as dialysate white blood cell (WBC) counts were obtained and the animal was sacrificed. At two week intervals, dialysate cultures and WBC counts were performed on 2 ml of fluid obtained through sterile abdominal puncture with a silicone catheter (Venflon, Becton Dickinson, Erembodegem-Aalst, Belgium) under halothane anesthesia 4 hours after the last dialysate injection. Antibiotics were discontinued 16 hours before sampling. WBC counts were performed in a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a dialysate WBC count higher than 1000 /mm$^3$.

*Peritoneal transport studies*

After 8 weeks of dialysate exposure, rats were anesthetized with thiobutabarbitral (Inactin, RBI, Natick, USA, 100 mg/kg s.c.). The trachea was intubated, a jugular vein was cannulated for continuous infusion of isotonic saline, and a carotid artery was cannulated for blood sampling. After 30 min, a silicone catheter was inserted in the abdomen and 15 mL of 3.86 % Dianeal was infused. Plasma and dialysate samples were collected at t=0, 30, 60, 120 min, for determination of creatinine and urea levels. After 120 min, dialysate was recovered through the silicone catheter and samples were obtained for culture and WBC counts. The abdomen was opened by midline incision for collection of the rest of the dialysate and for tissue sampling. The tunnelled polyurethane catheter was removed in a sterile way and the tip was cultured. The transport of low molecular weight solutes was evaluated by calculating the D/P ratio for urea and creatinine at 0, 30, 60 and 120 min.

*Study of peritoneal morphology*

One sample of parietal and visceral peritoneum was obtained in each experimental animal, fixed in 4% neutral buffered formalin and embedded in paraffin. Five µm sections were cut for histology and immunohistochemistry.

The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparafinized, rehydrated and stained briefly with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen.
Vascular density was evaluated with an immunostaining for endothelial NO synthase (eNOS). Sections were deparaffinized, rehydrated, incubated in 3 % H₂O₂ in PBS for 15 min to block endogenous peroxidase and washed in 10 % normal horse serum (Sigma, St.Louis, MO) in PBS for 20 min to block non-specific binding. Subsequently, they were incubated with the primary antibody (anti-human eNOS, Transduction Laboratories, Lexington, Kentucky), a biotinyla-ted IgG (Vector Laboratories, Burlingame, California) and streptavidine-peroxidase, for 45 min each. 3,3’-diaminobenzidine (DAB) was used as the chromogenic substrate to visualize immunolabelling, resulting in a brown precipitate.

**Morphometric analysis**

Morphometric measurements were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification x200. For each sample of parietal or visceral peritoneum, two sections were analyzed.

The Picro Sirius Red staining was evaluated quantitatively with a computerized image analysis system (Zeiss, Oberkochen, Germany). A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss, Oberkochen, Germany). Each pixel in a color image was divided into three color components (hue, saturation and intensity). The threshold for each color component of the staining was defined and kept constant throughout the analysis. In a predefined area covering the tissue within 500 µm of the mesothelium, the Picro Sirius Red staining was measured and expressed as a percentage.

The immunostaining for eNOS was also quantified using image analysis. Random fields (a mean of 6 per section) were selected and digitized as mentioned above. Labelled blood vessels were counted as N/field.

**Statistical analyses**

The results are expressed as mean +/- SEM. Statistical analysis was performed using ANOVA and where appropriate the Tukey test was used as multiple comparison t-test. The significance level was set at P≤0.05.
RESULTS

Technique survival and infection rate

No differences in body weight were observed between the experimental groups at the different time points (data not shown). Technique survival was 92 % in group A and 100 % in the remaining groups. The drop-out in group A was due to necrosis of the skin in the port area.

In 5 animals of group A, one or more dialysate cultures and WBC counts were indicative of infection (Table 1). None of the animals of the other groups had peritonitis at any time point (Table 1).

Peritoneal transport studies

The transport rates of urea and creatinine were elevated in the experimental animals that were exposed to peritoneal dialysate in the absence of antibiotic prophylaxis, as compared to controls (Figure 1). Similarly, net ultrafiltration was decreased in these animals (Figure 2). The mean WBC count in the dialysate of group A, B and C correlated with net ultrafiltration ($r^2=0.19$, $P=0.01$) and with D/P creatinine ($r^2=0.1$, $P=0.05$), but not with D/P urea ($r^2=0.08$, $P=0.1$).

Fibrosis and neoangiogenesis

The degree of fibrosis was increased in the animals that received dialysate without antibiotics, as compared to all other groups (Figure 3 and 4). In contrast, the number of blood vessels was higher in both groups that were exposed to dialysate, as compared to group C and D (Figure 5 and 6). Although neoangiogenesis tended to be more pronounced in group A, the difference with group B was not significant. The mean WBC count in the dialysate of group A, B and C correlated with fibrosis ($r^2=0.34$, $P=0.003$) and neoangiogenesis ($r^2=0.21$, $P=0.005$).
### Table 1: Dialysate cultures (DC), Dialysate WBC counts (WBC), and catheter tip cultures (CTC) in the experimental groups at different time points after catheter implantation. Episodes considered positive for infection are in boldface.

<table>
<thead>
<tr>
<th>Group</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DC</td>
<td>WBC</td>
<td>DC</td>
<td>WBC</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1410</td>
<td>-</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>560</td>
<td>-</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>510</td>
<td>ECl++</td>
<td>2470</td>
<td>ECl+/CoNS++</td>
</tr>
<tr>
<td>4</td>
<td>SA++</td>
<td>1390</td>
<td>Skin necrosis around the port.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>270</td>
<td>-</td>
<td>140</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>580</td>
<td>S</td>
<td>260</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>360</td>
<td>CoNS++</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>500</td>
<td>-</td>
<td>1310</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>EC++</td>
<td>4230</td>
<td>ECl++</td>
<td>3625</td>
</tr>
<tr>
<td>10</td>
<td>670</td>
<td>-</td>
<td>230</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>SA++</td>
<td>12000</td>
<td>SA++</td>
<td>11500</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>-</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>190</td>
<td>-</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>620</td>
<td>-</td>
<td>220</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>660</td>
<td>-</td>
<td>270</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>530</td>
<td>SA</td>
<td>740</td>
<td>SA++</td>
</tr>
<tr>
<td>6</td>
<td>840</td>
<td>-</td>
<td>260</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td>-</td>
<td>380</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>220</td>
<td>-</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>680</td>
<td>-</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>350</td>
<td>-</td>
<td>390</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>320</td>
<td>-</td>
<td>440</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>530</td>
<td>-</td>
<td>380</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>-</td>
<td>340</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>-</td>
<td>450</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>470</td>
<td>-</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>590</td>
<td>-</td>
<td>470</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>-</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>140</td>
<td>-</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>310</td>
<td>-</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>290</td>
<td>-</td>
<td>190</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>280</td>
<td>-</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>360</td>
<td>-</td>
<td>250</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>120</td>
<td>-</td>
<td>620</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>60</td>
<td>-</td>
<td>430</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>140</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>490</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>390</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>260</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>450</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>200</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>280</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>170</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>170</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>250</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>370</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

ECl = Enterobacter cloacae; CoNS = coagulase-negative staphylococcus; EC = Escherichia coli; SA = Staphylococcus aureus; SV = Streptococcus viridans; Ent = Enterococcus NA = data not available. Group A, 10 ml 3.86% glucose dialysate 2x daily, group B, oxacillin 2.5 mg/day and gentamicin 0.04 mg/day added to dialysate; group C, injected 2x daily with identical dose of antibiotics dissolved in 1 ml buffer solution; group D, untreated. Infusion under narcosis on day 14 and day 39. Infusion under narcosis on day 5.
Chapter 3: Antibiotics in animal models of PDF exposure

Figure 1: The transport rates for urea and creatinine after 120-minute dwell of 15 ml 3.86% glucose dialysate, defined as the area under the curve (AUC) of the dialysate-to-plasma ratio (D/P) curve in groups A, B, C and D. *p < 0.05 versus D. [Group A (n=12), 10ml 3.86% glucose dialysate 2x daily; group B (n=12), oxacillin 2.5 mg/day and gentamicin 0.04 mg/day added to dialysate; group C (n=12), injected 2x daily with identical dose of antibiotics in 1ml of buffer solution; group D (n=12), untreated]

Figure 2: Net ultrafiltration after a 120-minute dwell of 15 ml 3.86% glucose dialysate in groups A, B, C and D. *p < 0.05 versus D.
**Figure 3**: Picro Sirius Red staining for fibrosis in the visceral peritoneum (x200). Fibrous tissue deposition was most prominent after 8 weeks’ infusion of dialysate without antibiotics (group A, panel A). When antibiotics were added to the dialysate (group B, panel B) or EBSS (group C, panel C), fibrous tissue deposition was comparable to untreated rats (group D, panel D).

**Figure 5**: Staining for eNOS in the visceral peritoneum (x200). Vascular density increased in the animals that received dialysate without (group A, panel A), or with (group B, panel B) antibiotics for 8 weeks, compared to the group that was infused with EBSS and antibiotics (group C, panel C) and the untreated animals (group D, panel D).
Chapter 3: Antibiotics in animal models of PDF exposure

Figure 4: Fibrosis was evaluated with Picro Sirius Red staining. The areas staining red were quantified by image analysis and expressed as percentage. Significant fibrosis was present only in the animals treated with dialysate without antibiotics (group A). In groups B and C, the Picro Sirius Red staining was not different from control animals (group D). *p < 0.05 versus group B, C, and D.

Figure 6: Vascular density was measured as the number of blood vessels staining for eNOS in a predefined area. Neoangiogenesis was present in animals exposed to dialysate with (group B) or without (group A) antibiotics. Vascular density was not elevated in rats that received only antibiotics (group C). *p < 0.05 versus groups C and D.
DISCUSSION

Long-term PD affects the functional and structural characteristics of the peritoneal membrane. Structure-function relationship studies have shown that in patients on long-term PD loss of ultrafiltration capacity is associated with submesothelial fibrosis, presence of a hyalinizing vasculopathy and neoangiogenesis. The continuous exposure to the unphysiologic conventional peritoneal dialysate fluid (PDF) has been implicated as an important pathogenetic factor. Recently, more biocompatible PDF have been developed, with the potential to interfere less with peritoneal membrane homeostasis.

Diverse animal models have been used to research the effects of conventional and new PDF on different aspects of peritoneal membrane pathophysiology. Although substantial progress has been made in the development of these models, a common problem remains the high incidence of intraperitoneal infection. Peritonitis causes fibrosis and neoangiogenesis. Further, adenoviral-mediated gene transfer of interleukin-1β and tumor necrosis factor-α in the rat peritoneal membrane resulted in an upregulation of vascular endothelial growth factor and transforming growth factor-β1 and was associated with the development of fibrosis and neoangiogenesis, along with an increased permeability for small solutes and loss of ultrafiltration capacity. If unrecognized, the presence of intraperitoneal infection may thus substantially interfere with study results.

In the present study, absence of antibiotic prophylaxis was associated with peritonitis in 42% of the animals that were exposed to conventional PDF. Functionally, the peritoneal membrane in these animals was characterized by an elevated transport of small solutes and a decreased ultrafiltration rate. Structurally, severe submesothelial fibrosis was found. The mean WBC count in the dialysate, as a parameter of intraperitoneal inflammation, correlated with net ultrafiltration and the transport rate of creatinine, as well as with the extent of fibrosis.

The administration of antibiotics adequately prevented the occurrence of infection in the present animal model of dialysate exposure. Importantly, no significant increase in small solute transport and fall in ultrafiltration capacity was recorded. In addition, fibrosis did not develop in this animal group. It should be noted that 8 weeks exposure may have
been too short for significant dialysate-related alterations to develop. This does, however, not detract from the finding that the presence of intraperitoneal infection in animals exposed to PDF results in functional and structural alterations of the peritoneal membrane that cannot be solely attributed to the PDF exposure.

Neoangiogenesis was present in both animal groups that were exposed to PDF. Vessel density tended to be higher in the group that received no antibiotics as compared to the group that did, but the difference was not significant. On the other hand, the mean WBC count in the dialysate correlated with vessel density. These results indicate that neoangiogenesis can be mainly attributed to the effect of PDF, although intraperitoneal infection plays a contributory role.

The administration of antibiotics in the absence of PDF was not associated with alterations of the function and structure of the peritoneal membrane. Ultrafiltration rate tended to be lower and small solute transport tended to be higher, but were not significantly different from the values in untreated controls. These mild effects can most likely be attributed to the injection of fluid in the peritoneal cavity, although an effect of antibiotics cannot be entirely excluded. We have chosen untreated animals as control group, since injection of buffer without antibiotics would introduce bacteria and therefore cease to be a good control.

In conclusion, peritonitis is a frequent complication in animal models of dialysate exposure. The presence of peritonitis affects the function and structure of the peritoneal membrane and may thus interfere with study results. Prophylactic administration of antibiotics adequately prevents intraperitoneal infection and the associated alterations of the peritoneal membrane. There is no evidence that antibiotics per se may significantly alter the peritoneal membrane in animal models of peritoneal dialysate exposure.

ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance. The work was supported by a grant from Baxter Healthcare Co.
REFERENCES


Chapter 4

Membrane alterations after chronic exposure to conventional and new peritoneal dialysis fluids
4.1

Long-term Exposure to New Peritoneal Dialysis Solutions: Effects on the Peritoneal Membrane

Mortier S, Faict D, Schalkwijk CG, Lameire NH, De Vriese AS

Kidney Int 2004; 66: 1257-1265
Chapter 4: Effects of new PDF on peritoneal morphology and function

ABSTRACT

Chronic exposure to peritoneal dialysis fluid (PDF) affects the peritoneum, but precise causative factors are incompletely understood. We examined the effects of standard and new PDF on peritoneal function and structure.

Female Wistar rats received twice daily intraperitoneal infusions of a standard lactate-buffered 3.86 % glucose PDF at pH 5.5 (Dianeal®, n=12), a low GDP bicarbonate/lactate-buffered 3.86 % glucose PDF at pH 7.4 (Physioneal®, n=12), a lactate-buffered amino acid-based PDF at pH 6.7 (Nutrineal®, n=12) or Earle's Balanced Salt Solution at pH 7.4 (EBSS, n=12) during 12 weeks.

Net ultrafiltration was lower after treatment with standard PDF, but not with low-GDP bicarbonate/lactate-buffered and amino acid-based PDF, compared to EBSS. Peritonea exposed to standard PDF were characterized by an increased expression of vascular endothelial growth factor, microvascular proliferation as well as submesothelial fibrosis, which were not observed in other groups. Staining for methylglyoxal-adducts was prominent in the standard PDF exposed group, mild in the low GDP bicarbonate/lactate-buffered group and absent in the other groups. Standard PDF induced accumulation of advanced glycation end products (AGE) and upregulation of the receptor for AGE (RAGE). AGE accumulation was absent and RAGE expression was only modestly increased in low-GDP bicarbonate/ lactate-buffered and amino acid-based PDF.

Long-term in vivo exposure to standard PDF adversely affects peritoneal function and structure. A low-GDP bicarbonate/lactate-buffered and amino acid-based PDF better preserved peritoneal integrity and may thus improve the longevity of the peritoneal membrane. GDPs and associated accelerated AGE formation are the main causative factors in PDF-induced peritoneal damage.

INTRODUCTION

The performance of the peritoneum as a dialyzing membrane can be compromised by the development of various structural and functional alterations occurring in the course
of long-term PD treatment. Morphologically, a chronically exposed peritoneal membrane is characterized by interstitial fibrosis, reduplication of basement membrane of the mesothelium and the blood vessels, hyalinization of the blood vessel media and neoangiogenesis. Functionally, increased transport of small solutes and a loss of ultrafiltration capacity may develop, for which an increased effective vascular surface area is held responsible.

Evidence is mounting that standard PDF play a pathogenic role in the development of peritoneal changes. The relative contribution of the different PDF components in mediating peritoneal changes is, however, incompletely understood. As the described structural alterations resemble those that occur in diabetes, it was hypothesized that high glucose is an important contributing factor. In vitro exposure of endothelial and mesothelial cells to high glucose stimulates the expression of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-β). Chronic in vivo exposure of the peritoneal membrane to high glucose concentrations resulted in microvascular proliferation and submesothelial fibrosis, mediated by VEGF and TGF-β, respectively. Glucose carmelization during heat-sterilization and storage of PDF results in the formation of GDPs, such as formaldehyde, acetaldehyde, glyoxal, methylglyoxal, 3-deoxyglucosone and 3,4-dideoxyglucosone-3-ene. Methylglyoxal stimulates VEGF synthesis by peritoneal mesothelial and endothelial cells, which implies that GDPs may contribute to the vascular proliferation in the peritoneal membrane. Glucose and reactive carbonyl compounds, such as methylglyoxal, glyoxal and 3-deoxyglucosone have the potential to bind non-enzymatically to free amino-groups on proteins or lipids and form irreversible advanced glycation endproducts (AGEs). AGEs promote in vitro VEGF expression in several cell types. Vascular and interstitial AGE content correlated with the extent of vascular sclerosis and interstitial fibrosis in the peritoneal membrane of long-term PD patients. Functionally, AGE formation was associated with a decrease in ultrafiltration volume and an increased transport of various soluta. The best characterized of the AGE receptors has been termed the receptor for AGE (RAGE), although other AGE-binding structures have been described. Chronic exposure to high glucose concentrations greatly increased peritoneal expression of RAGE, most distinctly in
the mesothelium, submesothelial fibrotic tissue and blood vessel walls. The development of high glucose-induced interstitial fibrosis in the peritoneal membrane was prevented by an anti-RAGE antibody. Finally, the lactate buffer has been implicated in altering peritoneal structure. In vivo exposure of mice to 40 mmol/L lactate induced mesothelial damage, although not as pronounced as contact with standard PDF. Cultured peritoneal fibroblasts produced more collagen when exposed to increasing concentrations of lactate (10 mmol/L-40 mmol/L). Replacement of lactate by pyruvate in PDF prevented the development of both neoangiogenesis and fibrosis during chronic in vivo exposure in rats.

Low-GDP or non-glucose based PDF and PDF with alternative buffers are currently available. The aim of the present study was to investigate the effects of new PDF on the peritoneal membrane. More in particular, the relative contribution of glucose, GDPs and lactate in the development of functional and structural alterations in the peritoneum were examined. To this end, the effects of chronic exposure to a standard acidic lactate-buffered PDF, a double-chambered, physiological-pH bicarbonate/lactate-buffered PDF and a lactate-buffered amino acid-based PDF were compared.

**MATERIAL AND METHODS**

**Laboratory animals**

The studies were performed in 48 female Wistar rats (Iffacredo, Brussels, Belgium) with a mean body weight of 213 ± 1 g, receiving care in accordance with the national guidelines for care and use of laboratory animals. A subcutaneous port (PMINA-CBAS-C30 Soloport, Instech Solomon, Plymouth Meeting, USA) was implanted in the neck under halothane (Fluothane, Zeneca, Destelbergen, Belgium) anesthesia in sterile conditions. The attached polyurethane, heparin-coated catheter (Instech Solomon) was tunneled over the left flank to the peritoneal cavity. After surgery, the animals received an intramuscular injection of buprenorphine (0.1 ml/kg, Temgesic, Schering Plough NV/SA, Brussels, Belgium). The first week after implantation, catheters were flushed once daily with 1 ml of Earle's Balanced Salt Solution (EBSS, ICN Biomedicals Inc., Aurora,
Ohio, USA). Thereafter, 10 ml of PDF was administered twice daily during 12 weeks. Oxacilline (2.5 mg/day, Penstapho, Bristol-Myers Squibb, Brussels, Belgium) and gentamycine (0.04 mg/day, Geomycine, Shering-Plough, Brussels, Belgium) were added to all solutions. Laboratory technicians wore masks and gloves during manipulations. The area of the port was desinfected with ethanol 97% 20 seconds before puncture.

Study protocol

Animals received twice daily intraperitoneal infusions of 10 ml of a standard lactate-buffered 3.86% glucose PDF at pH 5.5 (Dianeal®, Baxter, SA, Lessines, Belgium, n=12), a low GDP bicarbonate/lactate-buffered 3.86% glucose PDF at pH 7.4 (Physioneal®, Baxter, n=12), a lactate-buffered amino acid-based PDF at pH 6.7 (Nutrineal®, Baxter, n=12) or Earle’s Balanced Salt Solution at pH 7.4 (EBSS, n=12) (Table 1). The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice daily. In case of catheter obstruction, an attempt was made to infuse fluids under halothane anaesthesia. In case of persistent catheter obstruction, skin lesions or severe weight loss, dialysate and catheter tip cultures as well as dialysate white blood cell (WBC) counts were obtained and the animal was sacrificed. At four week intervals, dialysate cultures and WBC counts were performed on 2 ml of fluid obtained through a sterile abdominal puncture with a silicon catheter (Venflon, Becton Dickinson, Erembodegem-Aalst, Belgium) under halothane anaesthesia 4 hours after the last dialysate injection. WBC counts were performed in a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a dialysate WBC count higher than 1000/mm³.

Study of peritoneal function

After 12 weeks of dialysate exposure, rats were anaesthetized with thiobutabarbitral (Inactin, RBI, Natick, USA, 100 mg/kg s.c.). The trachea was intubated and a jugular vein was cannulated for continuous infusion of isotonic saline. After 30 min, a silicone catheter was inserted in the abdomen and 15 mL of 3.86% Dianeal was infused. After 120 min, dialysate was recovered through the silicone catheter and samples were obtained for culture.
and WBC counts. The abdomen was opened by midline incision to collect the rest of the dialysate for determination of net ultrafiltration and to sample tissue. The tunneled polyurethane catheter was removed in a sterile way and the tip was cultured.

### Table 1: Composition of the solutions. GDP levels (a mean of 3 different batches) were taken from Schalkwijk et al. 37. < : below limit of detection, NA : data not available.

<table>
<thead>
<tr>
<th></th>
<th>Dianeo®</th>
<th>Physioneal®</th>
<th>Nutrineal®</th>
<th>EBSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.5</td>
<td>7.4</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Osmolality (mOsmol/L)</td>
<td>483</td>
<td>483</td>
<td>367</td>
<td>290</td>
</tr>
<tr>
<td>Osmotic agent (mmol/L)</td>
<td>glucose (214)</td>
<td>glucose (214)</td>
<td>Amino acids (87)</td>
<td>glucose (5.6)</td>
</tr>
<tr>
<td>Buffer</td>
<td>Lactate</td>
<td>lactate/bicarbonate</td>
<td>lactate</td>
<td>bicarbonate</td>
</tr>
<tr>
<td>GDP content (µmol/L) :</td>
<td>High</td>
<td>Low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>- 3-deoxyglucosone</td>
<td>525 ± 51</td>
<td>253 ± 30</td>
<td>&lt; 0.1</td>
<td>NA</td>
</tr>
<tr>
<td>- methylglyoxal</td>
<td>6.9 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>- glyoxal</td>
<td>9.4 ± 2.2</td>
<td>6.0 ± 1.6</td>
<td>0.8 ± 0.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Study of peritoneal morphology**

One sample of visceral and parietal peritoneum was obtained in each experimental animal in a standardized manner. Parietal peritoneum was obtained at the right hand side of the linea alba and visceral peritoneum was taken from the most distal loop of the small bowel. The samples were fixed in 4% neutral buffered formalin and embedded in paraffin. Five µm sections were cut for histology and immunohistochemistry.

The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparaffinized, rehydrated, and stained briefly with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen.

Immunostaining for endothelial NO synthase (eNOS), VEGF, methylglyoxal (MGO)-adducts, AGE and RAGE were performed. Sections were deparaffinized,
rehydrated, incubated in 3% H$_2$O$_2$ in PBS for 15 min to block endogenous peroxidase and washed in 10% normal horse serum (Sigma, St. Louis, MO, USA) in PBS for 20 min to block non-specific binding. Subsequently, they were incubated with the primary antibody, mouse anti-human eNOS (Transduction Laboratories, Lexington, Kentucky, USA), mouse anti-human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human methylglyoxal-adduct, mouse anti-human AGE (6D12, Cosmo Bio Ltd, Tokyo, Japan) and goat anti-human RAGE (Research Diagnostics, Flanders, NJ, USA), respectively. Thereafter, a biotinylated IgG (Vector Laboratories, Burlingame, California) and streptavidine-peroxidase were applied for 45 min each. 3,3' diaminobenzidine (DAB) was used as the chromogenic substrate to visualize immunolabelling, resulting in a brown precipitate.

The mouse monoclonal antibody specific for MGO-adducts was developed using MGO-modified keyhole limpet hemocyanin (KLH), that was prepared by the reaction of MGO (10mM) with KHL for 7 days at 37°C, as antigen for the immunization of mice. Ten days after the final booster, antisera were tested with MGO-albumin and the mouse with the highest titer was used for fusion. One out of 40 clones was further characterized. The monoclonal antibody is an IgG1 and has a 10-fold preference for 5-hydro-5-methyl-4-imidazolone and/or tetrahydro-pyrimidine as compared to agrypyrimidine, which are three of the characterized epitopes that are formed after the reaction of MGO with arginine. The monoclonal anti-AGE antibody 6D12 recognizes carboxymethyllysine (CML)-like structures, as well as carboxyethyllysine (CEL) and several unidentified AGE epitopes.

Morphometric analysis

Morphometric measurements of the Picro Sirius Red staining and the eNOS, VEGF, MGO-adduct, AGE and RAGE immunostaining were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification x200. For each sample of peritoneum, two sections were analyzed quantitatively with a computerized image analysis system (Zeiss, Oberkochen, Germany). A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss, Oberkochen,
Technique survival and infection rate of laboratory animals

Body weight was similar in the different experimental groups at all time points (data not shown). Technique survival was 83 % in the standard PDF, the low-GDP bicarbonate/lactate-buffered PDF and EBSS group and 75 % in the amino-acid based PDF group. Drop-out was caused by open abdominal wound with catheter damage (n=2) and leakage around the catheter port (n=7). No episodes of infection, defined as a positive dialysate culture and a dialysate WBC count > 1000/mm³, were diagnosed (data not shown).

Ultrafiltration

Net ultrafiltration was significantly lower in the animals that were exposed to the standard PDF, as compared to those treated with low-GDP bicarbonate/ lactate-buffered PDF, amino-acid based PDF and EBSS (Figure 1). Net ultrafiltration was not different among the latter three groups.
Chapter 4: Effects of new PDF on peritoneal morphology and function

Figure 1: Net ultrafiltration after a 120-minute dwell of 15 mL of 3.86% glucose dialysate in experimental animals exposed to the standard peritoneal dialysis fluid (PDF) (N=10, full bars), low-glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (N=10, hatched bars), amino-acid based PDF (N=9, open bars) and Earle's Balanced Salt Solution (EBSS) (N=10, squared bars). *P<0.05 versus EBSS, low-GDP bicarbonate/lactate-buffered PDF, and amino-acid based PDF.

Peritoneal morphology

Standard PDF exposure caused an upregulation of eNOS expression and increased vascular density, as compared to all other groups (table 2, figure 2). No differences in eNOS expression and vascular density were observed between animals treated with low-GDP bicarbonate/lactate-buffered PDF, amino-acid based PDF and EBSS. Similarly, VEGF expression was increased in standard PDF-exposed peritonea, most distinctly in the mesothelial cell layer and in the blood vessel wall (table 2, figure 3). VEGF immunostaining was not different between the other groups (table 2, figure 3). An inverse correlation was observed between net ultrafiltration and the number of blood vessels/area (Pearson r = -0.3696, p < 0.05). In addition, vascular density and VEGF expression correlated positively (Pearson r = 0.6745, p<0.0001). Submesothelial fibrosis was more pronounced in animals exposed to standard PDF, than in the other experimental groups (table 2, Figure 4). No correlation was observed between net ultrafiltration and the degree of fibrosis in the peritoneal tissue (Pearson r = -0.1716, p = 0.31).
Figure 2: Immunostaining for endothelial nitric oxide synthase (eNOS) of the visceral peritoneum. (A) Prominent eNOS staining was present after 12 weeks' exposure to standard peritoneal dialysis fluid (PDF) (x200). (B) Detail of the eNOS staining in the vascular endothelium of a standard PDF-treated rat (x630). Animals exposed to a low-glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (C), and amino acid-based PDF (D) showed an eNOS staining comparable to the Earle's Balanced Salt Solution (EBSS)-exposed group (E) (x200). No specific staining is observed when sections are incubated without primary antibody (F) (x200).
Figure 3: Immunostaining for vascular endothelial growth factor (VEGF) staining of the visceral peritoneum. (A) VEGF staining was prominent in the mesothelial cells and vascular wall of standard peritoneal dialysis fluid (PDF)-exposed rats (x200). (B) Detail of the mesothelium and blood vessel of a rat exposed to standard PDF (x630). No pronounced staining was observed in rats exposed to low-glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (C), amino acid-based PDF (D), and Earle’s Balanced Salt Solution (EBSS) (E) (x200). No specific staining is observed when sections are incubated without primary antibody (F) (x200).
Chapter 4: Effects of new PDF on peritoneal morphology and function

Figure 4: Picro Sirius Red staining of the visceral peritoneum. (A) Animals exposed to standard peritoneal dialysis fluid (PDF) for 12 weeks showed a prominent deposition of fibrous tissue (x200). Detail of the submesothelial fibrotic tissue in a standard PDF-treated animal (x630) (B). Almost no fibrosis was present in the animals exposed to low glucose degradation product bicarbonate/lactate-buffered PDF (C), amino acid-based PDF (D), and Earle’s Balanced Salt Solution (EBSS) (E) (x200).
Table 2: Histological and immunohistochemical analysis of the peritoneum

<table>
<thead>
<tr>
<th></th>
<th>Dianeal® (n=10)</th>
<th>Physioneal® (n=10)</th>
<th>Nutrineal® (n=9)</th>
<th>EBSS (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels, N/mm²</td>
<td>174 ± 17a</td>
<td>97 ± 9</td>
<td>102 ± 2</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>ENOS staining, %</td>
<td>0.28 ± 0.09b</td>
<td>0.17 ± 0.09</td>
<td>0.17 ± 0.3</td>
<td>0.18 ± 0.2</td>
</tr>
<tr>
<td>VEGF staining, %</td>
<td>1.41 ± 0.22 b</td>
<td>0.83 ± 0.15</td>
<td>0.53 ± 0.15</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td>Picro Sirius Red staining, %</td>
<td>4.11 ± 2.39a</td>
<td>1.47 ± 1.09</td>
<td>1.44 ± 0.79</td>
<td>0.87 ± 0.52</td>
</tr>
<tr>
<td>Methylglyoxal staining, %</td>
<td>3.75 ± 0.75b</td>
<td>2.28 ± 0.72c</td>
<td>1.40 ± 0.97</td>
<td>1.33 ± 0.32</td>
</tr>
<tr>
<td>AGE staining, %</td>
<td>1.70 ± 0.23b</td>
<td>0.92 ± 0.04</td>
<td>0.96 ± 0.13</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>RAGE staining, %</td>
<td>2.48 ± 0.31b</td>
<td>1.58 ± 0.25d</td>
<td>1.47 ± 0.27d</td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>Mesothelial damage, %</td>
<td>8.04 ± 2.04b</td>
<td>2.24 ± 1.29</td>
<td>0.20 ± 0.13</td>
<td>1.28 ± 0.82</td>
</tr>
</tbody>
</table>

Abbreviations are: eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; AGE, advanced glycation end products; RAGE, receptor of AGE.

\*P<0.005 versus EBSS, low-GDP bicarbonate/lactate-buffered PDF and amino acid-based PDF, \^P<0.05 versus EBSS, low-GDP bicarbonate/lactate-buffered PDF and amino acid-based PDF, \P<0.01 versus EBSS.

Long-term exposure to standard PDF was associated with prominent staining for MGO-adducts, especially in the vascular wall and mesothelium. A more modest staining for MGO-adducts was visible in the low-GDP bicarbonate/lactate buffered PDF group. No MGO-adducts accumulation was present in the peritoneum of rats treated with amino-acid-based PDF and EBSS (table 2, figure 5). The staining for MGO-adducts correlated positively with VEGF expression and vascular density (Pearson r = 0.5043, P<0.001 and Pearson r = 0.4089, P<0.01, respectively).

AGE strongly accumulated in peritoneal tissue exposed to standard PDF, which was most obvious in the mesothelial layer and the areas of neoangiogenesis. Virtually no AGE staining was observed in the other groups (table 2, figure 6). RAGE expression was higher in all PDF-exposed peritonea, but the most prominent expression was observed in animals treated with standard PDF and was localized in the mesothelial layer, submesothelial fibrotic tissue and blood vessel walls (table 2, figure 7). MGO-adducts accumulation significantly correlated with the staining for AGE and RAGE (Pearson r = 0.4471, P<0.005 and Pearson r = 0.3751, P<0.05). In addition, fibrosis correlated positively with the staining for MGO-adducts, AGE and RAGE (Pearson r = 0.4809, P<0.005,
Pearson r = 0.4843, P<0.005 and Pearson r = 0.4455, P<0.01, respectively). The staining for AGE and RAGE also correlated with VEGF expression (Pearson r = 0.4928, P<0.005 and Pearson r = 0.4015, P<0.05, respectively), but not with vascular density.

Standard PDF caused significant mesothelial damage as compared to all other groups (table 2).

**DISCUSSION**

Chronic exposure of the rat peritoneal membrane to a high-glucose standard PDF results in mesothelial damage, development of submesothelial and interstitial fibrosis and pronounced neoangiogenesis, the latter evidenced by an increased eNOS expression and vascular density. Functionally, the membrane is characterized by a prominent loss of ultrafiltration capacity. These functional and structural characteristics of the peritoneal membrane are similar to those found in patients on long-term PD, thus underlining the relevance of our experimental model. The salient observation of the present study is that the ultrafiltration failure as well as the diverse structural alterations were absent or much less pronounced with an amino acid-based PDF or a lactate/bicarbonate buffered PDF with a low GDP content.

Both the standard PDF and the bicarbonate/lactate-buffered PDF with low GDP content use glucose as the osmotic agent and both the standard PDF and the amino acid-based PDF are characterized by a non-physiologic pH and use lactate as the buffer. Only the standard PDF, however, contains a high level of GDPs. The double chamber design of the bicarbonate/lactate-buffered PDF allows glucose to be sterilized at a very low pH, substantially reducing GDP formation. As the amino acid-based PDF is non-glucose based, it contains no GDPs at all. Taken together, the present results point to GDPs as the principal causative factor for peritoneal damage.

The causality of GDPs in disturbing viability and normal function in cultured mesothelial cells is a well-established phenomenon. A better preservation of the integrity of the mesothelial cell layer with the bicarbonate/lactate-buffered PDF than with standard PDF has previously been observed, but the results could not be ascribed with certainty to differences in GDP content, as high lactate concentrations are also known to affect
Chapter 4: Effects of new PDF on peritoneal morphology and function

Figure 5: Immunostaining for methylglyoxal (MGO) adducts of the visceral peritoneum. (A) MGO adducts strongly accumulate in peritoneal tissue exposed to standard peritoneal dialysis fluid (PDF) (x200) and to a lesser extent in low glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (B) (x200). No MGO adduct accumulation was observed in animals exposed to amino acid-based PDF (C) (x200) or Earle’s Balanced Salt Solution (EBSS) (D) (x200). No specific staining is observed when sections are incubated without primary antibody (E) (x200).
Figure 6: Immunostaining for advanced glycation end products (AGE) of the visceral peritoneum. (A) Standard peritoneal dialysis fluid (PDF) exposure resulted in AGE accumulation in peritoneal tissue (x200). Detail of AGE localization in the standard PDF-exposed blood vessels (B) and mesothelium (C) (x630). In contrast, almost no AGE accumulation was present in tissue exposed to low glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (D), amino acid-based PDF (E), and Earle’s Balanced Salt Solution (EBSS) (F) (x200). No specific staining is observed when sections are incubated without primary antibody (G) (x200).
Figure 7: Immunostaining for receptor for advanced glycation end products (RAGE) of the visceral peritoneum. (A) RAGE staining was elevated in the standard peritoneal dialysis fluid (PDF)-exposed animals compared to the other PDF-treated groups (x200). Detail of RAGE-stained vascular tissue (B) and mesothelium (C) in a standard PDF-treated rat (x630). Low-grade staining was detected in animals treated with low glucose degradation product (GDP) bicarbonate/lactate-buffered PDF (D) and amino acid-based PDF (E) which was significantly higher than those exposed to Earle’s Balanced Salt Solution (EBSS) (F) (x200). No specific staining is observed when sections are incubated without primary antibody (G) (x200).
mesothelial cell viability in vitro. The amino acid-based PDF did not convey prominent mesothelial damage, although it also contains high lactate concentrations. The present findings thus strongly incriminate GDPs as responsible for PDF-induced in vivo mesothelial cell loss.

The involvement of GDPs in peritoneal neoangiogenesis has been suggested repeatedly, though direct evidence has not been provided yet. So far, only one study demonstrated upregulation of VEGF expression by MGO in mesothelial and endothelial cells in vitro, as well as in the peritoneal membrane in vivo. VEGF is a known promotor of peritoneal neoangiogenesis. Several lines of recent evidence have supported a strong causative link between peritoneal neoangiogenesis and loss of ultrafiltration capacity. In the present study, exposure to standard PDF resulted in an increased staining for VEGF, an increased vascular density and a lower ultrafiltration rate, with correlations between these four parameters. While these findings are descriptive and do not demonstrate causality, they support the previously formulated hypothesis that VEGF mediates neoangiogenesis and subsequent ultrafiltration failure. Although the potential of high glucose concentrations to upregulate VEGF expression in several cell types is well-established and to cause angiogenesis in vivo, exposure to high glucose bicarbonate/lactate buffered PDF was counter-intuitively not associated with increased VEGF expression or vascular density. In contrast with our findings, chronic exposure to a bicarbonate/lactate buffered PDF was associated with a higher vascular density compared to the control group, though not to the same extent as a standard PDF. It should be noted that the control group was left untreated. The present results, however, suggest that GDPs may be the principal mediators of peritoneal neoangiogenesis. Comparison of heat- versus filter-sterilized PDF could further strengthen this hypothesis.

GDPs are known to promote AGE generation in vitro. MGO-adducts strongly accumulated in the peritonea exposed to standard PDF. Only mild staining for MGO-adducts was present in the low-GDP bicarbonate/lactate buffered PDF group, corresponding with low MGO levels in this PDF. No staining for MGO-adducts was observed in the amino-acid based PDF and EBSS group. AGE accumulation was indeed prominent in the peritonea exposed to standard PDF, mainly in the mesothelial layer where contact with the PDF is most intense, but also in areas of neoangiogenesis and correlated
Chapter 4: Effects of new PDF on peritoneal morphology and function

with MGO-adduct accumulation. The results are commensurate with clinical studies demonstrating progressive AGE accumulation in the peritoneal membrane of long-term PD patients.\textsuperscript{30,31} Virtually no AGE formation was seen in the low-GDP PDF groups, suggesting that high glucose exposure by itself is not sufficient to produce AGEs. The pathogenicity of AGEs hinges on their ability to form cross-links with matrix proteins, resulting in an increased tissue thickness and rigidity. Moreover, they trigger a variety of signal transduction pathways through interaction with specific cellular receptors. RAGE, defined as the receptor for AGE, is best characterized as these AGE-binding structures. Human peritoneal mesothelial cells are known to express functional RAGE \textit{in vitro}. In a normal peritoneal membrane, RAGE expression is low-grade, as it is in other tissues in the absence of disease processes. Conditions characterized by cellular activation, including diabetes, uremia and inflammation are associated with strong upregulation of RAGE expression. In addition, AGE ligands themselves are potent inducers of RAGE gene transcription. RAGE expression was profoundly enhanced in the peritonea exposed to standard PDF, but a less pronounced expression was also found in the low-GDP bicarbonate/lactate-buffered and the amino acids-based PDF groups. The etiology nor the consequences of the latter observation are presently clear.

Both AGE accumulation and RAGE expression correlated strongly with the degree of fibrosis in the peritoneal membrane. The fibrotic properties of AGEs are well-known. We have previously demonstrated that binding of AGE to RAGE results in peritoneal fibrosis with TGF-\(\beta\) as down-stream mediator.\textsuperscript{7} AGEs have been implicated in PDF-induced neoangiogenesis, by virtue of their ability to up-regulate VEGF expression in diverse cell types.\textsuperscript{11,35} The correlation between AGE accumulation and VEGF expression makes it tempting to speculate that they function as up-stream mediators of VEGF-induced neoangiogenesis, though no causality was demonstrated. Inhibition of AGE-RAGE interaction did not prevent peritoneal angiogenesis.\textsuperscript{7} AGEs may, however, up-regulate VEGF expression through binding with other receptors or non-receptor mediated mechanisms.

Although GDPs and the associated accelerated AGE formation appear as the principal causative factors of PD-related membrane alterations, a role for combined low pH and high lactate concentrations is not entirely ruled out. Equilibration of PDF during the
dialysis procedure results in a neutralization of the pH within 30 min and a reduction of the lactate concentration to 12.5 mmol/L within the first hour of dialysis \(^{36}\), thus weakening their potential to provoke persevering membrane damage. Chronic exposure to an acidic lactate buffer without glucose, however, was associated with peritoneal angiogenesis, but the control group was left untreated \(^{28}\). It thus remains unclear to what extent these observations were related to the combined effect of high lactate concentrations and low pH or to the repeated fluid instillation, mechanical trauma and potential introduction of infection.

In conclusion, long-term exposure of the peritoneal membrane to low-GDP or non-GDP PDF was not associated with the development of structural and functional alterations that were prominently present after exposure to standard PDF. As GDPs and associated accelerated AGE formation appear to be the main pathogenetic factors in the progressive membrane deterioration, technological and pharmacological strategies reducing GDP and/or AGE formation have the potential to better preserve long-term peritoneal membrane integrity.

ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance. The work was supported by a grant from Baxter Healthcare Co.
Chapter 4: Effects of new PDF on peritoneal morphology and function

REFERENCES


Chapter 4: Effects of new PDF on peritoneal morphology and function


24. Park MS, Kim JK, Holmes C, Weiss MF. Effects of bicarbonate/lactate solution on
Chapter 4: Effects of new PDF on peritoneal morphology and function


37. Schalkwijk CG, ter Wee PM, Teerlink T. Reduced 1,2-dicarbonyl compounds in bicarbonate/lactate-buffered peritoneal dialysis (PD) fluids and PD fluids based on glucose polymers or amino acids. *Perit Dial Int* 2000; 20: 796-798
4.2

Benefits of switching from a conventional to a Low-GDP Bicarbonate/Lactate-Buffered Peritoneal Dialysis Solution in a Rat Model

Mortier S, Faict D, Lameire NH, De Vriese AS

Submitted
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

ABSTRACT

Long-term exposure to standard PDF results in alterations in peritoneal morphology and function. Studies investigating the long-term effects on the peritoneum of a low-GDP bicarbonate/lactate-buffered PDF demonstrated its superior biocompatibility. We examined the potential of the low-GDP bicarbonate/lactate-buffered solution to reverse or reduce standard PDF-induced peritoneal alterations.

Female Wistar rats received twice daily intraperitoneal infusions with either a lactate-buffered solution with 3.86% glucose at pH 5.5 (Dianeal®, referred to as standard PDF) or a low-GDP bicarbonate/lactate-buffered solution with 3.86% glucose at physiologic pH (Physioneal®, referred to as bicarbonate/lactate PDF) for different periods of time: 1) 12 weeks Dianeal® (n=9); 2) 12 weeks Physioneal® (n=9); 3) 20 weeks Dianeal® (n=11); 4) 20 weeks Physioneal® (n=10); 5) 12 weeks Dianeal® followed by 8 weeks Physioneal® (n=10).

Chronic standard PDF exposure resulted in loss of ultrafiltration capacity, increased VEGF expression and vascular density, higher AGE accumulation, up-regulation of TGF-β expression and development of fibrosis compared to low-GDP bicarbonate/lactate-buffered PDF. The PDF-induced alterations were time-dependent. Cross-over from standard PDF to low-GDP bicarbonate/lactate PDF resulted in a less impaired UF, less pronounced VEGF expression and neoangiogenesis and less severe AGE accumulation, TGF-β expression and fibrosis compared to continuous standard PDF exposure for 20 weeks.

Low-GDP bicarbonate/lactate-buffered PDF has the potential to slow down standard PDF-induced peritoneal membrane damage.

INTRODUCTION

Loss of ultrafiltration capacity is an important cause of treatment failure in peritoneal dialysis (PD), requiring the transfer of patients to hemodialysis or the introduction of an episode of peritoneal rest. Long-term treatment of PD patients with
conventional PDF is associated with fibrosis and neoangiogenesis, that is especially pronounced in patients with ultrafiltration failure.\(^1\)\(^-\)\(^3\).

*In vitro* studies demonstrated that low-GDP, bicarbonate/lactate-buffered PDF better preserved viability and functions of peritoneal leukocytes, mesothelial cells and fibroblasts than conventional solutions.\(^4\)\(^-\)\(^6\) In experimental animal models the low-GDP bicarbonate/lactate-buffered PDF was associated with less functional and structural alterations of the peritoneal membrane than standard PDF.\(^7\)\(^;\)\(^8\). In clinical trials alleviation of infusion pain, improved ultrafiltration, increases in CA125 concentrations and a decrease in hyaluronic levels were observed.\(^9\)\(^;\)\(^10\).

While several lines of evidence indicate that these new PDF have the potential to induce less peritoneal membrane damage than standard PDF, their ability to reverse or reduce standard PDF-associated structural and functional alterations of the peritoneum has not been examined. If true, a switch to a more biocompatible PDF could be an alternative to peritoneal resting or transfer to hemodialysis in patients with peritoneal membrane dysfunction. We therefore studied in a standardized rat model of chronic PDF exposure the potential reversibility of standard PDF-induced alterations during treatment with a low-GDP bicarbonate/lactate-buffered PDF.

**MATERIAL AND METHODS**

**Laboratory animals**

The studies were performed in 49 female Wistar rats (Iffacredo, Brussels, Belgium) with a mean body weight of 214 ± 1 g, receiving care in accordance with the national guidelines for care and use of laboratory animals. A subcutaneous port (PMINA-CBAS-C30 Soloport, Instech Solomon, Plymouth Meeting, USA) was implanted in the neck under halothane (Fluothane, Zeneca, Destelbergen, Belgium) anesthesia in sterile conditions. The attached polyurethane, heparin-coated catheter (Instech Solomon) was tunnelled over the left flank to the peritoneal cavity.\(^11\) After surgery, the animals received an intramuscular injection of buprenorphine (0.1 ml/kg, Temgesic, Schering Plough NV/SA, Brussels, Belgium). The first week after implantation, catheters were flushed once
daily with 1 ml of Earle's Balanced Salt Solution (EBSS, ICN Biomedicals, Inc., Aurora, Ohio, USA). Thereafter, 10 ml of PDF was administered twice daily during 12 or 20 weeks. Oxacilline (2.5 mg/day, Penstapho, Bristol-Myers Squibb, Brussels, Belgium) and gentamycine (0.04 mg/day, Geomycine, Shering-Plough, Brussels, Belgium) were added to all solutions. Laboratory technicians wore masks and gloves during manipulations. The area of the port was desinfected with ethanol 97% 20 seconds before puncture.

Study protocol

Five groups of animals were exposed to either a lactate-buffered solution with 3.86% glucose at pH 5.5 (Dianeal®, Baxter, SA, Lessines, Belgium), referred to as standard PDF, or to a low-GDP bicarbonate/lactate-buffered solution with 3.86% glucose at physiologic pH (Physioneal®, Baxter), referred to as bicarbonate/lactate PDF, for different periods of time: 1) 12 weeks standard PDF (n=9); 2) 12 weeks bicarbonate/lactate PDF (n=9); 3) 20 weeks standard PDF (n=11); 4) 20 weeks bicarbonate/lactate PDF (n=10); 5) 12 weeks standard PDF followed by 8 weeks bicarbonate/lactate PDF (n=10). The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice daily. In case of catheter obstruction, an attempt was made to infuse fluids under halothane anaesthesia. In case of persistent catheter obstruction, skin lesions or severe weight loss, dialysate and catheter tip cultures as well as dialysate white blood cell (WBC) counts were obtained and the animal was sacrificed. At four week intervals, dialysate cultures and WBC counts were performed on 2 ml of fluid obtained through a sterile abdominal puncture with a silicon catheter (Venflon, Becton Dickinson, Erembodegem-Aalst, Belgium) under halothane anaesthesia 4 hours after the last dialysate injection. WBC counts were performed in a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a dialysate WBC count higher than 1000/mm³.  

11,12
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

Study of peritoneal function

After the predefined period of dialysate exposure, rats were anaesthetized with thiobutabarbital (Inactin, RBI, Natick, USA, 100 mg/kg s.c.). The trachea was intubated and a jugular vein was cannulated for continuous infusion of isotonic saline. After 30 min, a silicone catheter was inserted in the abdomen and 15 mL of 3.86 % Dianeal was infused. After 120 min, dialysate was recovered through the silicone catheter and samples were obtained for culture and WBC counts. The abdomen was opened by midline incision to collect the rest of the dialysate for determination of net UF and to sample tissue. The tunnelled polyurethane catheter was removed in a sterile way and the tip was cultured.

Study of peritoneal morphology

One sample of visceral and parietal peritoneum was obtained in each experimental animal, fixed in 4% neutral buffered formalin and embedded in paraffin. Five µm sections were cut for histology and immunohistochemistry.

Immunostaining for endothelial NO synthase (eNOS), vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β) and advanced glycation endproducts (AGE) were performed. Sections were deparaffinized, rehydrated, incubated in 3% H2O2 in PBS for 15 min to block endogenous peroxidase and washed in 10% normal horse serum (Sigma, St. Louis, MO, USA) in PBS for 20 min to block non-specific binding. Subsequently, they were incubated with the primary antibody, mouse anti-human eNOS (Transduction Laboratories, Lexington, Kentucky, USA), mouse anti-human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-human AGE (6D12, Cosmo Bio Ltd, Tokyo, Japan) respectively. Thereafter, a biotinylated IgG (Vector Laboratories, Burlingame, California) and streptavidine-peroxidase were applied for 45 min each. 3,3'diaminobenzidine (DAB) was used as the chromogenic substrate to visualize immunolabelling, resulting in a brown precipitate.

The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparaffinized, rehydrated, and stained briefly
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen.

**Morphometric analysis**

Morphometric measurements of the eNOS, VEGF, TGF-β, AGE and Picro Sirius Red staining were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification x200. For each sample of peritoneum, two sections were analyzed quantitatively with a computerized image analysis system (Zeiss, Oberkochen, Germany). A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss, Oberkochen, Germany). Each pixel in a color image was divided into three color components (hue, saturation and intensity). The threshold for each color component of the staining was defined and kept constant throughout the analysis. In a predefined area eNOS, VEGF, TGF-β, AGE and Picro Sirius Red staining was measured and expressed as a percentage. In addition, eNOS-labelled blood vessels were counted as N/field.

**Statistical analyses**

The results are expressed as mean +/- SEM. Statistical analysis was performed using ANOVA and, where appropriate, the Tukey test was used as multiple comparison t-test. The significance level was set at P<0.05.

**RESULTS**

**Technique survival and infection rate of laboratory animals**

Body weight was similar in the different experimental groups at all time points (data not shown). Technique survival was 100 % in all groups. Except for one transient episode of infection, defined as a positive dialysate culture and a dialysate WBC count >
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

1000/mm³, in an animal exposed to the standard PDF, no infection was diagnosed (data not shown).

**Ultrafiltration**

After 12 weeks of PDF exposure, net UF did not differ significantly between animals exposed to standard PDF and to bicarbonate/lactate PDF, although a trend towards a better UF in the bicarbonate/lactate PDF group was present (Figure 1). At 20 weeks net UF was significantly lower in the animals exposed to standard PDF than in the cross-over group and in bicarbonate/lactate PDF-treated group. Net UF was not different between the cross-over and the bicarbonate/lactate PDF group (Figure 1).

![Figure 1](image-url)  

**Figure 1:** Net ultrafiltration after a 120 min dwell of 15 mL of 3.86 % glucose dialysate in experimental animals exposed to standard lactate-buffered PDF for 12 or 20 weeks (n=9 or n=11, closed bars), low-GDP bicarbonate/lactate-buffered PDF (n=9 or n=10, open bars) for 12 or 20 weeks and standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (n=10, squared bar). *P* < 0.01 vs Dianeal (20 weeks).
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

**Peritoneal morphology**

Vascular density was higher in the standard PDF than in the bicarbonate/lactate PDF group, both after 12 weeks and 20 weeks of PDF exposure (Table 1, Figure 2). In the cross-over group, vascular density was lower than in the standard PDF group and did not differ significantly from that of the bicarbonate/lactate PDF group (Table 1, Figure 2). After 12 weeks of PDF exposure, eNOS expression was not different in the standard PDF and bicarbonate/lactate PDF group (Table 1, Figure 2). At 20 weeks, eNOS was upregulated in the standard PDF group as compared with the bicarbonate/lactate PDF group. Intermediary values were measured in the cross-over group (Table 1, Figure 2).

VEGF expression was more pronounced in animals exposed to standard PDF compared to those exposed to bicarbonate/lactate PDF, both at 12 and 20 weeks (Table 1, Figure 3). At 20 weeks, VEGF expression in the cross-over group did not differ significantly from that in other experimental groups (Table 1, Figure 3). Vascular density correlated positively with both eNOS (Pearson r=0.6646, p<0.0001) and VEGF expression (Pearson r=0.6004, p<0.0001).

AGE accumulation was more pronounced in the standard PDF group than in the other experimental groups at 12 and 20 weeks (Table 1, Figure 4). No difference in AGE accumulation was found between the cross-over group and the bicarbonate/lactate PDF-treated group (Table 1, figure 4). After 12 weeks of PDF exposure, TGF-β expression was not different in the standard PDF and bicarbonate/lactate PDF group (Table 1, Figure 5). At 20 weeks, TGF-β was upregulated in the standard PDF group as compared with the bicarbonate/lactate PDF group. Intermediary values were measured in the cross-over group (Table 1, figure 5). Fibrosis was more pronounced after 12 and 20 weeks exposure to standard PDF compared to bicarbonate/lactate PDF (Table 1, Figure 6). The degree of fibrosis in the cross-over group did not differ significantly from both the standard PDF and the bicarbonate/lactate PDF treated groups (Table 1, Figure 6). Fibrosis correlated positively with TGF-β expression (Pearson r=0.3129, p<0.05). Furthermore, AGE accumulation and VEGF expression show a positive correlation (Pearson r=0.3229, p<0.05).
**Chapter 4: Benefits of a bicarbonate/lactate-buffered solution**

**Table 1: Histological and immunohistochemical analysis of the peritoneum**

<table>
<thead>
<tr>
<th></th>
<th>Dianeal 12 weeks</th>
<th>Physioneal 12 weeks</th>
<th>Dianeal 20 weeks</th>
<th>Cross-over</th>
<th>Physioneal 20 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels, N/mm²</td>
<td>183.93 ± 16.84</td>
<td>120.75 ± 6.34</td>
<td>299.72 ± 9.10</td>
<td>255.46 ± 16.06b</td>
<td>219.53 ± 11.70a</td>
</tr>
<tr>
<td>eNOS staining, %</td>
<td>0.36 ± 0.05</td>
<td>0.36 ± 0.06</td>
<td>0.88 ± 0.10</td>
<td>0.58 ± 0.04cd</td>
<td>0.39 ± 0.05c</td>
</tr>
<tr>
<td>VEGF staining, %</td>
<td>1.04 ± 0.12</td>
<td>0.62 ± 0.09</td>
<td>1.40 ± 0.12</td>
<td>1.13 ± 0.09</td>
<td>0.89 ± 0.10f</td>
</tr>
<tr>
<td>PSR staining, %</td>
<td>2.46 ± 0.28</td>
<td>1.80 ± 0.14</td>
<td>3.76 ± 0.28</td>
<td>3.24 ± 0.16</td>
<td>2.95 ± 0.21g</td>
</tr>
<tr>
<td>AGE staining, %</td>
<td>3.41 ± 0.88</td>
<td>1.06 ± 0.12</td>
<td>3.88 ± 0.21</td>
<td>1.22 ± 0.28g</td>
<td>1.41 ± 0.19f</td>
</tr>
<tr>
<td>TGF-β staining, %</td>
<td>0.47 ± 0.04</td>
<td>0.58 ± 0.13</td>
<td>1.55 ± 0.30</td>
<td>1.23 ± 0.14a</td>
<td>0.77 ± 0.15g</td>
</tr>
</tbody>
</table>

Abbreviations: eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; PSR, picro sirius red; AGE, advanced glycation end product; TGF, transforming growth factor.

*a P<0.005 versus Dianeal (12 weeks), b P<0.05 versus Dianeal (20 weeks), c P<0.01 versus Dianeal (20 weeks), d P<0.05 versus Physioneal (20 weeks), e P<0.05 versus Dianeal (12 weeks), f P<0.005 versus Dianeal (20 weeks), g P<0.0001 versus Dianeal (20 weeks).

**DISCUSSION**

Chronic exposure of the rat peritoneal membrane to standard PDF is characterized by progressive fibrosis and neoangiogenesis associated with upregulation of TGF-β and VEGF, respectively. In addition, a pronounced AGE accumulation was observed. Functionally, the membrane is characterized by a loss of ultrafiltration capacity. Although the present observations are descriptive and do not prove causal relations, they support a key pathogenic role for TGF-β and VEGF in the development of fibrosis and neoangiogenesis. The importance of these growth factors has previously been demonstrated in animal models [13-16], but direct evidence in PD patients is presently lacking. Furthermore, AGE accumulation is involved in the development of peritoneal alterations, as their capacity to promote TGF-β expression through interaction with RAGE has been evidenced [16] and they are known to upregulate VEGF [17;18]. The peritoneal changes tend to progress with time, confirming the results obtained in cross-sectional and longitudinal studies in PD patients [2;3;19].

Long-term treatment of the rat peritoneal membrane with the low-GDP bicarbonate/lactate-buffered PDF induced less neoangiogenesis with a concomitant lower eNOS and VEGF expression and was associated with less AGE accumulation, TGF-β...
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

Figure 2: Immunostaining for eNOS of the visceral peritoneum (x200) exposed to standard lactate-buffered PDF for 20 weeks (A, n=11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (B, n=10) and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (C, n=10).

Figure 3: VEGF expression, expressed as percentage staining, was evaluated by a VEGF immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (A, n=11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (B, n=10) and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (C, n=10).

Figure 4: AGE accumulation, expressed as percentage staining, was evaluated by an AGE immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (A, n=11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (B, n=10) and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (C, n=10).
Chapter 4: Benefits of a bicarbonate/lactate-buffered solution

Figure 5: TGF-β expression, expressed as percentage staining, was evaluated by a TGF-β immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (A, n=11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (B, n=10) and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (C, n=10).

Figure 6: Fibrosis was evaluated with a Picro Sirius Red staining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (A, n=11), standard lactate-buffered PDF for 8 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF (B, n=10) and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (C, n=10).
expression and fibrosis, compared to standard PDF. In addition, ultrafiltration capacity was better preserved. These findings confirm previous observations in chronic rat models of peritoneal exposure. Clinical studies found increased ultrafiltration, elevated CA125 and decreased hyaluronan levels in patients treated with bicarbonate/lactate-buffered PDF, suggestive of a better preservation of peritoneal membrane homeostasis. Unfortunately, but for obvious reasons, no direct comparisons of peritoneal membrane morphology are available. In our prior work, peritoneal membrane function and structure was similar after 12 weeks exposure to bicarbonate/lactate PDF and a buffer solution, suggesting that high glucose concentrations in the absence of other bioincompatible factors were not harmful to the peritoneum at that point. However, the present results at 20 weeks of study demonstrate that fibrosis and neoangiogenesis progress with time, also in the low-GDP bicarbonate/lactate-buffered treated animals, suggesting that glucose by itself is capable of inducing these changes. The present study thus indicates that the PDF-induced changes are time-dependent and underlines the importance of sufficiently long exposure periods in experimental animal models in order to reveal long-term PDF-induced effects.

The salient observation of the present study, however, is that cross-over from the standard PDF to low-GDP bicarbonate/lactate-buffered PDF was associated with less peritoneal membrane deterioration than continuous exposure to standard PDF. In PD patients, cross-over from a conventional PDF to a glucose-free regimen or low-GDP lactate-buffered PDF was associated with improvement of different markers of peritoneal integrity: CA125 levels increased, whereas hyaluronan levels, local VEGF production and circulating AGE levels decreased. If the present observations are confirmed in clinical trials, a switch to low-GDP bicarbonate/lactate-buffered PDF may be an alternative to peritoneal resting or transfer to hemodialysis for the management of patients with peritoneal membrane dysfunction.

In conclusion, the present study confirms the superior biocompatibility of the low-GDP bicarbonate/lactate-buffered PDF during a long-term treatment period. Furthermore, this new low-GDP, bicarbonate/lactate-buffered PDF with physiological pH has the potential to slow down peritoneal damage induced by the standard PDF.
ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance.
REFERENCES


Chapter 4: Benefits of a bicarbonate/lactate-buffered solution


4.3

Inhibition of the Interaction of AGE-RAGE Prevents Hyperglycemia-Induced Fibrosis of the Peritoneal Membrane

De Vriese AS, Flyvbjerg A, Mortier S, Tilton RG, Lameire NH

J Am Soc Nephrol 2003, 14: 2109-2118
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

ABSTRACT

The peritoneal membrane of long-term peritoneal dialysis patients is characterized by a loss of ultrafiltration capacity, associated morphologically with submesothelial fibrosis and neoangiogenesis. Exposure to high glucose concentrations in peritoneal dialysate and the resultant advanced glycation end-products (AGEs) accumulation have been implicated in the development of these changes, but their exact pathophysiological role is unknown. We examined the effect of the interaction of AGEs with one of their receptors (i.e. RAGE) on the function and structure of the peritoneum exposed to high ambient glucose concentrations. Streptozotocin-induced diabetic rats and control rats were treated during 6 weeks with either neutralizing monoclonal anti-RAGE antibodies or control antibodies. The expression of RAGE was strongly enhanced in the peritoneal membrane of the diabetic animals. The diabetic peritonea were characterized by an elevated transport of small solutes, lower ultrafiltration rates, a higher vascular density and an upregulation of eNOS expression. These parameters were unaffected by treatment with anti-RAGE antibodies. In contrast, anti-RAGE but not control antibodies prevented upregulation of TGF-β, development of submesothelial fibrosis and fibronectin accumulation in the peritoneum of diabetic animals. In conclusion, binding of AGEs to RAGE increases the expression of TGF-β and contributes to the development of submesothelial fibrosis. Neoangiogenesis and the resultant loss of ultrafiltration capacity are mediated by different pathogenetic pathways.

INTRODUCTION

Long-term peritoneal dialysis (PD) is associated with progressive functional and structural alterations of the peritoneal membrane. Prominent findings in peritoneal biopsies of PD patients are interstitial fibrosis, the presence of a hyalinizing vasculopathy and neoangiogenesis. Functionally, the peritoneal membrane in long-term PD is characterized by a loss of ultrafiltration capacity, which can generally be attributed to an increased effective vascular surface area. Evidence is mounting that chronic contact with
bioincompatible conventional PD solutions – in particular, exposure to the high glucose concentrations and the resultant advanced glycation end product (AGE) formation – is central in the loss of peritoneal membrane integrity.

AGEs have been detected immunohistochemically in the mesothelium, submesothelial stroma and vascular wall of PD patients. Circumstantial evidence implicates AGEs in the genesis of ultrafiltration failure. Peritoneal staining for AGEs was associated with a higher permeability to various solutes. In another study, the degree of interstitial fibrosis and vascular sclerosis correlated with interstitial and vascular AGE accumulation, respectively. An inverse relation was found between these peritoneal histological changes and ultrafiltration volume. However, the exact pathophysiological role of AGE accumulation in the peritoneal membrane remains unknown.

The pathogenicity of AGEs relates to their ability to form cross-links that alter the architecture and mechanical properties of the extracellular matrix, resulting in increased rigidity and thickness. Another mechanism through which AGEs exert their effects is the binding to specific cellular receptors with activation of signal transduction pathways, leading to the synthesis and release of cytokines and growth factors, including interleukin-1, platelet derived growth factor, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β), expression of adhesion molecules and the induction of procoagulant factors. The best characterized of the AGE receptors has been termed the receptor for AGE (RAGE), although other AGE-binding structures have been described.

Human peritoneal mesothelial cells have been reported to express RAGE in vitro, but the in vivo localization of RAGE in the peritoneal membrane in physiological and pathophysiological conditions is unknown.

The aim of the present study was to examine the contribution of AGE-RAGE interactions to the pathophysiological events observed in the peritoneal membrane exposed to high glucose concentrations present in PD solutions. A major hurdle in studying the effects of PD fluid in animal models is the inevitable interference of trauma and infection, which by themselves may cause fibrosis and neoangiogenesis. To circumvent these difficulties, we have previously used experimental diabetes as a model to study the
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

mechanisms of glucotoxicity to the peritoneal membrane. Using the same model, we examined the effects of inhibition of the AGE-RAGE interaction with a neutralizing monoclonal anti-RAGE antibody (Ab) on the function and structure of the peritoneal membrane exposed to high ambient glucose concentrations.

RESEARCH DESIGN AND METHODS

Laboratory animals

The studies were performed in 36 adult female Wistar rats (Iffa Credo, Brussels, Belgium), that received care in accordance with the national guidelines for animal protection. Diabetes was induced by a single i.v. injection of streptozotocin (30 mg/kg BW) (Pfanstiel, Davenham, UK) dissolved in citrate buffer (n=20). Age-matched control rats (n=16) received an i.v. injection of citrate buffer solution. The procedures were performed under anesthesia with halothane (Fluothane, Zeneca, Destelbergen, Belgium). Experiments were carried out 6 weeks after the injection of streptozotocin or citrate buffer. Diabetic rats were either treated with neutralizing monoclonal anti-RAGE Ab (n=10) or with murine IgG (n=10). Control rats were either treated with anti-RAGE Ab (n=8) or with murine IgG (n=8). The Ab (1 mg dissolved in 1 ml Earle's Balanced Salt Solution) were injected intraperitoneally 3 times per week during 6 weeks, starting 2 days after streptozotocin or citrate buffer administration.

Neutralizing monoclonal anti-RAGE Ab

The preparation and characterization of the neutralizing monoclonal anti-RAGE Ab followed procedures described previously in detail for the preparation of other neutralizing monoclonal Ab. The human RAGE extracellular domain encompassing residues 23-340 (sRAGE) was expressed and purified from E. coli using the pET thioredoxin system (Novagen, Madison, WI, USA). Female 8-week-old BALB/c mice were immunized, then boosted 3 times, 21 days apart, by intraperitoneal and subcutaneous injections of 100 µg of sRAGE protein in Complete Freund’s adjuvant for the primary immunization and an additional 50 µg of sRAGE in Incomplete Freund’s adjuvant for secondary immunizations.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

Each of the mouse Ab titers was measured with ELISA using 50 ng of rhRAGE coated on Immulon 4 96-well dishes overnight at 4°C. The dishes were blocked with 5% BSA in PBS for 2 hr at room temperature. The anti-sera were diluted using 1/10 dilutions, starting at 1/100 to 1/100,000 dilutions, then incubated at room temperature for 2 hr while shaking. After washing with PBS containing 0.05% Tween 20, and 3 washes with PBS, secondary Ab (goat anti-mouse gamma chain-specific, alkaline phosphatase-conjugated) was added at 1/2000 dilution for 45 min at room temperature while shaking. After washing with TBS containing 0.05% Tween 20, and 3 washes with TBS, PNPP substrate was added for 1 hr and absorbance was read at 405 nm. The mouse with the highest serum titer to rhRAGE was injected intravenously with an additional 30 µg of immunogen in PBS, 21 days after the last immunization. Three days later, spleen cells were harvested for production of hybridomas to rhRAGE using previously described techniques (11). The hybridoma cell line with highest Ab titer and neutralizing Ab activity was selected after cloning 3-4 times by limiting dilution in 96-well microtiter plates, then grown in a Cellmax Bioreactor (Spectrum; Rancho Dominguez, CA, USA) using DMEM culture media. Purified IgG was prepared by Protein A chromatography. The isotype (IgG\textsubscript{\textkappa}) and light chain composition (kappa) of the Ab were determined as described previously\textsuperscript{10}.

Characterization of anti-RAGE Ab neutralizing activity

A NF-\êB reporter-gene assay using N\textsuperscript{\textcarboxymethyl}lysine-modified human serum albumin (CML-HSA) as a ligand for the RAGE receptor was used to measure neutralizing activity of the monoclonal Ab. Details of the preparation of CML-HSA as well as the reporter gene assay have been published previously\textsuperscript{12}. THP-1 cells were seeded at 5 x 10\textsuperscript{6} cells per 100-mm dish in 10 ml of serum-free medium (SFM) the day before transfection. Transient transfection was performed using the DEAE-dextran method as described previously\textsuperscript{13}. Cells were washed once with SFM and resuspended in 1 ml of the same medium containing 2 µg of NF-\êB-Luc reporter plasmid (Clontech, Palo Alto, CA, USA) and 200 µg/ml of DEAE-dextran (Promega, Madison, WI, USA). The cell-DNA mixture was incubated at room temperature for 20-30 min prior to washing, centrifugation, and
resuspension into fresh SFM. Transfected cells were seeded into 96-well plates at 70,000 cells/well for recovery. After 24 h, cells were pretreated with 10 to 100 µg/ml anti-RAGE Ab for 1 hour, then treated with 200-600 µg/ml CML-modified albumin for 1-6 hours before the reporter assay. Equivalent amounts of cell lysates, normalized for total protein (Bradford protein assay; Bio-Rad, Palo Alto, CA, USA), were used for measurement of luciferase activity. Luciferase assays were performed using the Steady-Glo luciferase assay system according to the manufacturer’s instructions (Promega, Madison, WI, USA), and luminescence was detected in a TopCount microplate scintillation counter using a single-photon monitor program (Packard Instrument Company, Meriden, CT, USA).

Anti-RAGE Ab serum levels

To determine serum levels of the anti-RAGE Ab, serum samples were obtained after 6 weeks of treatment. Binding studies were performed using a commercially available ELISA kit with the capture Ab being rat anti-mouse IgG₃ (BD PharMingen #553404, San Diego, CA). Briefly, individual wells of a 96-well plate were coated with 50 µl of 1 µg/ml capture Ab overnight at 4 °C, blocked using heat-inactivated 10 % FCS PBS containing 0.05 % sodium azide overnight at 4 °C, then washed 3 times with PBS. Anti-RAGE Ab standards (50 µl of 10, 2, 0.4, and 0.08 µg/ml diluted in 5 % FCS/PBS) and plasma samples (50 µl of 1/25, 1/100, and 1/1000 serum dilutions) were added to appropriate wells, placed on a shaker for 2 hours at 22 °C, then incubations were terminated by washing wells once with 400 µl of ice cold PBS + 0.05 % Tween 20 followed by 4 more rinses with 200 µl of ice-cold PBS. Biotinylated rat anti-mouse IgG₃ (BD PharMingen #553401, 0.5 mg/ml, San Diego, CA) diluted 1/500 in TBS + 5 % FCS was mixed with an equal volume of ExtraAvidin alkaline phosphate (E2636, Sigma, St. Louis, MO, USA) diluted 1/1500 in TBS + 5 % FCS for 15 minutes at 22 °C, then 50 µl of the complex was added to each well. After shaking for 1 hour at 22 °C, each well was rinsed once with TBS + 0.05 % Tween 20 and 3 times with TBS, followed by the addition of 100 µl of 4-methylumbelliferyl phosphate liquid substrate (M3168, Sigma, St. Louis, MO, USA) and fluorescence was measured at excitation 360 nm / emission 440 nm.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

Urinary albumin excretion.

Rats were housed in metabolic cages for 24 hr and the urine was collected. Urine samples were stored at −20 °C until analysis. Urinary albumin concentration was determined by a rat albumin radioimmunoassay as previously described 14, using rabbit anti-rat albumin Ab RARa/Alb (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands) and globulin-free rat albumin for standard and iodination (Sigma, St. Louis, MO, USA).

Peritoneal transport studies

Peritoneal transport studies were performed as described previously 9. Rats were anesthetized with thiobutabarbitral (Inactin, RBI, Natick, USA) in a dose of 100 mg/kg s.c.. The trachea was intubated, a jugular vein was cannulated for continuous infusion of isotonic saline, and a carotid artery was cannulated for blood sampling. The saline infusion rate was matched with diuresis to maintain euvolemia. After 30 min, a silicon catheter (Venflon, Becton Dickinson, Erembodegem-Aalst, Belgium) was inserted in the abdomen and 15 ml of a 3.86 % glucose peritoneal dialysate solution (Dianeal, Baxter, Nivelles, Belgium) was infused. Plasma and dialysate samples were collected at t=0, 30, 60, 120 min, for determination of creatinine, urea and glucose levels. Fructosamine and total protein levels were determined on the first plasma sample only. Dialysate cultures were obtained at the end of the experimental dwell and animals were excluded from analysis if cultures were positive. After 120 min, the abdomen was opened by midline incision for collection of the dialysate fluid and for tissue sampling. The transport of low molecular weight solutes was evaluated by calculating the mass transfer area coefficient (MTAC) of urea and creatinine, using the Garred equation: MTAC = volume_out/dwell time x ln[volume_in x conc_plasma/volume_out x (conc_plasma-conc_dialysate_end)] (15). The initial peritoneal concentration of urea and creatinine is set at zero. The Garred formula is a simplified approach to calculate MTAC, assuming that the reflection coefficient of the solute is zero and that the average solute concentration in the membrane equals the plasma concentration. The magnitude of the transport of small solutes is determined by the effective vascular surface area, which is dependent on the number of perfused peritoneal capillaries 16.
Histologic and immunohistochemical analyses

A sample of parietal and visceral peritoneum was obtained in each experimental animal, fixed in 4 % neutral buffered formalin and embedded in paraffin. Five µm sections were cut for histology and immunohistochemistry.

The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparafinized, rehydrated and stained briefly with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen. Morphometric measurements were made by a masked operator with a Zeiss Axiophot microscope and a computerized image analysis system (Zeiss, Oberkochen, Germany) at magnification ×200. For each sample of peritoneum, two sections were analyzed. A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss, Oberkochen, Germany). Each pixel in a color image was divided into three color components (hue, saturation and intensity). The threshold for each color component of the staining was defined and kept constant throughout the analysis. In a predefined area covering the tissue within 500 µm of the mesothelium, the Picro Sirius Red staining was measured and expressed as a percentage.

Vascular density was evaluated with an immunostaining for endothelial NO synthase (eNOS). Sections were deparafinized, rehydrated, incubated in 3 % H₂O₂ in PBS for 15 min to block endogenous peroxidase and washed in 10 % normal horse serum (Sigma, St.Louis, MO, USA) in PBS for 20 min to block non-specific binding. They were subsequently incubated with the primary Ab (anti-human eNOS, Transduction Laboratories, Lexington, KY, USA), a biotinylated IgG (Vector Laboratories, Burlingame, CA, USA) and streptavidine-peroxidase, for 45 min each. 3,3’-diaminobenzidine (DAB) was used as the chromogenic substrate to visualize immunolabelling, resulting in a brown precipitate. The immunostaining for eNOS was quantified as described above. The tissue staining for eNOS was measured and expressed as a percentage. In addition, labelled blood vessels were counted as N/field.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

The expression of TGF-β was investigated using a rabbit anti-human TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody. The localisation of RAGE was evaluated using a goat anti-human RAGE (Research Diagnostics, Flanders, USA) as the primary antibody. The immunostaining procedure and quantification were performed as described above.

Immunoblotting for eNOS and fibronectin

The remaining visceral peritoneum was harvested, snap frozen in liquid nitrogen and stored at –80 °C until use. Approximately 50 mg of peritoneal tissue was sonicated for 20 s in chilled 50 mM Tris, 1 mM EDTA, pH 7.5 buffer. Samples (100 ìg protein per lane) of tissue homogenates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5 % acrylamide) and transferred by electroelution onto nitrocellulose paper (Schleicher & Schuell, Munich, Germany). The blots were blocked overnight at 4 °C in 5 % nonfat milk and incubated for 12 hr at 4 °C with a monoclonal eNOS Ab (Transduction Laboratories, Lexington, KY, USA) in a dilution of 1/1000, or with a polyclonal fibronectin Ab (Biogenesis Ltd., New Fields, Pooke, UK) in a dilution of 1/6500. After washing in TBS-Tween (0.1 %), the membranes were incubated with an anti-mouse IgG Ab (for eNOS) or an anti-rabbit IgG Ab (for fibronectin) conjugated with horseradish peroxidase (both from Pierce, Rockford, IL, USA) for 1 hr at room temperature in a dilution of 1/10000 and 1/75000, respectively. The bands were visualized by chemiluminescence (BioWest™, UPV Inc., Upland CA, USA) and quantified by a UPV BioImaging System (UPV Inc., Upland CA, USA).

Statistical analysis

The data are presented as mean ± SEM. Analysis of variance and unpaired t-tests were used as appropriate to test statistical significance. The significance level was set at P<0.05.
RESULTS

Characteristics of laboratory animals

Diabetic animals had higher plasma glucose and fructosamine levels as compared to the age-matched control rats (Table 1). There were no differences in metabolic control between anti-RAGE and control Ab-treated diabetic rats. Body weights were lower in diabetic rats as compared to age-matched control rats, but did not differ between the diabetic groups. Albuminuria was significantly elevated in diabetic animals treated with control Ab compared to control rats. Treatment with anti-RAGE Ab normalized albuminuria in diabetic animals (Table 1).

Table 1: Clinical and biochemical characteristics of the experimental groups

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Body Weight (g)</th>
<th>Plasma Glucose (mmol/l)</th>
<th>Plasma Fructosamine (µmol/g total protein)</th>
<th>Albuminuria (µg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes+anti-RAGE Ab (n=10)</td>
<td>223±9*</td>
<td>27.3±4.5**</td>
<td>10.2±0.8**</td>
<td>352±91§</td>
</tr>
<tr>
<td>Diabetes+control Ab (n=10)</td>
<td>206±8*</td>
<td>29.0±4.4**</td>
<td>11.8±0.5**</td>
<td>1503±525§</td>
</tr>
<tr>
<td>Control+anti-RAGE Ab (n=8)</td>
<td>248±2</td>
<td>7.9±0.3</td>
<td>4.3±0.2</td>
<td>243±36</td>
</tr>
<tr>
<td>Control+control Ab (n=8)</td>
<td>255±4</td>
<td>7.6±0.3</td>
<td>4.0±0.1</td>
<td>224±51</td>
</tr>
</tbody>
</table>

*P<0.02 vs. controls; **P<0.001 vs. controls; #P<0.05 vs. controls; §P<0.05 vs. diabetes+control Ab

Serum anti-RAGE-Ab levels

Serum IgG levels were significantly elevated in animals treated with anti-RAGE Ab, while they were low in the groups treated with murine IgG (Figure 1).
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

Figure 1: Serum IgG levels (µg/ml) in diabetic rats + anti-receptor for advanced glycation endproducts (RAGE) antibody (Ab) (n=10), diabetic rats + control Ab (n=10), control rats + anti-RAGE Ab (n=8), and control rats + control Ab (n=8) after 6 weeks of treatment. *P<0.01 versus diabetic + control Ab and control + control Ab. Data are given as mean ± SEM.

Localisation of RAGE

The staining for RAGE was greatly enhanced in the peritonea of the placebo-treated diabetic animals, as compared to the placebo-treated control rats. The expression was most prominent in the mesothelium, the submesothelial fibrotic tissue and the vascular wall (Figure 2).

Peritoneal transport studies

Small solute transport rates were higher in diabetic rats than in age-matched controls, indicating the presence of a larger effective vascular surface area in the diabetic peritoneum (Figure 3). Treatment with anti-RAGE Ab did not affect the elevated transport of small solutes in diabetic rats (Figure 3). Net ultrafiltration was significantly lower in the diabetic animals as compared to controls, without differences between diabetic rats treated with anti-RAGE and control Ab (Figure 4).
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

Figure 3: The mass transfer area coefficient (MTAC) for urea (open bars) and creatinine (hatched bars) was measured after 2h of 3.86% glucose dialysate in the peritoneal cavity in diabetic rats + anti-RAGE Ab (n=10), diabetic rats + control Ab (n=10), control rats + anti-RAGE Ab (n=8), and control rats + control Ab (n=8). *P<0.05 versus control + anti-RAGE and control + control Ab. Data are given as mean +SEM.

Figure 4: Net ultrafiltration rate (ml) was measured after 2h of 3.86% glucose dialysate in the peritoneal cavity in diabetic rats + anti-RAGE Ab (n=10), diabetic rats + control Ab (n=10), control rats + anti-RAGE Ab (n=8), and control + control Ab (n=8). *P<0.05 versus control + anti-RAGE Ab and control + control Ab, #P<0.05 versus control + anti-RAGE Ab, $P=0.06 versus control + control Ab. Data given as mean + SEM.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

TGF-β expression

The expression of TGF-β was increased in the diabetic animals that were treated with control Ab, but not in those that were treated with anti-RAGE Ab. The staining was especially enhanced in the vascular endothelium, as well as in the mesothelium and fibrotic tissue (Figure 5, Table 2).

Table 2: Histological and immunohistochemical analyses of the peritoneum

<table>
<thead>
<tr>
<th></th>
<th>Diabetes+anti-RAGE Ab (n=10)</th>
<th>Diabetes+control Ab (n=10)</th>
<th>Control+anti-RAGE Ab (n=8)</th>
<th>Control+control Ab (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β staining, %</td>
<td>2.77±0.42</td>
<td>5.76±1.19*</td>
<td>2.35±0.53</td>
<td>2.78±1.06</td>
</tr>
<tr>
<td>Picro Sirius Red staining, %</td>
<td>1.77±0.48**</td>
<td>5.14±1.45**§</td>
<td>0.45±0.14</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td>Blood vessels, N/mm²</td>
<td>357.1±78.6#</td>
<td>266.2±39#</td>
<td>153.0±23.3</td>
<td>136.2±23.0</td>
</tr>
<tr>
<td>eNOS staining, %</td>
<td>1.49±0.38#</td>
<td>1.32±0.23#</td>
<td>0.40±0.07</td>
<td>0.44±0.11</td>
</tr>
</tbody>
</table>

*P<0.05 vs. controls and diabetes+anti-RAGE Ab, **P<0.01 vs. controls, §P<0.05 vs. diabetes+anti-RAGE Ab, #P<0.05 vs. controls

Fibrosis and fibronectin expression

Submesothelial fibrosis, as evaluated with a Picro Sirius Red staining, was more pronounced in diabetic rats than in control animals (Figure 6, Table 2). Exposure to anti-RAGE Ab significantly impaired the development of fibrosis in diabetic animals (Figure 6, Table 2). The fibronectin content of the mesenteric tissue was increased in diabetic animals treated with control Ab as compared to all other experimental groups (Figure 7). Treatment of diabetic rats with anti-RAGE Ab resulted in a fibronectin expression that was not different from control values (Figure 7).

Neoangiogenesis

The density of blood vessels was higher in diabetic animals than in control groups, with no difference between those treated with anti-RAGE or control Ab (Figure 8, Table 2). The expression of eNOS, measured both by immuno-histochemistry and immunoblotting.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

Figure 2: RAGE staining of the visceral peritoneum. (A) In diabetic rats, the expression of RAGE is strongly increased in the mesothelium, submesothelial fibrotic tissue, and vascular wall (x200). (B) Detail of the staining in the mesothelial cells and submesothelial tissue of diabetic rats (x630). (C) Low level of staining in the peritoneum of control animals (x200).

Figure 5: TGF-β staining of the visceral peritoneum. (A) In diabetic rats treated with anti-RAGE Ab, the intensity of the staining is similar to that in control animals (x200). (B) The expression of TGF-β is upregulated in placebo-treated diabetic animals. It is localized mainly in the vascular endothelium, as well as in the mesothelium and fibrotic tissue (x200). (C) Detail of the staining in mesothelial and endothelial cells in diabetic rats treated with control Ab (x630). (D) Low levels of staining in control animals treated with anti-RAGE Ab (x200) and (E) in control animal treated with control Ab (x200).
Figure 6: Picro Sirius Red staining of the visceral peritoneum. (A) Anti-RAGE Ab treatment prevents the development of fibrosis in diabetic animals (x200). (B) The Picro Sirius Red staining is strongly enhanced in the diabetic animals that were treated with control Ab (x200). (C) Detail of the submesothelial fibrotic tissue in diabetic rats treated with control Ab (x630). (D) No significant fibrosis is present in the peritonea of control rats treated with anti-RAGE Ab (x200) and (E) control rats treated with control Ab (x200).
Figure 8: Staining for endothelial nitric oxide synthase (eNOS) of the visceral peritoneum. (A) In diabetic rats treated with anti-RAGE Ab (x200) as well as in (B) diabetic rats treated with control Ab (x200), the intensity of the eNOS staining and the vascular density are higher than in control animals. (C) Detail of the staining in the vascular endothelium of placebo-treated diabetic animal (x630). (D) In control rats treated with anti-RAGE Ab (x200) and (D) control rats treated with control Ab (x200), the staining is low-grade.
was more pronounced in diabetic animals than in controls (Figure 8, Figure 9, Table 2). Anti-RAGE Ab did not affect eNOS expression in diabetic rats (Figure 8, Figure 9, Table 2).

Figure 7: (A) Representative immunoblot for fibronectin (230kD) in the peritoneum of diabetic rats + anti-RAGE Ab (DR), diabetic rats + control Ab (DC), control rats + anti-RAGE Ab (CR) and control rats + control Ab (CC). (B) Densitometry analysis of the fibronectin immunoblot in diabetic rats + anti-RAGE Ab (n=10), diabetic rats + control Ab (n=10), control rats + anti-RAGE Ab (n=8) and control rats + control Ab (n=8). *P<0.003 versus diabetic + anti-RAGE Ab, control + anti-RAGE Ab, and control + control Ab. Data are given as mean ± SEM.
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis

A

![Image of immunoblot for eNOS (140 kD) in the peritoneum of diabetic rats + anti-RAGE Ab (DR), diabetic rats + control Ab (DC), control rats + anti-RAGE Ab (CR), and control rats + control Ab (CC).]

B

**Figure 9:** (A) Representative immunoblot for eNOS (140 kD) in the peritoneum of diabetic rats + anti-RAGE Ab (DR), diabetic rats + control Ab (DC), control rats + anti-RAGE Ab (CR), and control rats + control Ab (CC). (B) Densitometry analysis of the eNOS immunoblot in diabetic rats + anti-RAGE Ab (n=10), diabetic rats + control Ab (n=10), control rats + anti-RAGE Ab (n=8) and control rats + control Ab (n=8). *P*<0.01 *versus* control + anti-RAGE Ab and control + control Ab, #*P*<0.05 *versus* control + anti-RAGE Ab, $P=0.07$ *versus* control + control Ab. Data are given as mean ± SEM.

**DISCUSSION**

Exposure of the peritoneal membrane to hyperglycemia results in the development of neoangiogenesis, as evidenced by an increased vascular density and an upregulation of the eNOS expression, as well as in an increased expression of TGF-β and a submesothelial collagen and fibronectin accumulation. Functionally, high glucose exposure causes an
increased small solute transport, indicative of an enlarged effective peritoneal vascular surface area and resulting in loss of ultrafiltration capacity. These functional and structural characteristics of the peritoneal membrane are similar to those found in patients on long-term PD, thus incriminating the high glucose concentrations in peritoneal dialysate in the development of these changes. The salient observation in the present study is that inhibition of the AGE-RAGE interaction with neutralizing monoclonal anti-RAGE Ab prevented the upregulation of TGF-β and the extracellular matrix accumulation, but had no effect on the neoangiogenesis and the resultant rapid small solute transport and ultrafiltration failure.

RAGE is an integral membrane protein from the immunoglobulin superfamily and is the best characterized signal transduction receptor for AGEs. RAGE is expressed in a variety of cell types, including endothelial cells, vascular smooth muscle cells, macrophages, mesangial cells and neurons. One study confirmed mRNA production and expression of functional RAGE in human peritoneal mesothelial cells in culture, but the localization of RAGE in the peritoneal membrane in vivo has not been reported previously. RAGE expression is low during homeostasis, but is strikingly enhanced in conditions characterized by cellular activation, including diabetes, uremia and inflammation. In the present study, the expression of RAGE was greatly increased in the peritoneum of diabetic animals. It was mainly found in the mesothelium, submesothelial fibrotic tissue and blood vessel walls. Antagonism of RAGE had no apparent adverse effects in control rats, commensurate with the low expression level of RAGE in these animals.

Several lines of evidence support the fibrogenic properties of AGEs. In cultured mesangial cells and vascular smooth muscle cells, exposure to both high glucose concentrations and AGEs induced extracellular matrix synthesis, as evidenced by increased production of fibronectin, laminin or collagen. The effects of high glucose or AGEs appear to be mediated by TGF-β, as neutralizing anti-TGF-β antibodies prevented the increased production of extracellular matrix components. Exposure to ambient high glucose concentrations also induced fibronectin production in HPMC, with TGF-β as a down-stream mediator. The effects of AGEs per se on extracellular matrix production in
the peritoneal membrane are, however, less well understood. The present study provides the first in vivo evidence for a pivotal role of AGE-RAGE interaction in high glucose-induced peritoneal fibrogenesis. Further, the results point towards TGF-β as a important downstream effector of the profibrotic effects of AGE-RAGE. The development of peritoneal fibrosis in the diabetic animals was associated with an upregulation of TGF-β expression, and both phenomena were prevented by treatment with neutralizing anti-RAGE Ab. Interaction of AGEs with RAGE and the resultant TGF-β-mediated myofibroblast transdifferentiation contributed to interstitial fibrosis in diabetic nephropathy. It is tempting to speculate that a similar mechanism may be responsible for the high glucose-induced fibrosis in the peritoneal membrane.

AGEs are known to upregulate VEGF expression and have angiogenic properties that are mediated by VEGF. We have previously reported that VEGF is a key player in hyperglycemia-induced neoangiogenesis. It was thus tempting to speculate that AGEs might be upstream mediators of VEGF-induced neoangiogenesis in the peritoneal membrane. In the present study, however, inhibition of the AGE-RAGE interaction had no effect on vascular density, eNOS expression, small solute transport rate and ultrafiltration capacity. The present results thus do not support a role for the AGE-RAGE interaction in peritoneal neoangiogenesis, but they do not exclude an potential effect of AGEs on vascular proliferation through other actions, i.e. binding with other receptors or non-receptor mediated mechanisms. Alternatively, other pathways may mediate the high glucose-induced VEGF expression in the peritoneal membrane, including reactive oxygen species, protein kinase C and reductive stress.

Inhibition of fibrosis with anti-RAGE did not portent significant improvement in the transport characteristics of the peritoneal membrane. The results are in line with the observation that decorin, a TGF-β-inhibiting proteoglycan, decreased peritoneal collagen content but did not improve ultrafiltration rate in a rat model of peritoneal dialysate exposure. In contrast, angiostatin, an inhibitor of angiogenesis, significantly reduced peritoneal vascular density and increased net ultrafiltration. Taken together, these results support
the tight link between peritoneal neoangiogenesis and ultrafiltration failure and the absence of prominent functional consequences of submesothelial fibrosis.

Streptozotocin-induced diabetes was used as an experimental model for chronic exposure of the peritoneum to high glucose concentrations, in order to avoid the difficulties associated with chronic dialysate infusion models. While succeeding in this respect, the diabetes model also has limitations. In PD, the higher glucose load and the exposure to reactive carbonyl groups derived from the uremic state and the presence of glucose degradation products may result in different and accelerated AGE formation. The present results, therefore, need to be confirmed in experimental models combining chronic dialysate exposure and uremia.

In conclusion, exposure of the peritoneal membrane to high glucose concentrations results structurally in fibrosis and neoangiogenesis and functionally in increased transport of small solutes and ultrafiltration failure. Action of AGEs through binding with RAGE, with TGF-β as downstream mediator, seems to be central in the development of submesothelial fibrosis, while neoangiogenesis and the resultant loss of ultrafiltration capacity are mediated by different pathogenetic pathways.

ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Karen Mathiassen, Nele Nica, Kirsten Nyborg, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance. Financial support was provided by the Fund for Scientific Research Flanders, the Fund for Research of the Ghent University, the Danish Diabetes Association, the Frænkels Memorial Foundation, the Danish Medical Research Council and Institute of Experimental Clinical Research at University of Aarhus, Denmark.
REFERENCES


257
Chapter 4: AGE-RAGE interaction and hyperglycemia-induced fibrosis


4.4

Glucotoxicity of the Peritoneal Membrane: the Case for VEGF

De Vriese AS, Mortier S, Lameire NH

Nephrol Dial Transpl 2001, 16: 2299-2302
Chapter 4: Glucotoxicity of the peritoneal membrane

INTRODUCTION

Although nephrologists are well aware of the devastating consequences of chronic hyperglycemia in patients with diabetes, the continuous exposure of the peritoneum to the high glucose concentrations present in peritoneal dialysate has been approached with aloof concern. This is rather surprising, as dialysate glucose concentrations are 15 to 40 times the physiological levels and peritoneal dialysis (PD) patients may be exposed to the equivalent of 100 kg of glucose per year.

Long-term PD is associated with the progressive development of functional and structural alterations of the peritoneal membrane. Longitudinal reports suggest that ultrafiltration tends to decrease with time on dialysis \(1^{12}\). In the majority of cases, loss of ultrafiltration capacity can be attributed to a rise in small solute transport, resulting in a rapid dissipation of the transperitoneal osmotic gradient. The presence of high small solute transport rates points to an expansion of the effective peritoneal vascular surface area \(3\). Its morphological substrate may be the neoangiogenesis that has been observed in the peritoneum of long-term PD patients \(4^{6}\). Other structural changes include reduplication of the basal lamina of the mesothelium and the blood vessels, interstitial fibrosis and hyalinization of the blood vessel media, with preferential deposition of collagen type IV \(4^{9}\). The diabetiform nature of these morphological changes has bolstered the hypothesis that the continuous exposure to the high glucose concentrations in dialysate is an important pathogenetic factor.

The recognition that glucotoxicity contributes to the development of the functional and structural alterations of the peritoneum is essential, as the initial clinical approach to a patient with failing ultrafiltration capacity is to increase the number of hypertonic exchanges or to switch to cyclic continuous PD. These interventions further increase the cumulative glucose exposure and the patient may thus be precipitated into a vicious circle with progressively worsening peritoneal membrane damage. The present communication briefly reviews the extant evidence that glucose, either directly or indirectly through the generation of glucose degradation products (GDPs) or the formation of advanced glycation end-products (AGEs) is responsible for these changes. More in particular, the role of vascular endothelial growth factor (VEGF) as a downstream mediator is highlighted.
GLUCOSE

VEGF is a heparin-binding cytokine that plays a prominent role in physiological and pathological angiogenesis and potently induces microvascular hyperpermeability\(^{10}\). Several lines of evidence implicate VEGF as a mediator of glucose-induced tissue damage. VEGF expression is upregulated in diverse cell types cultured in a high glucose environment, as well as in various tissues of experimental animals and humans with diabetes. Microvascular dysfunction induced by topical application of elevated glucose levels in a granulation skin chamber was attenuated by administration of neutralizing anti-VEGF antibodies\(^{11}\). Finally, VEGF blockade prevented early renal dysfunction in experimental diabetes\(^{12}\).

Considering its biological properties and its upregulation by high ambient glucose concentrations, VEGF is an attractive candidate to provide a mechanistic link between chronic glucose exposure on the one hand, and peritoneal neoangiogenesis with resultant loss of ultrafiltration on the other. Human peritoneal mesothelial cells harvested from spent dialysate and cultured \textit{in vitro} have the capacity to produce substantial amounts of VEGF, but no correlations were found between supernatant VEGF levels and time on PD, solute transport characteristics, ultrafiltration rate or accumulated dose of glucose\(^{13}\). Other cell types present in the peritoneal cavity that are capable of producing VEGF include peritoneal macrophages\(^{14}\) or capillary endothelial cells\(^{6}\). The concentration of VEGF in peritoneal effluent was higher than what could be attributed to transport from the circulation, implying local production\(^{15}\). The amount of locally synthesized VEGF correlated with the MTAC of creatinine and urate, glucose absorption and transcapillary ultrafiltration, and increased with time on PD\(^{15}\). In addition, VEGF effluent concentrations decreased after switching to glucose-free dialysate, with a commensurate effect on the parameters of effective peritoneal surface area\(^{16}\).

Although these studies present ample circumstantial evidence that VEGF mediates glucose-induced damage of the peritoneal membrane, they do not provide proof of causality. We have studied experimental diabetes as a model for chronic exposure of the peritoneum to high ambient glucose levels\(^{17}\). The peritoneal microcirculation in early streptozotocin-induced diabetes in the rat is characterized by pronounced neoangiogenesis,
associated on the functional level with an elevated small solute transport rate, similar to the alterations found in long-term PD patients. The hyperglycemia-induced microvascular alterations were largely prevented by treatment with a monoclonal anti-VEGF antibody, whereas treatment with an isotype-matched control antibody was without effect (Figure 1). These results are thus the first to support an etiologic role for glucose in the development of peritoneal neoangiogenesis and identify VEGF as an important down-stream mediator.

**Figure 1:** The vascular density of the visceral peritoneum was significantly higher in diabetic rats than in control animals. The vascular proliferation was prevented by chronic treatment with anti-VEGF antibodies (Ab), but not by control Ab. VEGF blockade did not decrease vascular density in control rats. * $P<0.01$ versus controls; ** $P<0.01$ versus diabetes and diabetes + control Ab.

**GDPs**

Glucose in PD fluid is known to degrade during heat-sterilization and to a lesser extent during prolonged storage, giving rise to a variety of GDPs. The toxic effects of these components on viability and function of peritoneal leukocytes, fibroblasts and mesothelial cells are by now fully recognized. There is, however, a dearth of information on the potential vascular consequences of chronic exposure to GDPs. Cultured rat mesothelial and human endothelial cells expressed VEGF in response to methylglyoxal, but not to glyoxal or 3-deoxyglucosone. Intraperitoneal exposure to methylglyoxal increased VEGF expression in peritoneal tissue of experimental animals. Although these results need confirmation, they suggest that GDPs may augment local production of VEGF and thus contribute to peritoneal neoangiogenesis.
Glucose and a variety of other reactive carbonyl compounds have the potential to bind non-enzymatically to free amino groups on proteins or to lipids, resulting in the formation of AGEs. AGE formation is accelerated when the ambient glucose levels are elevated or when the prevailing oxidant stress is high, as for instance in uremia. Furthermore, GDPs are known to facilitate the formation of AGEs. As residual renal function deteriorates, low molecular weight AGEs are retained in the circulation and may be actively transported into the peritoneum. The peritoneal cavity of PD patients thus harbours optimal conditions for a dramatically accelerated AGE formation and accumulation. AGEs have, indeed, been detected immunocytochemically in the mesothelium, submesothelial stroma and vascular wall of PD patients.

The pathogenicity of AGEs relates to their ability to accumulate in tissues with the formation of cross-links, and to generate oxygen-derived free radicals. Another important biological action of AGEs is the induction of VEGF expression in diverse cell types. AGEs may thus have the potential to promote peritoneal neoangiogenesis.

Attempts have been made to correlate the extent of AGE accumulation with functional parameters. Peritoneal staining for AGEs increased along with time on PD and was associated with a higher permeability to various solutes. In another study, the degree of interstitial fibrosis and vascular sclerosis correlated with interstitial and vascular AGE accumulation, respectively. An inverse correlation was found between these peritoneal histological changes and ultrafiltration volume. The results incriminate AGE accumulation in the pathophysiology of ultrafiltration failure, although the precise mechanisms underlying this association remain unclear. It is generally acknowledged that the interstitium does not contribute importantly to the barrier function of the peritoneal membrane. It is unknown whether interstitial fibrosis can be associated with a decreased hydraulic permeability or with a more rapid transport of small solutes. On the other hand, a correlation has been reported between the degree of interstitial fibrosis and vascular density in the membrane of long-term PD patients. It may be more logical to postulate that peritoneal AGE accumulation is associated with both interstitial fibrosis and neoangiogenesis, while the latter phenomenon is primarily responsible for the increased...
small solute transport and loss of ultrafiltration capacity. Unfortunately, no morphometrical analysis of vascular density was performed in the studies on peritoneal AGE accumulation.\footnote{9,25-26}

**STRATEGIES TO REDUCE PERITONEAL EXPOSURE TO GLUCOSE AND GDPs**

Non-glucose-based dialysates, including icodextrin, glycerol and amino acids, as well as double-chamber dialysates with low GDP content have become available for clinical use. Whereas extensive *in vitro* testing has suggested that these new dialysates may be more biocompatible than conventional dialysate\footnote{18,30}, their superiority on *in vivo* peritoneal membrane function remains to be fully demonstrated.

Icodextrin and amino acid solutions contain lower concentrations of GDPs than conventional dialysate\footnote{31}. *Less in vitro* glycation and AGE formation occurs in icodextrin than in conventional heat-sterilized glucose-based dialysate\footnote{27,32-33}. In accordance, AGE accumulation was lower in the peritoneal membrane of experimental animals dialyzed with icodextrin than in those treated with glucose-based solutions\footnote{34}. A switch to icodextrin- and glycerol-based dialysis in small group of patients with severe UFF decreased pentosidine dialysate levels and modestly improved ultrafiltration rate\footnote{35}. In contrast, no difference in peritoneal transport characteristics and peritoneal membrane markers were found in CCPD patients randomized to either icodextrin or standard dialysate\footnote{36}.

Double-chamber dialysates with low GDP content induce lower in vitro AGE-formation than conventional dialysate\footnote{37}. In keeping with these findings, the peritoneum of rats exposed to these dialysates is characterized by lower staining for AGES and a better preserved ultrafiltration capacity\footnote{38}, as well as by less submesothelial thickening\footnote{39}.

**CONCLUSION**

Several lines of evidence support the involvement of dialysate glucose and GDP concentrations with resultant peritoneal AGE accumulation in the pathophysiology of peritoneal membrane alterations. High glucose concentrations, as well as GDPs and AGES
Chapter 4: Glucotoxicity of the peritoneal membrane

have the potential to upregulate VEGF expression. Even though the cell type that generates VEGF in response to dialysate exposure is unknown, strong evidence implicates local VEGF production in the pathogenesis of peritoneal neoangiogenesis and loss of ultrafiltration capacity. This knowledge has given impetus to the extensive testing of new dialysate solutions, either non-glucose based or with low GDP content. Whereas promising preliminary results have been obtained, further work is necessary to demonstrate that these new dialysates better preserve peritoneal membrane integrity in vivo.
REFERENCES


Chapter 4: Glucotoxicity of the peritoneal membrane


31. Schalkwijk CG, ter Wee PM, Teerlink T. Reduced 1,2-dicarbonyl compounds in bicarbonate/lactate-buffered peritoneal dialysis (PD) fluids and PD fluids based on glucose polymers or amino acids. *Perit Dial Int* 2000; 20: 796-798

32. Dawnay AB, Millar DJ. Glycation and advanced glycation end-product formation with icodextrin and dextrose. *Perit Dial Int* 1997; 17: 52-58


Chapter 5

Established rat models of chronic renal failure
Myofibroblast Transdifferentiation of Mesothelial Cells is Mediated by RAGE and Contributes to Peritoneal Fibrosis in Uremia

Mortier S, Tilton RG, Lameire NH, De Vriese AS

Submitted
ABSTRACT

Uremia is associated with fibrosis of the peritoneal membrane, even prior to the start of peritoneal dialysis. Increased carbonyl stress and the resultant formation of AGEs are potentially involved. Interaction of AGEs with their cell surface receptor RAGE induces sustained cellular activation, including the production of the fibrogenic growth factor TGF-β. TGF-β is pivotal in the process of epithelial-to-mesenchymal transition with the acquisition of myofibroblast characteristics. We investigated whether antagonism of RAGE prevents uremia-induced peritoneal fibrosis. In addition, we examined whether myofibroblast transdifferentiation of mesothelial cells contributes to peritoneal fibrosis in uremia.

Uremia was induced in rats by subtotal nephrectomy. Uremic and age-matched sham-operated rats were treated for 6 weeks with neutralizing monoclonal anti-RAGE antibodies or isotype-matched irrelevant IgG. Uremia resulted in accumulation of AGE, upregulation of RAGE and TGF-β and development of fibrosis in the peritoneal membrane. Prominent myofibroblast transdifferentiation of mesothelial cells was identified by colocalization of cytokeratin and α-smooth muscle actin in submesothelial and interstitial fibrotic tissue. Antagonism of RAGE prevented upregulation of TGF-β, epithelial-to-mesenchymal transition of mesothelial cells and fibrosis in uremia. In conclusion, ligand engagement of RAGE induces peritoneal fibrosis in chronic uremia. The process is mediated by upregulation of TGF-β and subsequent conversion of mesothelial cells into myofibroblasts.

INTRODUCTION

Data from the Peritoneal Biopsy Registry have indicated that peritoneal fibrosis appears in predialysis and hemodialysis patients and thus precedes the start of peritoneal dialysis (PD) \(^{1,2}\). Submesothelial and perivascular fibrosis were also observed in a rat model of uremia \(^{3}\). The thickness of the submesothelial compact zone was larger in PD patients with clinical problems than in those where biopsies were taken at random, suggesting that peritoneal fibrosis is clinically relevant \(^{2}\).
Chapter 5: RAGE-mediated myofibroblast transdifferentiation

Carbonyl stress is actively involved in the development of uremic complications. Reactive carbonyl compounds derived from either carbohydrates or lipids, accumulate in uremic plasma and contribute to the formation of advanced glycation end products (AGEs). AGE accumulation has been demonstrated in the peritoneum of PD patients and uremic rats, more specifically in the mesothelium, submesothelial stroma and blood vessels and correlated with the extent of vascular sclerosis and interstitial fibrosis. AGEs exert their biological effects by receptor-independent and receptor-dependent pathways. Several cell surface receptors, such as 80 K-H, OST-48, galactin-3, macrophage scavenger receptor and receptor for AGE (RAGE) have been identified, the latter of which is the best characterized. RAGE is a member of the immunoglobulin superfamily of cell surface molecules and interacts with diverse ligands, including AGEs, S100/calgranulins, amphoterin and amyloid-beta-peptid. This ligand-receptor interaction activates multiple cellular signal transduction pathways, leading to the secretion of inflammatory cytokines, upregulation of adhesion molecules and production of growth factors, such as VEGF and TGF-β.

Several in vivo studies support the importance of AGE-RAGE interaction in the development of diabetic complications. The interaction of AGE with RAGE induced a TGF-β-dependent epithelial-myofibroblast transdifferentiation in a proximal tubule cell line, providing a novel mechanism for tubulo-interstitial fibrosis in diabetic nephropathy. Exposure of cultured human peritoneal mesothelial cells to TGF-β resulted in the conversion of these cells into myofibroblasts, suggesting that a similar mechanism could be operative in the peritoneal membrane. In peritoneal biopsies of PD patients, fibroblast-like cells in the stroma were found to express mesothelial markers, supporting the contention that epithelial-to-mesenchymal transition of mesothelial cells could be a source of myofibroblasts in the peritoneum.

The present study evaluates the contributory role of AGE-RAGE interaction to peritoneal fibrosis in uremia and examines whether epithelial-to-mesenchymal transition of mesothelial cells is involved in the process of uremia-associated peritoneal fibrosis. We used a model of subtotal nephrectomy for the induction of chronic renal failure and antagonized RAGE with a neutralizing monoclonal anti-RAGE antibody (Ab).
MATERIAL AND METHODS

Laboratory animals

The studies were performed in 44 female Wistar rats (Iffacredo, Brussels, Belgium) with an initial mean body weight of 217 ± 1 g, receiving care in accordance with the national guidelines for care and use of laboratory animals. The rats were randomly assigned to uremic (n=24) or sham-operated groups (n=20). Uremia was induced using a standard procedure of subtotal nephrectomy as described earlier. Rats were anesthetized with halothane (Fluothane, Astra-Zeneca, Destelbergen, Belgium) and a flank incision was made to expose the left kidney. Subsequently, the upper and lower poles as well as the anterior and posterior lateral sides of the kidney were cryoablated. One week later a right nephrectomy was performed. Animals undergoing subtotal nephrectomy received buprenorphine (0.1 mg/kg IM/12h, Temgesic, Schering-Plough, Brussels, Belgium) post-surgically for 48 h and erythropoietin (100 IU/kg SC, Neo-Recormon, Boehringer Mannheim, Brussels, Belgium) twice weekly to correct anemia due to uremia. In sham-operated rats, flank incisions were made and the left and right kidney were manipulated without tissue destruction, respectively separated by one week. After the induction of uremia, rats were treated during 6 weeks with neutralizing monoclonal anti-RAGE Ab (n=12) or with isotype-matched irrelevant murine IgG (n=12). Similarly, sham-operated rats were treated with anti-RAGE Ab (n=10) or murine IgG (n=10). The Ab (1 mg dissolved in 1 ml saline) were injected intraperitoneally three times a week.

Neutralizing monoclonal anti-RAGE Ab

The preparation and characterization of the neutralizing monoclonal anti-RAGE Ab followed procedures described previously. The human RAGE extracellular domain encompassing residues 23-340 (sRAGE) was expressed and purified from E. coli using the pET thioredoxin system (Novagen, Madison, WI, USA). Female 8-week-old BALB/c mice were immunized, then boosted 3 times, 21 days apart, by intraperitoneal and subcutaneous injections of 100 µg of sRAGE protein in Complete Freund’s adjuvant for the primary immunization and an additional 50 µg of sRAGE in Incomplete Freund’s adjuvant for
secondary immunizations. The mouse with the highest serum titer to sRAGE as measured by enzyme-linked immunosorbent assay was injected intravenously with an additional 30 µg immunogen in PBS, 21 days after the last immunization. Three days later, spleen cells were harvested for production of hybridomas to sRAGE using previously described techniques. The hybridoma cell line with highest Ab titer and neutralizing Ab activity was selected after cloning 3-4 times by limiting dilution in 96-well microtiter plates, then grown in a Cellmax Bioreactor (Spectrum; Rancho Dominguez, CA, USA) using DMEM culture media. Purified IgG was prepared by Protein A chromatography. The isotype (IgG3) and light chain composition (κ) of the Ab were determined as described previously.

**Histology and immunohistochemistry**

One sample of parietal and visceral peritoneum was obtained in each experimental animal, fixed in 4% neutral buffered formalin and embedded in paraffin. Five µm sections were cut for histology. The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparaffinized, rehydrated and stained briefly with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen.

Immunostainings for AGE, RAGE, TGF-β, α-smooth muscle actin (α-SMA) and cytokeratin, as well as a double staining for α-SMA and cytokeratin were performed. Sections were deparaffinized, rehydrated, incubated in 3% H2O2 in PBS to block endogenous peroxidase and washed in 10% normal horse serum (Sigma, St. Louis, MO, USA) in PBS to block non-specific binding. Subsequently, they were incubated with the primary antibody: a mouse anti-human AGE (6D12, Cosmo Bio Ltd, Tokyo, Japan), a goat anti-human RAGE antibody (Research Diagnostics, Flanders, NJ), a rabbit anti-human TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-human α-SMA (clone 1A4, DAKO, Glostrup, Denmark) and a polyclonal rabbit anti-cytokeratin (wide spectrum screening, DAKO). For the AGE, RAGE and TGF-β staining, a biotinylated IgG (Vector Laboratories, Burlingame, California) and streptavidine-peroxidase were applied and 3,3'diaminobenzidine (DAB) was used as the chromogenic substrate, resulting in a brown precipitate. For the α-SMA staining, a peroxidase-labelled IgG (rabbit anti-mouse
IgG/HRP, DAKO) was applied and immunolabelling was visualised with DAB. For the cytokeratin staining, an alkaline phosphatase-labelled IgG (goat anti-rabbit IgG/AP, DAKO) was applied and Fast Red (DAKO) was used as chromogenic substrate, resulting in a red precipitate. For the double $\alpha$-SMA/cytokeratin immunostaining, tissues were pretreated with a Tris/EDTA epitope retrieval solution (pH 9.0, DAKO) and incubated with the polyclonal rabbit anti-cytokeratin and the goat anti-rabbit alkaline phosphatase-conjugated IgG. After color development with Fast Red, the peritoneum was treated with 3% $\text{H}_2\text{O}_2$ to inactivate endogenous peroxidase, incubated with the mouse anti-human anti-$\alpha$-SMA and the rabbit anti-mouse peroxidase-conjugated IgG and developed with DAB.

Morphometric measurements of the AGE, RAGE, TGF-$\beta$ and Picro Sirius Red staining were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification x200. For each sample of peritoneum, two sections were analyzed quantitatively with a computerized image analysis system (Zeiss, Oberkochen, Germany). A camera sampled the image of the stained sections and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss, Oberkochen, Germany). Each pixel in a color image was divided into three color components (hue, saturation and intensity). The threshold for each color component of the staining was defined and kept constant throughout the analysis. In a predefined area RAGE, AGE, TGF-$\beta$ and Picro Sirius Red staining were measured and expressed as a percentage.

A semi-quantitative assessment of the double $\alpha$-SMA/cytokeratin staining was performed independently by two blinded operators with a light microscope at magnification x100. The whole tissue section was screened to estimate the extent and distribution of colocalization of $\alpha$-SMA and cytokeratin in each slide. Staining results were classified from 0-3: 0= no, 1= mild, 2= moderate and 3= severe colocalization. The results were calculated as the mean of the individual scores of both operators.
**Chapter 5: RAGE-mediated myofibroblast transdifferentiation**

**Statistical analysis**

The results are expressed as mean +/- SEM. Statistical analysis was performed using ANOVA and, where appropriate, the Tukey test was used as multiple comparison t-test. The significance level was set at P<0.05.

**RESULTS**

**Characteristics of laboratory animals**

The cumulative mortality, including the nephrectomy/sham procedure, was 0% in sham-operated rats vs. 33 % in uremic rats, which is in accordance with literature data 21-23. About 75 % of the observed mortality (6/8) occurred within ten days, most likely due to surgical complications. The other animals (2/8) died in the last two weeks before sacrifice, as a consequence of their uremic state. The body weight of the uremic rats was significantly lower than that of the sham-operated rats. Ab treatment did not affect body weight (Table 1). Erythropoietin treatment prevented anemia in uremic animals. Exposure to anti-RAGE Ab did not alter hematocrit levels (Table 1). Uremia was documented by significantly increased plasma ureum and creatinine levels in uremic rats compared to sham-operated animals (Table 1).

**Table 1:** Plasma creatinine and ureum levels and creatinine clearance in the different experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Sham + control Ab (n=10)</th>
<th>Sham + RAGE Ab (n=10)</th>
<th>Uremia + control Ab (n=7)</th>
<th>Uremia + RAGE Ab (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>275.4 ± 2.7</td>
<td>265.3 ± 4.7</td>
<td>223.0 ± 6.4</td>
<td>229.7 ± 12.2</td>
</tr>
<tr>
<td>Hematocrit level (%)</td>
<td>43.8 ± 0.6</td>
<td>44.4 ± 0.6</td>
<td>44.5 ± 2.1</td>
<td>43.1 ± 1.2</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/L)</td>
<td>26.8 ± 1.0</td>
<td>32.08 ± 0.7</td>
<td>68.4 ± 10.1</td>
<td>77.0 ± 9.2</td>
</tr>
<tr>
<td>Plasma ureum (mmol/L)</td>
<td>4.7 ± 0.1</td>
<td>4.0 ± 0.4</td>
<td>14.3 ± 1.4</td>
<td>16.24 ± 1.6</td>
</tr>
</tbody>
</table>

*P < 0.01 vs. sham, **P < 0.001 vs. sham

**Peritoneal morphology**

The staining for AGE and RAGE was greatly enhanced in the peritoneal tissue of placebo-treated uremic rats compared to placebo-treated sham rats (Table 2, Figure 1, 2).
Figure 1: AGE immunostaining of the visceral peritoneum (x200). AGEs strongly accumulate in the peritoneal tissue of placebo-treated uremic rats (A), compared to placebo-treated sham-operated rats (B).

Figure 2: RAGE immunostaining of the visceral peritoneum (x200). RAGE expression was strongly up-regulated in placebo-treated uremic rats (A), compared to placebo-treated sham-operated rats (B).
Chapter 5: RAGE-mediated myofibroblast transdifferentiation

Submesothelial and interstitial fibrosis, as evaluated by Picro Sirius Red staining, was more pronounced in the uremic rats than in the sham-operated rats (Table 2, Figure 3). Exposure to anti-RAGE Ab significantly reduced fibrosis in the uremic animals (Table 2, Figure 3). TGF-β expression was strongly up-regulated in uremic rats compared to the sham-operated groups (Table 2, Figure 4). Antagonism of RAGE partially prevented upregulation of TGF-β expression in uremic animals (Table 2, Figure 4).

Staining for the epithelial marker cytokeratin was confined to the mesothelial cell layer in sham-operated animals. In uremic animals, an extensive additional staining was observed in the submesothelial and interstitial fibrotic tissue (Figure 5). Staining for α-SMA was limited to the muscularis of the blood vessels in control rats, but was also found in fibrotic areas in uremic animals (Figure 5). Double α-SMA/cytokeratin staining was virtually absent in controls, but was prominent in uremic animals (Figure 5). In uremic animals treated with anti-RAGE antibodies, colocalization of α-SMA and cytokeratin was significantly lower than in those that received control antibodies (Table 2).

Table 2: Histological and immunohistochemical analyses of the peritoneum

<table>
<thead>
<tr>
<th></th>
<th>Sham + control Ab (n=10)</th>
<th>Sham + RAGE Ab (n=10)</th>
<th>Uremia + control Ab (n=7)</th>
<th>Uremia + RAGE Ab (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE staining, %</td>
<td>2.3 ± 0.2</td>
<td>-</td>
<td>3.7 ± 0.6ab</td>
<td>-</td>
</tr>
<tr>
<td>RAGE staining, %</td>
<td>3.4 ± 0.3</td>
<td>-</td>
<td>6.9 ± 0.4c</td>
<td>-</td>
</tr>
<tr>
<td>Picro Sirius Red staining %</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>3.3 ± 0.5c</td>
<td>2.0 ± 0.3cd</td>
</tr>
<tr>
<td>TGF-β staining, %</td>
<td>1.8 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td>6.1 ± 0.6c</td>
<td>3.8 ± 0.4cd</td>
</tr>
<tr>
<td>Double staining, grade</td>
<td>1.0 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>2.6 ± 0.2c</td>
<td>1.8 ± 0.2c</td>
</tr>
</tbody>
</table>

*aP<0.05 vs. sham, bP<0.0001 vs. sham, cP<0.001 vs. sham, dP<0.05 vs. uremia + control antibody, eP<0.01 vs. uremia + control antibody

DISCUSSION

Chronic uremia results in fibrosis of the peritoneal membrane, in accordance with findings in human biopsies and other experimental studies. A pronounced deposition of AGEs was observed in uremic peritonea. Accumulation of AGEs is known to enhance the
surface expression of RAGE. A prominent upregulation of RAGE was indeed evident in the peritoneal membrane of uremic animals, while its expression was low-grade in sham-operated animals. The salient observation in the present study is that inhibition of the AGE-RAGE interaction with a neutralizing monoclonal anti-RAGE antibody prevented uremia-induced fibrosis. Several lines of evidence support that the fibrogenic properties of AGEs are mediated by upregulation of TGF-β. Uremia resulted in upregulation of TGF-β, which was partially prevented by antagonism of RAGE. The results support the contention that ligand engagement of RAGE induces expression of TGF-β and results in peritoneal fibrosis.

Further experiments were conducted to unravel the pathophysiologic mechanism underlying peritoneal fibrosis in uremia. Exposure of a proximal tubular cell line to AGE-BSA induced tubular-to-myofibroblast transdifferentiation through interaction with RAGE and resultant generation of TGF-β. Similarly to smooth muscle cells, myofibroblasts are characterized by the presence of contractile fibers and stain positively for α-SMA. They secrete cytokines and growth factors and are major producers of extracellular matrix molecules, such as collagen, fibronectin and glycosaminoglycans. Activation and proliferation of myofibroblasts, when unchecked, invariably results in tissue fibrosis. The origin of myofibroblasts is not certain, but they have been postulated to arise from resident fibroblasts, perivascular cells or circulating precursor cells. Recently, mesothelial cells and the process of epithelial-to-mesenchymal transition has been implicated as a potential source of myofibroblasts in peritoneal tissues. Administration of TGF-β to cultured human peritoneal mesothelial cells was associated with ultrastructural changes, the appearance of α-SMA myofilaments and the deposition of extracellular matrix typical for a myofibroblast phenotype. Mesothelial cells isolated from the effluent of PD patients showed phenotypical changes characteristic of an epithelial-to-mesenchymal transition process. In peritoneal biopsies of PD patients, fibroblast-like cells in the stroma stained positive for epithelial cell markers, thus revealing their mesothelial origin. In the present study, we performed a double staining for cytokeratin and α-SMA to examine potential conversion of mesothelial cells into myofibroblasts. Cytokeratin is a typical epithelial marker and thus allows the identification of mesothelial cells. As expected, the
Figure 3: Picro Sirius Red staining of the visceral peritoneum (x200). Prominent fibrosis was observed in uremic animals treated with placebo (A) or anti-RAGE-Ab (B), compared to sham-operated animals, treated with placebo (C) or anti-RAGE-Ab (D). RAGE-Ab partially prevented the development of fibrosis in uremic animals.

Figure 4: TGF-β immunostaining of the visceral peritoneum (x200). TGF-β expression was increased in the peritoneum of placebo-treated uremic rats (A). Antagonism of RAGE partially prevented the upregulation of TGF-β in uremic rats (B). TGF-β expression was virtually absent in the sham-operated groups with placebo (C) or RAGE-Ab (D).
Figure 5: Immunostaining of the visceral peritoneum for α-SMA (A, D, G), immunostaining for cytokeratin (B, E, H) and double immunostaining for α-SMA and cytokeratin (C, F, I) of serial sections (x200). In sham-operated animals, only vascular smooth muscle cells stain for α-SMA (A), only mesothelial cells stain for cytokeratin (B) and virtually no α-SMA/cytokeratin colocalization (C) occurs. In the peritoneal membrane of uremic animals, α-SMA (D, G) and cytokeratin (E, H) staining is additionally found in submesothelial and interstitial fibrotic tissue, where they colocalize (F, I).
cytokeratin staining was confined to the mesothelial cell layer in the peritoneal membrane of the control animals. α-SMA stains smooth muscle cells and is thus found in the vasculature of a normal peritoneal membrane. It is also characteristic for myofibroblasts and is often used as a marker for these cells. We detected a pronounced colocalization of cytokeratin and α-SMA in the submesothelial and interstitial fibrotic tissue of uremic peritonea. These results indicate that mesothelial cells migrate towards the interstitium and acquire a myofibroblastic phenotype. Importantly, inhibition of RAGE was not only associated with a lower TGF-β expression and less fibrosis, but also with less α-SMA/cytokeratin colocalization.

In conclusion, chronic uremia results in the development of submesothelial and interstitial fibrosis of the peritoneal membrane. We identified interaction of AGE with RAGE and subsequent upregulation of TGF-β and myofibroblast transdifferentiation of mesothelial cells as a potential pathogenic pathway for peritoneal fibrosis.

ACKNOWLEDGEMENTS

The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance. Siska Mortier is supported by a grant from the Bijzonder onderzoeksfonds (BOF/056) and An De Vriese by a grant from the Fonds voor Wetenschappelijk onderzoek-Vlaanderen (B/02476/05).
REFERENCES


292
Chapter 5: RAGE-mediated myofibroblast transdifferentiation


Chapter 6

Summary and future perspectives
SUMMARY AND FUTURE PERSPECTIVES

Although peritoneal dialysis (PD) has evolved to a standard form of renal replacement therapy, it still has to cope with a high level of technique failure, mainly owing to ultrafiltration failure (UFF) and recurrent peritonitis. An emerging consensus exists that structural and functional disturbances of the peritoneal integrity induced by the repeated exposure to the bioincompatible standard PDF are -at least partially- responsible for the deterioration of PD adequacy on the long-term. In the present thesis, it was demonstrated that PDF and their components played a contributory role in 1) the impairment of peritoneal host defense, 2) the development of an increased effective vascular surface area and 3) fibrosis.

Experimental models

The acute and chronic biocompatibility of standard and new PDF were evaluated using in vivo rat models of PDF exposure. Previous studies in our laboratory established the intravital microscopy method as a valuable technique to study acute functional and structural alterations of the PM. This technique was further developed specifically to evaluate hemodynamic changes of the peritoneal microcirculation (Chapter 2, 2.1) and peritoneal leukocyte recruitment (Chapter 2, 2.2).

In addition, a rat model of chronic PDF exposure was worked out, allowing a valid interpretation of chronic biocompatibility effects, unbiased by technical confounders. Evaluating different modes of heparin administration elucidated that intraperitoneal administered heparin, although reducing the incidence of infection, was not capable to prevent catheter obstruction and eventually still resulted in technique failure. Additionally, heparin also accumulated in the PM and thus may exert -in addition to its anticoagulant effect- a variety of biological actions leading to reinforcement or prevention of PDF-induced effects. The use of a heparin coated catheter, though, maintained a proper peritoneal access and hence increased technique survival, but had to cope with a high incidence of peritoneal infection (Chapter 3, 3.1). Combining the use of the heparin-coated catheter with prophylactic antibiotic administration prevented the occurrence of peritoneal infection and reduced infection-related fibrosis, increased solute transport and fall in UF
volume. No evidence was found that antibiotics per se altered the physiology of the PM (Chapter 3, 3.2).

The above described animal model was further utilized as a standard animal model for peritoneal PDF exposure for a period up to 20 weeks and was associated with a minimal technique failure (Chapter 4, 4.1 and 4.2).

**Impaired peritoneal host defense**

The integrity of local peritoneal host defense mechanisms plays a key role in the prevention of and recovery from bacterial peritonitis. To obtain a clear overview of previous studies performed on the effect of PDF on peritoneal host defense, the available literature, consisting largely of in vitro and ex vivo experiments, was reviewed (Chapter 2, 2.3). Very little attention has been paid to the effects of PDF on peritoneal host defense in vivo. We demonstrated that conventional PDF suppresses the in vivo physiologic host response when the PM was concomitantly exposed to an infectious stimulus. The different steps of leukocyte recruitment -rolling, adhesion and extravasation- were all dramatically affected. Despite the fact that PDF equilibrates progressively during the dwell time, spent PDF depressed leukocyte recruitment to a similar extent as fresh PDF. In contrast, acute exposure to a pH-neutral, bicarbonate-buffered PDF with low glucose degradation product (GDP) content exerted much less suppressive effects on peritoneal leukocyte kinetics (Chapter 2, 2.2). As efficient leukocyte recruitment to the area of infection is a prerequisite for the exertion of their effector functions, it was hypothesized that repeated PDF exposure persistently impairs host defense and increases patients' sensitivity to peritonitis. A controlled prospective randomized study should determine whether the improved biocompatibility of new PDF is associated with a clinical benefit regarding the incidence of infectious complications.

**Increased effective vascular surface area**

Adequate PD is also a matter of achieving a good UF volume in PD patients. Long-term PD is associated with a progressive loss of UF capacity, which can be attributed in most cases to an increased effective vascular surface area. As the incidence of UFF
increases with time spent on PD, repeated exposure to bioincompatible PDF was incriminated in the loss of UF capacity\(^1\).

Acute exposure to conventional PDF exerts vasoactive effects on the rat peritoneal microcirculation. Besides a reversible maximal vasodilation of the mesenteric arteries, conventional PDF induced a doubling of arteriolar flow with a commensurate increase in the number of perfused capillaries. This capillary recruitment could play a contributory role in increasing the effective vascular surface area and thus may impair UF function (Chapter 2, 2.1). Chronic treatment of the rat PM with conventional PDF resulted in a decreased UF volume. Morphologically, this correlated with a pronounced neoangiogenesis, associated with an increased eNOS and VEGF expression (Chapter 4, 4.1). These data support the etiologic role for neoangiogenesis in ultrafiltration, evidenced recently by De Vriese et al and Margetts et al.\(^2\)\(^3\). Neutralizing anti-VEGF antibody and angiostatin therapy, respectively, prevented neoangiogenesis and associated loss of ultrafiltration capacity or rise in small solute transport.

This knowledge has given onset to extensive in vivo testing of new PDF, either non-glucose based with low GDP content or with an alternative buffer system. Acute exposure to a bicarbonate-buffered PDF with low GDP content had much less hemodynamic effects on the peritoneal microcirculation (Chapter 2, 2.1). The chronic study revealed that UFF as well as neoangiogenesis and associated eNOS and VEGF expression were absent when the PM was exposed to an amino-acid based PDF or a bicarbonate/lactate-buffered PDF with low GDP content (Chapter 4, 4.1). In addition, a cross-over study demonstrated that conventional PDF-induced neoangiogenesis and the commensurate decrease in UF were less pronounced when the PM was subsequently exposed to the bicarbonate/lactate-buffered PDF with low GDP content (Chapter 4, 4.2).

Whether these new dialysates will also be associated with better UF rates in patients needs to be determined in large clinical trials, taking in account the large intra- and interindividual variations in drained volumes.

The development of fibrosis

Peritoneal fibrosis is a common finding in long-term PD patients. A chronic in vivo rat model of peritoneal exposure indeed demonstrated that the PM, chronically
exposed to conventional PDF, developed pronounced fibrosis, whereas new PDF better preserved peritoneal integrity (Chapter 4, 4.1). Furthermore, a cross-over study from standard PDF to low-GDP bicarbonate/lactate buffered PDF, demonstrated that the development of fibrosis was slowed down compared to continuous standard PDF exposure (Chapter 4, 4.2). In the latter study, fibrosis correlated positively with TGF-β. TGF-β is known to play a central role in the development of fibrosis, as adenovirus-mediated gene transfer of the active form of TGF-β leads to fibrosis of the peritoneum 4.

Although some studies found a correlation between fibrosis and ultrafiltration 5,6, the causal relation between peritoneal fibrosis and loss of UF is not clear. In contrast, gene transfer of decorin, a proteoglycan that binds and inactivates TGF-β, in a rat model of daily dialysate exposure reduced the fibrogenic process, however, had no impact on solute transport 3. Similarly, we demonstrated in a diabetic rat model that reduction of fibrosis by inhibiting AGE-RAGE interaction had no effect on small solute transport and ultrafiltration failure (Chapter 4, 4.3). The ubiquitous nature of fibrosis in PD patients confirms the clinical suspicion that the development of fibrosis is a consequence of the repeated exposure to injurious agents, though, at first sight its functional impact on the process of PD seems inferior compared to the impact of vascular alterations.

Relative contribution of PDF components to peritoneal membrane deterioration

Chronic exposure to conventional PDF implies chronic exposure to a mixture of bioincompatible components, which may exert, either isolated or combined, damaging effects on the peritoneal membrane. As stated above, the changes of the peritoneal membrane observed in vivo in our animal model confirm the findings of clinical studies, thus underlining the clinical relevance of our experimental model. Though next to the evaluation of the effects of the commercially available PDF, a primary goal of our studies was to shed some more light on the relative responsibility of the different PDF components in the observed PDF-induced changes (Flow diagram).

The combination of low pH and lactate has been shown in in vitro studies to be extremely toxic to peritoneal cells, because it decreases the intracellular pH, which is partly irreversible 7. In our acute model of peritoneal exposure, however, adjustment of the PDF
pH to 7.4 demonstrated that low pH *per se* did not play an essential role in affecting peritoneal hemodynamics and leukocyte recruitment (Chapter 2, 2.1 en 2.2).

Although comparing the conventional PDF with commercially available new PDF- a pH neutral bicarbonate/lactate-buffered high glucose PDF and a lactate-buffered amino-acid based PDF at near neutral pH- in our chronic model did not allow to rule out a contributory role for pH entirely, a primary role for low pH in the observed changes was doubted as *in vivo* acidity is rapidly corrected after infusion of the PDF in the abdominal cavity. Furthermore, a potential role for lactate to the peritoneal damage was suggested as it was demonstrated to induce mesothelial damage 8,9, collagen production 10, neoangiogenesis and fibrosis 11. Present findings demonstrated an isolated effect of lactate *per se* as it transiently induced vasodilation and caused a partial inhibition of the recruitment of leukocytes to an area of infection. The use of a bicarbonate as buffer anion appeared hemodynamically inert and better preserved host defense (Chapter 2, 2.1 en 2.2). The absence of salient functional and structural alterations of the peritoneal membrane after long-term exposure to lactate-buffered amino-acid based PDF, compared to the conventional lactate-buffered PDF, more or less excludes lactate as a direct factor for long-term membrane deterioration (Chapter 4,4.1). However, we did not investigate a possible combined effect of lactate and glucose through the polyolpathway, which is known to be hyperglycemia-driven and promoted by the presence of lactate 12, in causing the observed membrane alterations.

Histologic data suggested that the structural changes observed during long-term peritoneal dialysis are reminiscent of those observed in diabetic patients. Therefore, it was widely assumed that chronic exposure to high glucose concentrations is, at least in part responsible for the development of these changes. *In vitro* exposure of peritoneal cells to high glucose stimulates the expression of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β), growth factors which mediate neoangiogenesis and fibrosis respectively 13,14. No causative role was found for glucose *per se* or its associated hyperosmolality in the PDF-induced hemodynamic effects as a bicarbonate-buffered PDF was hemodynamically neutral despite its similar glucose concentration and osmolality (Chapter 2, 2.1). In contrast, glucose was found to partially impair leukocyte recruitment. Replacement of glucose by mannitol in order to obtain similar osmolality as the
conventional PDF, allowed to ascribe the observed partial inhibition to the hyperosmolality rather than to glucose per se (Chapter 2, 2.2). Similarly to the observed hemodynamic effects, the presence of high glucose concentrations, associated with hyperosmolality, was not the predominant causative factor of long-term alterations in the peritoneal membrane (Chapter 4, 4.1).

Glucose, however, also has the potential to affect the peritoneal membrane indirectly through the formations of glucose degradation products (GDP) formed during heat-sterilization of the conventional PDF. In vitro GDPs revealed a significant cytotoxic potential towards peritoneal fibroblasts and mesothelial cells. Furthermore, intraperitoneal exposure to the GDP methylglyoxal increased VEGF expression in the peritoneal tissue of experimental animals, suggesting GDPs may contribute to peritoneal neoangiogenesis. In our studies we evaluated the isolated effects of GDPs on peritoneal hemodynamics and leukocyte recruitment by exposure of the peritoneal membrane to a resterilized bicarbonate-buffered PDF. In contrast to the regular bicarbonate-buffered PDF which preserved peritoneal hemodynamic and host defense, the resterilized counterpart induced a peritoneal vasoreactivity and impairment of leukocyte recruitment similar to that caused by the conventional PDF (Chapter 2, 2.1 and 2.2). Chronic exposure of the rat peritoneal membrane to a conventional PDF was characterized by loss of ultrafiltration capacity, pronounced neoangiogenesis and development of fibrosis. In contrast, ultrafiltration failure as well as the diverse structural alteration were absent or much less pronounced with amino-acid based PDF or lactate/bicarbonate buffered PDF with low GDP content. As the most remarkable difference between the new PDF and the conventional PDF evaluated in our chronic study is the absence or significant reduction in GDP content in the former PDF, the present findings strongly imply a contributory role for GDPs in the observed changes. Furthermore, the increased VEGF expression associated with the observed neoangiogenesis after conventional PDF exposure supports the VEGF upregulating properties of GDPs (Chapter 4, 4.1).

Finally, glucose and GDPs can damage the peritoneal tissue by inducing non-enzymatic glycosylation of tissue proteins, which leads to AGE formation. AGEs have been demonstrated to promote VEGF expression in vitro. Although interstitial fibrosis and vascular sclerosis in the peritoneum correlated with interstitial and vascular AGE
Chapter 6: Summary and future perspectives

Accumulation, respectively, its precise pathophysiologic role underlying this association remained unclear. AGE accumulation indeed was prominently present in peritonea exposed to standard PDF, associated with an increased expression for the receptor of AGE, RAGE. In addition, it is tempting to speculate that AGEs function as an upstream mediator of fibrosis and VEGF-induced neoangiogenesis, as AGE accumulation correlated with the degree of fibrosis and VEGF expression. To evaluate the effective contribution of AGEs in these structural alterations occurring, AGE-RAGE interaction was inhibited in a streptozotocin-mediated diabetic rat model, in which the occurring changes were similar to those found in long-term PD patients. Inhibition of the AGE-RAGE interaction prevented the upregulation of TGF-β, development of submesothelial fibrosis and fibronectin accumulation, indicating that binding of AGE to RAGE induces TGF-β expression and associated development of fibrosis. Despite the known angiogenic properties of AGEs, no contributory role for AGE-RAGE interaction was found in neoangiogenesis. In addition, AGE-RAGE interaction was also found to play a pathogenic role in uremia-induced fibrosis.

General conclusions and future perspectives

These new data cast some light on the relative responsibility of the different PDF components on peritoneal membrane deterioration. Although the evidence is at times circumstantial, together with the existing literature, the following can be expounded: high glucose is the major determinant of PDF-induced damage of the PM, with the notion that it rather exerts its effects indirectly, through GDP and AGE formation. New PDF with alternative osmotic agents or with low GDP content, thus have the potential to better preserve peritoneal integrity.

Future work will be conducted to elucidate whether animals treated chronically with new bicarbonate/lactate buffered PDF clear peritonitis more quickly than animals treated with conventional PDF. Further, a chronic uremic model will be used to evaluate the combined effects of PD treatment and uremia on the PM.
Flow diagram: Relative contribution of peritoneal dialysis fluid (PDF) components to PDF-induced membrane alterations and PD technique failure.

- - - : demonstrated relations, - - - - : hypothesized relations.
SAMENVATTING EN TOEKOMSTPERSPECTIEVEN

Hoewel peritoneal dialyse (PD) reeds geëvolueerd is tot een gevestigde nierfunctie-vervangende therapie, is techniek falen, als gevolg van UF falen (UFF) en recidiverende peritonitis nog een frequent probleem. Herhaalde blootstelling aan peritoneale dialysaat oplossingen (PDO) wordt verantwoordelijk geacht voor het ontstaan van structurele en functionele veranderingen van het peritoneum en het daarmee geassocieerde verlies van PD efficiëntie. In deze thesis werd aangetoond dat PDO en hun bestanddelen een rol spelen in 1) de onderdrukking van het peritoneale immuunsysteem, 2) de toename van het effectief vasculair oppervlak en 3) de ontwikkeling van fibrose.

Experimentele modellen

De acute en chronische biocompatibiliteit van standaard en nieuwe PDO werd bestudeerd aan de hand van in vivo rat modellen. Voorgaande studies van onze groep hebben aangetoond dat de intravitaal microscopie techniek een waardevolle methode is om acute functionele en structurele veranderingen van de peritoneale membraan (PM) te bestuderen. Deze techniek werd verder op punt gesteld voor de evaluatie van hemodynamische veranderingen in de peritoneale microcirculatie (Hoofdstuk 2, 2.1) en van de leukocyte-endotheliale interacties (Hoofdstuk 2, 2.2).

Daarnaast werd een rat model van chronisch peritoneale expositie verder uitgewerkt om zo een valiede interpretatie van chronische biocompatibiliteits-effecten toe te laten zonder interferentie door technische tekortkomingen. De studie van verschillende heparine toedienings schema's toonde aan dat intraperitoneaal toegediende heparine, hoewel het optreden van infectie verminderde, niet in staat was om catheter obstructie volledig te voorkomen en uiteindelijk toch nog resulteerde in techniek falen. Daarnaast accumuleerde het heparine in het peritoneale weefsel, waar het biologische effecten zou kunnen uitoefenen die leiden tot versterking of preventie van de PDO-geïnduceerde veranderingen. Met een heparine-gecoate catheter daarentegen, bleef de peritoneale toegang goed open en was er een betere techniek overleving, echter met een hoge incidentie van peritoneale infectie (Hoofdstuk 3, 3.1). De combinatie van een heparine-gecoate catheter met profylactische antibiotica therapie bleek echter efficiënt de infectie, de infectie-
gerelateerde fibrose, het toegenomen soluta transport en de verminderde ultrafiltratie (UF) te vermijden. Antibiotica op zich hadden geen invloed op de fysiologie van de PM (Hoofdstuk 3, 3.2).

Het op punt gestelde dierexperimenteel model werd vervolgens gebruikt als standaard model voor chronische peritoneale PDO expositie (tot 20 weken) en ging gepaard met een minimum aan techniek falen (Hoofdstuk 4, 4.1 en 4.2).

Onderdrukking van het peritoneale immuunsysteem

Het lokale peritoneaal immuunsysteem is zeer belangrijk voor preventie en recuperatie van peritonitis. Om een duidelijk overzicht te krijgen van voorgaande studies die de effecten van PDO op het lokale immuunsysteem onderzochten, werd een overzicht gemaakt van de reeds beschikbare studies die voornamelijk in vitro en ex vivo uitgevoerd werden (Hoofdstuk 2, 2.3). Tot nu toe werd weinig aandacht besteed aan de effecten van PDO op het peritoneale immuunsysteem in vivo. In deze thesis werd aangetoond dat de conventionele PDO de in vivo immuun respons onderdrukt wanneer de PM gelijktijdig werd blootgesteld aan een inflammatoire stimulus. Zowel leukocyten rolling, adhesie als extravasatie werden drastisch onderdrukt. Ondanks het feit dat PDO equilibreren naarmate de verblijf tijd vordert, onderdrukte gebruikt dialysaat de leukocyt-endotheliale interacties in dezelfde mate als vers dialysaat. Acute expositie aan pH neutrale, bicarbonaat-gebufferde PDO met lage glucose degradatie product (GDP) concentratie, daarentegen, had minder onderdrukkende effecten op de leukocyten (Hoofdstuk 2, 2.2). Aangezien efficiënte leukocyt en migratie naar een infectiehaard vereist is voor een goede uitvoering van hun effector functies, kunnen we stellen dat repetitieve PDO blootstelling het immuunsysteem aanhoudend onderdrukt en de patiënt-gevoeligheid voor peritonitis verhoogt. Om na te gaan of de verbeterde biocompatibiliteit van de nieuwe PDO het aantal infectieuze complicaties zal verminderen, is een gecontroleerde prospectieve gerandomiseerde studie noodzakelijk.

Toename in effectief vasculair oppervlak

Efficiënte PD betekent ook een adequate UF capaciteit. Lange termijn PD gaat gepaard met een progressief verlies van UF, dat in de meeste gevallen kan toegeschreven worden...
Chapter 6: Summary and future perspectives

worden aan een toename in het effectief vasculair oppervlak. Aangezien het voorkomen van UFF toeneemt met de behandelingsduur, werd herhaalde blootstelling aan PDO geïmpliceerd in het verlies van UF capaciteit 1.

Acute expositie aan conventionele PDO beïnvloedde de vasculaire reactiviteit van de peritoneale microcirculatie in de rat. Naast een reversibele maximale vasodilatatie van de mesenterische arteriën, inducerde de conventionele PDO een verdubbling van de arteriële bloedstroom en een recrutering van capillairen. Deze recrutering van capillairen verhoogt het effectief vasculair oppervlak, waardoor de UF functie zou kunnen verminderen (Hoofdstuk 2, 2.1). Chronische behandeling van de PM met conventionele PDO resulteerde in een afgenomen UF volume. Morfologisch was dit gecorreleerd met een uitgesproken neoangiogenese en een toegenomen eNOS en VEGF expressie (Hoofdstuk 4, 4.1). Deze bevindingen ondersteunen een causale rol voor neoangiogenese in ultrafiltratie falen, wat recentelijk werd aangetoond door De Vriese et al. en Margetts et al. 2-3. Aan de hand van respectievelijk een VEGF-neutraliserend antilichaam en angiostatine therapie kon neoangiogenese en daarmee geassocieerd ultrafiltratie falen of stijging in soluta transport verhinderd worden.

Deze kennis heeft aanleiding gegeven tot het intensief in vivo testen van nieuwe PDO: PDO zonder glucose, met lage GDP concentratie of met een alternatief buffersysteem. Acute expositie aan een bicarbonaat-gebufferde PDO met lage GDP inhoud oefende veel minder hemodynamische effecten uit op de peritoneale microcirculatie (Hoofdstuk 2, 2.1). De chronische studie toonde aan dat zowel UFF als neoangiogenese en de geassocieerde eNOS en VEGF expressie afwezig waren als de membraan brootgesteld werd aan aminozuur PDO of bicarbonaat/lactaat-gebufferde PDO met lage GDP concentratie (Hoofdstuk 4, 4.1). Daarnaast toonde een cross-over studie aan dat de conventioneel PDO-geïnduceerde neoangiogenese en de overeenkomstige afname in UF minder uitgesproken waren wanneer de PM vervolgens brootgesteld werd aan een bicarbonaat/lactaat-gebufferde PDO met lage GDP concentratie (Hoofdstuk 4, 4.2).

Of deze nieuwe PDO de UF capaciteit bij patiënten beter zullen bewaren, moet nog bevestigd worden in grote klinische trials, rekening houdend met een grote intra- en interindividuele variatie in gedraineerde volumes.
Ontwikkeling van fibrose

Tijdens een langdurige behandeling met PD ontstaat peritoneale fibrose. Chronische expositie aan conventionele PDO veroorzaakte inderdaad peritoneale fibrose in de rat, terwijl nieuwe PDO de peritoneale integriteit beter bewaarden (Hoofdstuk 4, 4.1). Verder toonde een cross-over studie van een standaard PDO naar een laag-GDP bicarbonaat/lactaat gebufferde PDO aan dat de fibrose ontwikkeling vertraagd werd in vergelijking met een continue standaard PDO expositie (Hoofdstuk 4, 4.2). In deze laatste studie werd een positieve correlatie aangetroffen tussen fibrose en TGF-β, een gekende mediator van fibrose. De centrale rol van TGF-β in het fibrotische proces werd reeds aangetoond via adenovirus-gemedieerde gentransfer van de actieve vorm van TGF-β in ratten, wat leidde tot de ontwikkeling van fibrose in het peritoneum 4.

Hoewel in sommige studies een correlatie gevonden werd tussen fibrose en UF 5,6, blijft het oorzakelijke verband tussen peritoneale fibrose en het verlies van UF onduidelijk. Gentransfer van decorin, een proteoglycaan dat TGF-β bindt en inactiveert, in een rat model van dagelijkse dialysaat expositie zorgde voor een reductie van het fibrogene proces, maar had echter geen impact op soluta transport 3. Wij stelden een gelijkaardig resultaat vast: een vermindering van fibrose door het inhiberen van de AGE-RAGE interactie in een rat model ging niet gepaard met een overeenkomstig effect op soluta transport en ultrafiltratie falen (Chapter 4, 4.3).

Alhoewel het veelvuldig voorkomen van fibrose in PD patiënten het klinische vermoeden bevestigt dat dit een gevolg is van repetitieve blootstelling aan beschadigende agentia, lijkt de functionele impact op het PD proces in eerste instantie gering vergeleken met de impact van de vasculaire veranderingen.

Relatieve bijdrage van PDO bestanddelen in peritoneale membraan deterioratie

Chronische blootstelling van de peritoneale membraan aan een conventioneel PDO staat gelijk aan chronische blootstelling aan een mengsel van onfysiologische bestanddelen, die elk op zich of gecombineerd beschadigend kunnen zijn voor het peritoneum. Het voornaamste doel van onze studies was dan ook meer in detail na te gaan wat de relatieve
bijdrage was van de verschillende PDO componenten in de geobserveerde PDO-
geïnduceerde veranderingen (Flow diagram).

In vitro werd reeds aangetoond dat de combinatie van lage pH en lactaat extreem
toxisch is voor peritoneale cellen, omdat het een daling in intracellulaire pH veroorzaakt,
die gedeeltelijk irreversibel is. In ons acuut model echter toonde het neutraliseren van de
pH tot 7.4 aan dat lage pH per se geen essentiële rol speelde in het beïnvloeden van de
peritoneale hemodynamiek en leukocyten recrutering (Hoofdstuk 2, 2.1 en 2.2). Daarnaast
is van hoge lactaat concentraties reeds geweten dat zij mesotheliale schade, collageen
productie en fibrose kunnen induceren. Onze huidige bevindingen toonden inderdaad een geïsoleerd effect van lactaat op zich: het induceerde een transiënte
vasodilatatie van peritoneale bloedvaten en veroorzaakte een partiële inhibitie van
leukocyten recrutering. Het gebruik van bicarbonaat als buffer anion daarentegen bleek
hemodynamisch inert te zijn en zorgde voor een beter behoud van het peritoneale
immuunsysteem (Hoofdstuk 2, 2.1 en 2.2). De afwezigheid van uitgesproken functionele
en structurele veranderingen in de peritoneale membraan na chronische blootstelling aan de
lactaat-gebufferde aminozuur-bevattend PDO, vergeleken met de conventionele PDO,
spreken echter tegen dat lactaat rechtstreeks betrokken zou zijn bij de lange termijn
achteruitgang van de peritoneale membraan (Hoofdstuk 4, 4.1). Een mogelijk
gecombineerd effect van lactaat en glucose in het veroorzaken van de geobserveerde
membraan veranderingen via polyol pathway, waarvan reeds geweten is dat deze
hyperglycemie-gedreven is en bijkomend gestimuleerd kan worden door lactaat, werd
echter niet onderzocht.

De structurele veranderingen waargenomen tijdens chronische PD doen sterk
denken aan deze waargenomen bij diabetische patiënten. Vandaar werd verondersteld dat
de ontwikkeling van deze veranderingen tenminste gedeeltelijk kon worden toegeschreven
aan chronische blootstelling aan hoge glucose concentraties. In vitro blootstelling van
peritoneale cellen aan hoge glucose concentraties stimuleerde de expressie van vasculaire
endotheliale groeifactoren (VEGF) en transformerende groeifactor β (TGF-β), groeifactoren
die respectievelijk neoangiogenese en fibrose mediëren. Glucose per se of zijn
geassocieerde hyperosmolaliteit bleek echter niet bij te dragen tot de PDO-geïnduceerde

309
Chapter 6: Summary and future perspectives

hemodynamische effecten, aangezien de bicarbonaat-gebufferde PDO hemodynamisch neutraal was ondanks eenzelfde glucose concentratie en osmolaliteit (Hoofdstuk 2, 2.1). Leukocyten recrutering, daarentegen, werd wel beïnvloed door de aanwezigheid van glucose. Vervanging van glucose door mannitol met als doel het behouden van een gelijkwaardige osmolaliteit als de conventionele PDO toonde aan dat de geobserveerde partiële inhibitie van leukocyten recrutering eerder kon toegeschreven worden aan hyperosmolaliteit dan aan glucose per se (Hoofdstuk 2, 2.2). In overeenstemming met de hemodynamische effecten bleek glucose en de daarmee geassocieerde hyperosmolaliteit geen voormande factor in het veroorzaken van chronische veranderingen in de peritoneale membraan (Hoofdstuk 4, 4.1).

Glucose bezit echter ook de mogelijkheid om de peritoneale membraan indirect te beïnvloeden via de vorming van glucose degradatie producten (GDPs), die ontstaan tijdens hitte-sterilisatie van de conventionele PDO. In vitro werd reeds aangetoond dat deze GDPs extreem schadelijk zijn voor peritoneale fibroblasten en mesotheliale cellen 15. Daarnaast gaf intraperitoneale blootstelling aan het GDP methylglyoxal aanleiding tot een verhoogde VEGF expressie in het peritoneale weefsel van proefdieren, wat suggereerde dat GDPs peritoneale neoangiogenese kunnen stimuleren 16. In onze studies werd het geïsoleerde effect van GDPs bestudeerd op de peritoneale hemodynamiek en leukocyten recrutering via blootstelling van de membraan aan een gehersteriliseerd bicarbonaat-gebufferde PDO. In tegenstelling tot de gewone bicarbonaat-gebufferde PDO, die geen ongunstig effect uitoefende op de peritoneale hemodynamiek en het immuunsysteem, induceerde zijn gehersteriliseerde tegenhanger een peritoneale vasoreactiviteit en onderdrukking van de leukocyten recrutering, gelijkwaardig aan deze waargenomen met de conventionele PDO (Hoofdstuk 2, 2.1 en 2.2). Chronische blootstelling van de rat peritoneale membraan aan de conventionele PDO werd gekarakteriseerd door een verlies van ultrafiltratie capaciteit, uitgesproken neoangiogenese en de ontwikkeling van fibrose. Zowel ultrafiltratie falen als de diverse structurele veranderingen bleken echter afwezig te zijn of veel minder uitgesproken na blootstelling aan de aminozuur-bevattende PDO of de lactaat/bicarbonaat-gebufferde PDO met lage GDP inhoud. Aangezien deze laatste PDO’s zich voornamelijk onderscheiden van de conventionele oplossing door de afwezigheid van of een uitgesproken reductie in GDPs, werd verondersteld dat GDPs een belangrijke bijdrage
leverden in de waargenomen veranderingen. Daarnaast was de geobserveerde neoangiogenese na conventionele PDO blootstelling geassocieerd met een toegenomen VEGF expressie, wat de VEGF stimulerende eigenschappen van GDPs bevestigt (Hoofdstuk 4, 4.1).

Tenslotte is algemeen bekend dat glucose en GDPs het peritoneale membraan ook kunnen beschadigen door de inductie van non-enzymatische glycosylatie van weefselproteïnen, wat aanleiding geeft tot de vorming van AGEs, geavanceerde glycatie eindproducten. In vitro werd vastgesteld dat AGEs VEGF expressie promoten 17. Hoewel reeds werd waargenomen dat interstitiële fibrose en vasculaire sclerose in het peritoneum correleerden met interstitiële en vasculaire AGE accumulatie 6, blijft de precieze pathofysiologische link achter deze associatie onduidelijk. AGE accumulatie was inderdaad opvallend aanwezig in peritonea geëxposeerd aan standaard PDO en ging gepaard met een toegenomen expressie van de receptor voor AGE, RAGE (Hoofdstuk 4, 4.1). Daarnaast was het eveneens verleidelijk om te speculeren dat AGEs dienst doen als stroomopwaartse mediator van fibrose en VEGF-geïnduceerde neoangiogenese, aangezien AGE accumulatie zowel met de graad van fibrose als met VEGF expressie correleerde. De effectieve bijdrage van AGEs tot deze structurele veranderingen werd geëvalueerd via inhibitie van de AGE-RAGE interactie in een hoog glucose expositie model, waarin de geobserveerde veranderingen gelijkaardig waren aan deze waargenomen bij chronische PD-patiënten. Inhibitie van de AGE-RAGE binding verhinderde de up-regulatie van TGF-β, de ontwikkeling van submesotheliale fibrose en fibronectine accumulatie. Dit toont aan dat de interactie van AGE met RAGE TGF-β expressie en geassocieerde fibrose induceert. Ondanks de gekende angiogene eigenschappen van AGEs 18, kon geen bijdrage van de AGE-RAGE interactie in neoangiogenese vastgesteld worden (Hoofdstuk 4, 4.3). De AGE-RAGE interactie speelde eveneens een pathogene rol in uremie-geïnduceerde fibrose (Hoofdstuk 5, 5.1).

Algemene conclusies en toekomstperspectieven

Deze nieuwe bevindingen geven meer inzicht in de relatieve bijdrage van de verschillende PDO componenten tot de achteruitgang van de peritoneale membraan. Samen
met de bestaande literatuur kan de volgende hypothese vooropgesteld worden: glucose is
de voornaamste bepalende factor van de PDO-geïnduceerde schade. Glucose oefent hierbij
zijn effecten eerder indirect uit, met name via GDP en AGE vorming. Nieuwe PDO met
alternatieve osmotische agentia of met lage GDP inhoud, bezitten daarom dus het potentieel
om op lange termijn de peritoneale integriteit beter te bewaren.

In de toekomst zal getracht worden om op te helderen of dieren die chronisch
behandeld worden met de nieuwe bicarbonaat/lactaat-gebufferde PDO sneller herstellen
van peritonitis dan dieren behandeld met conventionele PDO. Daarnaast zal een chronisch
model van uremie toegepast worden om het gecombineerde effect van PDO expositie en
uremie op de PM na te gaan.
Flow diagram: Relatieve bijdrage van peritoneale dialyse oplossing (PDO) componenten tot PDO-geïnduceerd membraan veranderingen en PD techniek falen. ———— demonstrated relations, ———— hypothesized relations.
REFERENCES


DANKWOORD

Last but not least is dit de plaats om de mensen te bedanken die gedurende 4 jaar intensief hebben meegewerkt, direct of indirect, aan het realiseren van alle data die zich in dit gebundelde werk bevinden.

Indien ik alles op mijn eentje had moeten verwezenlijken, ben ik er 100 % van overtuigd dat ik meerdere keren zou blijven steken zijn in 'onoverkomelijke' problemen. Ik hoop dat ik in een paar zinnetjes duidelijk kan maken, hoe elk van jullie meer dan één steentje heeft bijgedragen tot deze doctoraatsthesis.

Prof. Dr. Norbert Lameire, mijn promotor, ik wil je graag bedanken voor de kansen die ik heb gekregen binnen de dienst Nefrologie en voor je interesse in het wetenschappelijke onderzoek omtrent peritoneaal dialyse. Dankzij de vrijheid die ik kreeg, kon ik meerdere malen nationale en internationale congressen bijwonen om mijn kennis uit te breiden en andere mensen uit hetzelfde onderzoeksgewijs te ontmoeten.

Prof. Dr. An De Vriese, mijn co-promotor. Jij hebt van mijn eerste werkervaring iets uniek gemaakt. Jouw aanstekelijk enthousiasme en kritische, eerlijke kijk op de dingen zijn alvast 2 zaken die ik zeker verder zal meedragen. Jij hebt mij stap voor stap binnengeleid in het wereldje van wetenschappelijk onderzoek, het opstellen van protocols, omgaan met tegenvallende experimenten, het schrijven van artikels, … en nog zoveel meer. Daarnaast bleef er ook nog tijd over voor een gezellige babbel tussendoor. Ik wil je daarom in het bijzonder bedanken voor jouw onuitputbare energie, die de drijvende motor achter dit werk was.

I would like to thank the members of the jury: Prof. Dr. Raymond Vanholder, Prof. Dr. Geert Leroux-Roels, Prof. Dr. Jean Plum, Prof. Dr. Johan Van De Voorde, Prof. Dr. Romain Lefebvre, Dr. Wim Van Biesen and Dr. Nicholas Topley for the critical evaluation of my thesis and the valuable suggestions.

A special thanks goes to Dr. Nicholas Topley. In particular for the opportunity you gave me to visit your laboratory in Wales, where Rachel McLoughlin taught me everything I needed to know about the use of cell free supernatans of Staphyloccoccus epidermidis as inflammatory stimulus in our rat models.

Prof. Dr. Ria Cornelissen en Leen Pieters zou ik eveneens willen bedanken. Jullie maakten ons wegwijs bij het zetten van onze eerste stapjes in de wondere wereld van de immunohistochemie. De meisjes van de pneumo, zoals wij jullie hier op de nefro wel eens durven te noemen, Els Palmans, Nele Vanacker, Ann Neessen, Indra De Borle, An D'huyst en Katy De Swert, hielden ons dan verder op weg bij de evaluatie van tal van immunokleuringen. Bedankt dat we regelmatig eens bij jullie mochten aankloppen voor uitleg over programma's voor het KS 4000 analyse systeem, probleempjes bij kleuringen en het lenen van producten die bij ons plots uitgeput waren.

Julien, het zonnetje en groot lawaai van blok B. Je bent echt een manusje van alles. Je zorgde er niet alleen voor dat alles wat het werk betreft op rolletjes liep, daarnaast nam je je ook het versterken van de innerlijke mens ter harte met koffie, fruit, chocola en andere
versnaperingen. Dankzij jouw inzet, samen met die van Mieke, Nele en Tommy konden de verschillende studies elkaar in een ‘vlot’ tempo opvolgen. Dank je wel voor de talloze keren dat jullie ratten implanteerden, injecteerden en uiteindelijk sacrifieerden. Mieke, bedankt voor de talloze WBC tellingen, het kritisch nalezen van de protocols en praktische tips. Nele, we hebben ons gaandeweg ingewerkt in de soms frustrerende wereld van de immunohistochemie, talloze kleuringsprotocols opgesteld en door analyses geworsteld, eerst samen, maar al snel kon je alles alleen klaarspelen en had je enkel op het einde van de kleuring nog eens mijn ‘OK’ nodig. Dank je wel voor de gezellige uren die we doorgebracht hebben aan de microscoop bij het uitzoeken van de foto’s. Marie-Anne, bedankt voor de hulp bij analyses in het labo. Henri, bedankt voor het uithalen van artikels en het transport van dringende zaken tussen K12 en Blok B door weer en wind.

Ingrid, Chantal, Isabelle, Christel, en Lucrèce. Hoewel jullie werk niet rechtstreeks terug te vinden is in dit boekje, waren jullie onmisbaar op het gebied van afspraken met Prof. Lameire, het regelen van buitenlandse trips, indienen van onkosten, het bevoorraden met labo materiaal …

Als het onderzoek al wou vlotten, dan waren er ook nog Dirk en Cyriel om ervoor te zorgen dat de techniek mee wilde. Dirk, dank je wel voor het temmen van tegenstribbelende computers, printers en videorecorders. Cyriel, dank je wel voor het ontwerpen van de microscooptafel, die meer dan eens zijn nut heeft bewezen, en voor het herstellen van allerlei uiteenlopende elektronische mankementen. Uiteraard dank ik ook de andere mensen van blok B die gedurende de voorbije 4 jaar mijn verblijf aangenaam maakten, hetzij door een gezellige babbel, praktische tips of nuttig advies.

Tenslotte gaat een zeer dikke dank je wel naar mijn ouders. Moeke en papa, ik kan jullie eigenlijk niet genoeg bedanken. Jullie hebben me niet alleen op de wereld gezet, maar hebben mij opgevoed tot de persoon die ik nu ben. Jullie gingen zonder tegenpruttelen mee in mijn toekomstdromen, die ik dankzij jullie steun tot nu toe stuk voor stuk heb kunnen realiseren. Wie weet stop ik ooit nog wel eens met ‘studeren’…

Francis, het is zover, mijn doctoraat is af! Of ik je niet langer met vreemde termen rond je oren zal zwaaien (waar je intussen toch al aardig begon in thuis te raken), je geduld niet langer op de proef zal stellen met uitleg over protocols, verkeerd gelopen experimenten, writer’s block en andere ‘ratte’nissen, betwijfel ik. Ik zal altijd wel ten volle in iets moeten opgaan en mij daar ten gepaste tijde eens als een wervelwind over uitlaten, maar met je luisterend oor en relativerende kijk, komt dit altijd in orde!

Allemaal heel erg bedankt voor jullie niet aflatende inzet.

Siska