Pro inflammatory cytokines and neurogenic inflammation in peritoneal dialysis

Master thesis in Medicine

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Abstract

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Introduction: For patients with end stage renal disease (ESRD) peritoneal dialysis (PD) is a widely available and comparatively cheap method to mimic the lost functions of the kidney. However the longevity of the treatment is dependant upon the function of the peritoneal membrane to facilitate the removal of solutes from blood to the dialysate. PD triggers an inflammation, which gradually decreases this function of the peritoneal membrane. Earlier studies have shown expression of various inflammatory cytokines during PD, however the release mechanisms remain unknown. Recently is was shown that PD not only triggers release of pro inflammatory cytokines but also triggers a short neurogenic inflammation with release of the neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP), through activation of the nociceptor transient receptor potential vanilloid 1 (TRPV1). These neuropeptides are known to induce synthesis of pro inflammatory cytokines.

Objectives: Since PD is performed several times daily, neuropeptides are frequently released in to the peritoneal space. The aim of this study was to evaluate the connection between frequent neurogenic inflammations and a sustained, cytokine induced, inflammation. The hypothesis being that inhibition of the TRPV1 receptor decreases the synthesis of pro inflammatory cytokines thereby reducing fibrosis caused by inflammation, leading to a prolonged technique survival in PD.

Methods: Rats were subjected to PD with or without preceding i.v. TRPV1-antagonistic treatment (BCTC). After 4 hours of PD, pieces of connective tissue from the diaphragm was excised and homogenized. The mRNA expression of pro-inflammatory cytokines was measured via qPCR of the homogenate.

Results: The obtained data showed no statistic significance supporting the hypothesis.

Discussion and Conclusion: Although no significant results could be shown, the rats receiving antagonistic TRPV1 treatment before PD treatment generally showed a lower average expression of pro inflammatory cytokines than the treatment group receiving only PD, suggesting that the TRPV1 treatment was anti-inflammatory. Implications: Since one of the dominating causes of treatment failure in PD is fibrosis due to inflammation, a treatment that reduces inflammation would have great clinical implications. This study could not show data supporting the hypothesis, why the clinical implications become limited. However, the trends seen in this material deserves a closer look in a future study.

Populärvetenskaplig sammanfattning på Svenska

Njuren har bland annat till uppgift att rena kroppen från slaggprodukter och överbliven vätska, vilket sker via urinen. För personer vars njurar inte längre fungerar finns dialysbehandling. Det finns två huvudtyper av dialysbehandling; haemodialys (HD), där man renar blodet med hjälp av en maskin, och peritonealdialys (PD) där man via en slang tillför en lösning till bukhålan vilken samlar på sig slaggprodukter och vätska. Denna lösning töms efter några timmar ut igen varpå proceduren upprepas. PD har både för- och nackdelar jämfört med HD, en nackdel är att dialysen ger upphov till en låggradig inflammation i bukhålan. Med tiden leder inflammationen till förändringar i bukhålan vilket minskar effekten av PD-behandlingen och slutligen måste man byta till HD. Man har forskat mycket på vad som startar och underhåller inflammationen vid PD, man har sett att det frisätts inflammatoriska molekyler kallade cytokiner. Dessa cytokiner har många olika effekter på bukhålan och det är de som till slut gör att PD behandlingen slutar att vara effektiv. Man har dock inte kunnat visa vad som frisätter dem. Nyligen visade man i en studie att det vid PD förutom cytokiner också frisätts s.k. neuropeptider. Dessa neuropeptider har en rad kända effekter som påverkar bukhålan, effekten är dock kortvarig. Frågeställningen i den här studien var om dessa neuropeptider som frisätts vid PD möjligtvis kunde initiera bildandet av inflammatoriska cytokiner, alltså utgöra en länk mellan kortvarig retning vid PD och långvarig inflammation. För att undersöka detta delades råttor in i behandlingsgrupper där några råttor fick vanlig PD medan andra råttor först fick en behandling som hindrar frisättning av neuropeptider, varefter de fick PD. Det fanns också kontrollgrupper. Efter några timmars PD togs en bit vävnad från bukhålan och uttrycket av inflammatoriska cytokiner mättes. Resultaten visade inga säkerställda skillnader mellan de råttor som fick behandling innan PD och de råttor som endast fick PD. Trender i materialet indikerar dock att behandlingen verkligen haft effekt, det var dock för litet forskningsmaterial för att kunna dra några säkra slutsatser. Om man kunde hitta en behandling som minskar inflammation vid PD skulle det betyda att man sannolikt kan förlänga antalet år man kan få PD behandling. Detta skulle vara betydelsefullt främst i länder där tillgången på HD är begränsad, vilket den är i många utvecklingsländer. De trender som sågs i denna studie motiverar en större framtida studie vilket skulle ge mer data och skulle då eventuellt kunna visa med säkerhet att behandling som blockerar frisättningen av

neuropeptider också minskar frisättning av inflammatoriska cytokiner.

Introduction

Peritoneal dialysis (PD) is a well-established treatment of end stage renal disease (ESRD). It is divided into two subgroups, continuous ambulatory peritoneal dialysis (CAPD), where the patient undergoes several shorter treatments daily, and automated peritoneal dialysis (APD), where the patient undergoes one longer treatment per 24h, usually at night. APD consists of a series of shorter dialysis cycles managed by a machine. In PD, fluid is administrated into the abdominal cavity, accessed by a surgically placed catheter. Through the catheter the abdominal cavity is filled with approximately 2 litres of fluid that remains for some time, in CAPD typically 4-6 hours. Distributing dialysate fluid to the abdominal cavity creates an imbalance of concentration of solutes such as urea between the bloodstream and the cavity. The dialysate fluid also contains an osmotic agent, typically glucose, which by osmosis facilitates the transport of fluid from the bloodstream to the peritoneal cavity. This will increase the volume of fluid in the abdominal cavity, increasing the imbalance of solutes, which subsequently will diffuse from the bloodstream to the dialysate. In addition, excess water will be removed from the circulation. Fluid moving from surrounding capillaries to the peritoneal cavity is referred to as ultrafiltration (UF). When removed, the fluid will contain solutes. It is then replaced with sterile fluid and the process is repeated, each cycle is called a dwell. Normally the CAPD patient undergoes 3 to 5 dwells per day, each time depleting the body of solutes and water. Another type of dialysis is haemodialysis (HD), where the patient's blood is transferred to a machine that filters the blood and redistributes it to the patient. HD is dependant upon frequent in-hospital treatments each week, or access to a machine for home-based HD treatment, services available in most developed countries. However, availability in developing countries is limited and since PD is a less expensive treatment than HD, the number of patients using PD is still increasing in developing countries (1). Also, compared to HD, PD has advantages such as better preservation of residual renal function and better initial patient survival (2).

Function of the peritoneal membrane

Peritoneal dialysis depends on the peritoneum working as a filter, mimicking the filter effect of the kidneys, i.e. to remove water and waste products such as urea from the blood stream. The peritoneum consists of three distinct layers. The outermost cells are

the mesothelial cells, a monolayer of cells standing on the second layer, a thin basement membrane. The third layer is the connective tissue, consisting of extracellular matrix (ECM), collagen, proteoglycan gel and a network of capillaries and lymphatic vessels. Embedded in the ECM are cells such as macrophages, mast cells and fibroblasts. Endothelial cells lining the capillaries build up the most important barrier for solute transport during PD, the capillary membrane.

Complications to PD

Although peritoneal dialysis offers an affordable treatment for people with ESRD it has it´s shortcomings, the two main problems being ultrafiltration failure (UFF) and peritonitis. Even though progress has been made, UFF and in particular peritonitis still are dreaded complications and leading contributors to poor technique survival with subsequent transfer to HD, a technique not widely available in developing countries.

Peritonitis

Infectious peritonitis is caused by bacteria, which enter the peritoneum through the catheter or via the tunnel the catheter passes through. Peritonitis can be a dangerous infection and is therefore important to reveal in an early stage. The patient usually readily reveals an infection as opalescent fluid comes out after dialysis. Treatment is antibiotics, added to the dialysate or via systemic distribution. Frequent episodes of peritonitis increases the risk of maintaining a chronic inflammatory state, leading to functional and morphological changes of the peritoneum. Such changes may ultimately lead to the development of ultrafiltration failure (UFF).

Ultrafiltration failure (UFF)

Filtration through a semipermeable membrane or any filter that separates colloid solutions from crystalloids or separates particles of different size in a colloid mixture is called ultrafiltration (UF). Anything that tampers with this process can lead to ultrafiltration loss or failure (UFF). The two most recognized processes that contribute to UFF in PD are angiogenesis and fibrosis of the peritoneal membrane. Angiogenesis leads to changes in the vessel wall that increases the diffusive solute transport, which enhances reabsorption of glucose causing a decrease in osmotic pressure. Fibrosis thickens the ECM, this reduces the osmotic pressure-gradient and thereby UF (3).

Inflammation, whatever the cause, contributes to both fibrosis and angiogenesis. Frequent or persistent inflammation may therefore result in UFF.

Inflammation in PD

There are a number of causes to inflammation in PD such as the very presence of a catheter, the PD fluid, peritonitis, volume loading and uraemia. These stimuli will release several inflammatory mediators from the peritoneal cells (4). M*acrophages* produce interleukin 1 beta (IL-1β), tumor growth factor beta (TGF-β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin 8 (IL-8). *Mesothelial cells* produce vascular endothelial growth factor (VEGF), TNF-α and TGF-β. *Fibroblasts* is affected by cytokines to produce IL-8. *Mast cells* produce TNF-α, VEGF, TGF-β and IL-8. *Endothelial cells* will especially be affected by IL-1β and TNF-α, causing a production of pro-inflammatory substances such as prostaglandin I2 (PGI2), IL-1, IL-6, nitric oxygen (NO), IL-8 and monocyte chemo attractant protein 1 (MCP-1) whose effect among other things are recruiting leukocytes, and inducing cell death (5). When exposed to pro-inflammatory mediators, endothelial cells will also change their form, thereby affecting filtration.

The inflammatory mediators produced by these cells induce chemokine secretion which signals inflammation, attracting more macrophages along with neutrophils to the site. Inflammatory cells will release toxic substances to liquidate the pathogen, present or not. Once inflammation has started, damage to cells is inevitable. In addition to this cellular damage, inflammation influences surrounding cells to continue producing cytokines such as VEGF and TGF-β, known contributors of fibrosis and angiogenesis, thereby further affecting UF. Continuous inflammation is known to result in structural change of the peritoneum, promoting fibrosis and angiogenesis, which may result in technique failure and UFF.

Attempts to minimize inflammation in PD

Conventional PD fluid is characterized by high osmolality, low pH, high lactate- and high glucose- concentrations. Heat sterilization of PD-fluid results in the forming of advanced glycated end products (AGEs) and glucose degradation products (GDPs). These PD fluids are known to cause peritoneal inflammation in animals (6)(2). Research has so far mainly focused on finding more biocompatible PD fluids, thereby trying to diminish inflammation. With new fluids, several studies report beneficial results regarding residual renal function, peritonitis and pain, whereas impact on

peritoneal membrane function and UFF is more inconclusive (2). Regarding the inflammation however, even when using “bio-compatible” fluids inflammation is still present. This leads to the conclusion that the triggering mechanisms leading to a release of pro-inflammatory mediators are unknown.

Neurogenic inflammation could be one of, or the, trigger of peritoneal inflammation. We know that neurogenic inflammation triggers release of inflammatory mediators during PD in rats (7). Blocking mediators of neurogenic inflammation may prevent the release of pro-inflammatory mediators and thereby have a beneficial effect on inflammation in PD, contributing to prolonged technique survival.

Neurogenic inflammation

In the beginning of last century the discovery was made that activation of dorsal root ganglia resulted in vasodilation, leading to the suggestion that sensory nerves are not only afferent but also efferent (8). Further research put forth the hypothesis that nerves themselves could release substances that induces inflammation, so called neurogenic inflammation. Jancso et al (9) discovered that capsaicin-sensitive primary afferent nerve-fibres (CSPA) are responsible for a release of substances causing the neurogenic inflammation. Since then it has been confirmed that activation of sensory neuron terminals, either by depolarization, dorsal root reflexes or axonal reflexes, leads to a release of peptides capable of inducing inflammation. Sensory neurons express transient receptor potential (TRP) -channels, a group of trans membrane nociceptive ion channels susceptible for various stimuli such as mechanical, thermal and/or chemical stimulation. TRP-channels are all tetramers, several with high calcium permeability and cat ion-selective pores (10). Stimulation of TRP-receptors leads to a release of neuropeptides, which mediates the inflammatory effect (11). The ultimate effects of neurogenic inflammation are very similar to infectious inflammation. Vasoactive peptides such as NO, CGRP and vasoactive intestinal polypeptide (VIP) will cause vasodilation in capillaries and arteries, thereby creating warmth and redness. SP will affect endothelial cells to change their shape with a following increased extravasation of plasma, creating swelling. The peptides will affect hypersensitivity by altering the excitability of nearby neurons, creating increased pain-sensitivity.

The neuropeptides in focus in this study are substance P (SP) and calcitonin gene related peptide (CGRP), although other peptides such as Vasoactive Intestinal

Polypeptide (VIP), neurokinin A (NKA), NO and 5-HT are present during the neurogenic inflammation. Apart from the direct effects of inflammation mentioned above, these substances stimulate surrounding cells to release cytokines, chemokines and/or to change their normal appearance, thereby contributing to prolonged inflammation and structural changes of the peritoneum.

The TRPV1 receptor

The nociceptive transient receptor potential vanilloid (TRPV) receptors are a subgroup of the TRP receptors, known to play an important role in neurogenic inflammation. Activation of TRPV-receptors triggers a neurogenic inflammation, initiating interleukin release via neurotransmitters such as SP and CGRP (12). TRPV has many functions in the body; it plays a role in regulating body temperature, feeding and body weight as well as having beneficial effects on gastrointestinal and cardiovascular function (13, 14).

The TRPV1 receptor, predominantly expressed in sensory neurons, is the most studied receptor related to neurogenic inflammation. It is sensitive for capsaicin, a vanilloid found in hot peppers, and therefore often referred to as the “capsaicin receptor”. TRPV1-expressing nerves are found in the gastrointestinal wall, both in the muscles and surrounding the blood vessels (15). Stimulation of TRPV1 with capsaicin triggers release of SP and CGRP. Some lipids and substances such as extracellular protons have an allosteric effect on the TRPV1 channel, potentiating each other thereby increasing TRPV1 sensitivity to heat (16). Prolonged exposure to elevated levels of capsaicin hinders the effect of later distribution, presumably due to depletion of neurotransmitters (12). Antagonists to TRPV1 include capsazepine, iodo-resiniferatoxin (I-RTX) and BCTC (17).

Substance P

In neurogenic inflammation, SP is released from nerve fibres. The effect of SP can be mediated through activation of mast cells or as a direct effect of SP. SP will directly affect endothelial cells and blood vessels, inducing vasodilation and increased permeability for plasma (18). Via the neuro kinin (NK)-1 receptor, SP acts on multiple inflammatory cells such as monocytes, neutrophils, T-lymphocytes and mast cells, stimulating them to proliferate (T-lymphocytes) and to release cytokines (macrophages, mast cells) (19). SP stimulates the adhesion of leukocytes to endothelium thereby helping the recruitment to sites of inflammation. Via mast cells,

SP releases histamine, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and nerve growth factor (NGF)) and the growth factor VEGF, as shown *in vitro (12)*. *In vivo,* it is known that mast cells reside in proximity to SP-containing nerve fibres (20). Administration of SP (or agonists to SP) triggers neurogenic inflammation while pre-treatment with antagonists of SP cause a diminished inflammation (11). Inhibition of the NK-1 receptor has been shown to avert fibrosis after peritoneal surgery, suggesting that such treatment would be beneficial for the PD patients (21). Also, administration of an antagonist to SP has been shown to attenuate capsaicin-induced hyperalgesia, suggesting that SP is responsible for hyperalgesia in neurogenic inflammation (12). An effective SP antagonist is Spantide II. Spantide II is a NK antagonist with the dominating effect on NK-1 (22). Preadministration of Spantide II prevents upregulation of cytokines, thereby reverting SP-induced inflammation. It does not, however, interfere with the release of VIP or CGRP (23).

CGRP

Another neuropeptide released in neurogenic inflammation is calcitonin gene related peptide (CGRP). CGRP is among other things a vasodilator, considered the most potent vasodilator of the neuropeptides. Under both pathological and normal conditions CGRP is therefore believed to play an important role in the regulation of blood pressure and local blood flow in tissue organs (24). CGRP is found in the sensory nervous system, and is released after administration of capsaicin, bradykinin, prostaglandin E1 (PGE1) or whenever TRPV1 is activated. During inflammation, the release of CGRP from sensory neurons increases (25, 26). CGRP has been shown to affect mast cell degranulation and to potentiate SP, increasing vascular permeability (11, 27). As SP, CGRP (or agonists to CGRP) will produce symptoms of inflammation through release of inflammatory mediators. Also, high-dose treatment with agonists (capsaicin) can abolish the CGRP-effect due to depletion of transmitters in the neuron, which in the case of CGRP results in increasing development of hypertension, as shown in rats (24). CGRP receptor antagonists include $CGRP_{8-37}$, olcegepant and telcegepant (28).

The connection between the neurogenic inflammation and a sustained inflammatory response

When PD fluid is administrated into the peritoneum, neurogenic inflammation starts quite rapidly (7). The resulting release of neuropeptides, as stated above, is known to have a short, local effect. Even so, repeated distribution of dialysate fluid will cause several daily local inflammations. The discovery that SP via mast cells can induce the release of pro-inflammatory cytokines (12) provides a possible link between repeated neurogenic inflammations and chronic inflammation, characterized by fibrosis and angiogenesis leading to UFF in PD.

Cytokines that are relevant for peritoneal changes

VEGF

Vascular Endothelial Growth Factor (VEGF) is considered the most important factor contributing to angiogenesis and thereby UFF in PD. VEGF is a heparin-binding growth factor known to stimulate endothelial cell proliferation. It has the capacity to induce endothelium-dependent vasodilation thereby acting as a potent vascular permeabilizing agent (29). Mesothelial cells when exposed to pro-inflammatory substances such as IL-1β and TNF-α, in rats, express VEGF (4). VEGF is also expressed by activated mast cells along with several other factors including TGF-β, IL-8 and TNF-α (30). The expression of VEGF by other cells is increased in presence of TNF-α, TGF-β and IL-6, all these are cytokines that are present during inflammation. Another known stimuli for VEGF expression and subsequent angiogenesis is hypoxia. In summary, VEGF is released along with several other pro-inflammatory factors during inflammation, for example when peritoneum is exposed to high glucose solutions (31), or during peritonitis (32). It is known that inhibition of VEGF with anti-VEGF antibodies during exposure to GDPs and AGEs hinders peritoneal angiogenesis leading to normalized solute transport in PD (33). Recently it has been shown that in neurogenic inflammation, SP can induce the release of VEGF from mast cells (34).

IL-8 (CXCL1)

Discovered in 1987, interleukin 8 (IL-8 or CXCL1) was among the first found cytokines. IL-8 is a small (8-10 kDa), soluble, basic, heparin-binding protein, member of the CXC chemokine family. It is a strong chemo attractant for neutrophils and mast

cells. Expression is tightly regulated; therefore the level of IL-8 is normally undetectable or low in tissues (35). Although IL-8 acts predominantly on neutrophils, it has been shown that it has effects also on monocyte recruitment (adhesion) and smooth muscle proliferation (36, 37). Several chemokine-producing cells, such as mast cells, macrophages and neutrophils secrete IL-8 along with other substances, when appropriately stimulated (38). Also endothelial cells produce IL-8 when exposed pro-inflammatory cytokines (5). The receptors for IL-8, CXCR1 and CXCR2, are found on neutrophils, macrophages, lymphocytes and endothelial cells (35), allowing IL-8 to promote angiogenesis.

In animal studies, PD leads to recruitment of neutrophil granulocytes, probably due to release of IL-8. It has previously been shown that CXCL1 (also known as CINC-1), the rat equivalent to IL-8, is released during PD in rats (39), and that concentration of IL-8 was correlated to numbers of neutrophils. IL-8 is one of the first substances to be secreted during inflammation (27) and has a long list of effects in autoimmune and inflammatory diseases. Importantly in PD, IL-8 has been shown to promote angiogenesis (40). As for most pro-inflammatory substances, prolonged presence of IL-8 may result in tissue injury due to active neutrophils.

TNF-α

TNF-α belongs to the tumor necrosis factor (TNF) superfamily, signalling trough 29 known receptors and composed of at least 19 members, all of which are pro-inflammatory substances. The pro-inflammatory effect of TNF-α is mediated through activation of NF-KB, which regulates many cytokines, for example IL-8 and IL-6. TNF-α is capable of inducing cell death, and a key mediator of inflammation. Initially it was believed that TNF-α was produced primarily by macrophages but is has been clear that it is produced by many cell types such as endothelial cells, neurons and fibroblasts (41). Other cells expressing TNF-α are GDP-exposed mesothelial cells during PD (4) and activated mast cells (along with VEGF, TGF-β, and IL-8) (30, 42). Expression of TNF-α occurs in both a trans-membrane and a soluble form.

In peritoneal inflammation, activated macrophages release TNF-α along with other factors (IL-1, IL-6, IL-8 and MCP-1) (4). In the rat peritoneum, overexpression of TNF-α increases expression of TGF-β and VEGF thereby contributing to fibrosis and angiogenesis (43). Many members of the TNF-family are known to have both pro-

inflammatory as well as anti-inflammatory effects. As for TNF-α, it is known to both stimulate and inhibit angiogenesis. These findings have lead to the suggestion that the TNF-α effect is dose dependent. The angiogenic effect of TNF-α is mediated by VEGF, FGF and IL-8 (44). Neurons affected by TNF-α become hyper-excitable via an increase of membrane K^+ ion conductance (45). Among its effects TNF-α activates mesothelial- and endothelial cells, causing a production of IL-8 and enhanced expression of adhesion molecules for neutrophils on endothelial cells. In addition TNF-α itself is a potent chemoattractant for neutrophils.

Objectives

Earlier studies on animals have shown that peritoneal dialysis (PD) causes an acute release of neuropeptides to the abdominal cavity (7). Some of these peptides, such as SP, are known to induce a release of pro inflammatory cytokines thus providing a possible link between the short neurogenic reaction to PD-fluid and the chronic inflammation supported by cytokines. The hypothesis is that production of cytokines will decrease by pharmacological inhibition of the nociceptor TRPV1, thereby eliminating one mechanism behind the release of neuropeptides such as SP and CGRP. The aim is to see whether this affects the transcription of the pro inflammatory cytokines VEGF, TNF-α and IL-8 (CXCL1), factors known to affect fibrosis, angiogenesis and therefore UFF.

Material and Methods

The interactions between cells and signal substances in inflammation are complex. In order to evaluate inflammation during PD, dialysis was performed in vivo. The rat was the animal of choice since it is an established model animal in dialysis research. As this is a study of a research area not previously studied, the numbers of animals used were held to a minimum.

Animals

Male Sprague-Dawley rats weighing between 300 - 360 grams were used in the experiments. The rats were kept 5 by 5 in cages with free access to standard food (pellets) and water. The rats followed a 12h day/night cycle.

Ethics

When conducting experiments on animals it is always necessary to evaluate the possible benefits for future patients compared to the harm caused. If the hypothesis of this study should be proven it could have potential benefits for ESRD patients. Göteborg ethical committee approved the study protocol and the NIH Guide for the Care and Use of Laboratory Animals was adhered to.

Surgical procedures and anaesthesia

A 7 French silicone catheter (Renasil® SILO8O; Braintree Scientific Inc., Braintree. MA, USA) was implanted under sterile conditions and general anaesthesia one week before the experiment. To access the peritoneal cavity, an incision was made through the abdominal skin, and a 3 mm hole was made through linea alba. After being inserted 2,5 cm, the catheter was sutured to the superficial abdominal muscle fascia. The rest of the catheter was tunnelled subcutaneously to the neck where it was mobilised through the skin. After injecting 5 ml of saline, a stainless clip was used to close the catheter. The wounds were closed with agraffes. No antibiotics were administrated. Throughout the surgery, the animals were subject to general anaesthesia via inhalation of Isofluran Baxter (Baxter Medical AB, Kista, Sweden) in room air.

Experimental protocol

This study used a total of 15 rats. 3 different treatments were performed and compared with untreated rats (control). Two groups (n=9) were subjected to single 4-hour dwells of PD-fluid by infusion via the previously implanted catheter. The fluid of use was lactate buffered filter sterilized (Nalgene® 0,2 UM SFCA 150ml Nalgene NUNC International, New York, USA), with 2.5% glucose as osmotic agent. A total of 20 ml of fluid was added trough the catheter, the animals were then allowed to wake up. After four hours the animals were anesthetized and the central part of the diaphragm, consisting of connective tissue, was excised and immediately immersed in RNA later® buffer. The animal was then humanely killed by cutting of the thorax and heart, during anaesthesia.

Group name	Treatment	n
BCTC	Implanted PD catheter and PD-treatment following i.v. administration of BCTC (TRPV1 antagonist)	4
PD	Implanted PD catheter and PD-treatment	5

Control	Untreated	4
Catheter	Implanted PD catheter	2

Measurement of cytokines

The biopsies were homogenized mechanically, releasing intracellular substances. The homogenate was filtered separating DNA and RNA from other substances. The filter was rinsed to remove all non-DNA/RNA particles. DNAse was added to remove DNA. The filter was then rinsed with sterilized water to release the RNA from the filter. The obtained RNA was analysed with spectrophotometer to verify that the samples contained ample amount of RNA. qPCR was performed to ensure there was no DNA present in the samples. The RNA samples were then converted to cDNA via reverse transcriptase PCR. The obtained cDNA were analysed with relative qPCR. A standard curve per gene was made using qPCR on diluted series of the samples, thus measuring the efficacy of the qPCR, allowing comparison between different qPCR-sessions. To make sure the expression of genes of interest was comparative between samples, the expression of the reference-gene RPLP0 was measured and used as reference (housekeeping gene).

Statistics

To evaluate differences between samples, the student's t-test was used with a chosen level of significance at $p \leq 5$ %. Data presented as mean +/- SEM.

Results

All catheters were patent. Two rats were excluded from the study due to bad expression of the reference gene RPLP0, one from the Control group and one from the BCTC group.

The standard curves obtained after qPCR were of poor quality. Instead, to evaluate the data, the number of cycles required to reach the threshold level of DNA concentration (Ct–values) were used to calculate the amplification of each cytokine. The calculated amplifications of each cytokine were linearized ($0{,}5^{Ct}$) and normalized in relation to the reference gene RPLP0 in order to allow comparison between data from different samples.

No significant differences were seen regarding measured cytokines between the Control-, Catheter-, BCTC- or PD-group, however some trends were obvious. Average expression was highest in the PD group and lowest in the BCTC group regarding all cytokines (fig 1.1, 1.2, 1.3). The expression of cytokines in the Catheter group correlated to the Control group, except regarding VEGF where data showed a wider spread in the Catheter group.

Discussion

No significant differences could be detected in this material. This could have several reasons. There were some technical problems regarding the quality of the standard curves, some of them showing insufficient correlation. This could possibly be due to suboptimal primers or polluted samples. The reference gene of choice, RPLP0, showed bad expression in two rats that had to be excluded from the study. Since a small number of animals were included, the statistical power was small. This model used a pre-implanted catheter for administration of PD fluid. Such treatment is known to induce inflammation and it is therefore possible that the catheter in this study affected the peritoneal response to PD treatment (46). However, differences between the Control group and the Catheter group in this study were small, making it less likely that the implanted catheters helped trigger release of CXCL1, TNF-α and VEGF. This experimental model mimics the reality for humans with PD treatment and cytokine release due to method would therefore possibly mimic hypothetical tests on humans.

There were several interesting trends in the data. Regarding all cytokines the PD group showed the highest average expression while the BCTC group showed the lowest average expression. Regarding CXCL1 this suggests that TRPV1-antagonistic treatment decreased expression of CXCL1. Results from the PD group indicate that dialysis triggered synthesis of CXCL1, as seen in previous study (39). It is known that CXCL1 is co-expressed along with other pro inflammatory cytokines in response to various stimuli (38), so the expression of CXCL1 seen in the PD group might depend on release of other substances such as TNF-α (4). Regarding VEGF the data suggests that adding a TRPV1-antagonist decreases VEGF expression, presumably through the inhibition of neuropeptides SP and CGRP. Such results would be in line with recent data found in another study (34), and suggest that neurogenic inflammation, in

particular SP, could release VEGF. However, differences between BCTC- and PD-group was not significant (p=0,15) and the VEGF release in this study may have additional sources or trigger mechanisms. One possible reason to decreased expression of VEGF could be due to decreased release of TNF-α, which normally increases expression of TGF-beta and VEGF (30, 43). Regarding TNF-α the results suggests that the TRPV1 receptor, at least in part, could be responsible for the release of TNF-α. Since TNF-α is released together with CXCL1 by macrophages one could expect a similar pattern for the both substances (31). This was not the case in this study, presumably because also other cells release CXCL1.

It is known that the very presence of dialysate fluid itself, through mechanisms as distension and hyperosmolarity, causes release of pro inflammatory substances. As could be expected the PD group showed higher average expression rates than the Control group. Although not significant, the BCTC group showed the lowest average expression rates regarding all measured cytokines. This means that the i.v. treatment with TRPV1 antagonist (BCTC) inhibited the cytokine release induced by PD. Notably the average expression of inflammatory cytokines in the BCTC group was lower than in the untreated Control group. It is well established that the TRPV1 receptor causes a release of SP and CGRP. In an earlier study, inhibition of TRPV1 reduced osmotic ultrafiltration and reabsorption, but the study could not connect these findings with the release of SP and CGRP (7). The present study points in the direction that TRPV1-antagonistic treatment (BCTC) may have decreased the expression of measured inflammatory cytokines and therefore reduced inflammation.

Implications and Conclusions

Any improvement of PD leading to prolonged technique survival would be beneficial for patients with ESRD, especially in developing countries where the availability of HD is limited. Since one of the dominating causes of poor technique survival is fibrosis due to inflammation, treatment that reduces inflammation would have important clinical implications. This study could not prove decreased inflammation why the clinical implications become limited. However, the trends seen in this material deserves a closer look in a future study. Such a study would preferably use a larger number of animals in order to improve the statistical power of the results. It would also be interesting to redesign the primers used in PCR.

To the writers knowledge there are no other studies regarding cytokine release due to neurogenic inflammation in PD. It is well established that during PD, there is a release of several cytokines, including TNF-α, CXCL1 and VEGF. It is also likely that these substances are produced locally (47). However, the release mechanisms remain unknown.

To summarize there were no significant differences in the present data, although some interesting trends could be seen, pointing in the direction that inhibition of TRPV1 decreases cytokine release and thereby inflammation induced by PD.

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Figures

Figure 1.1

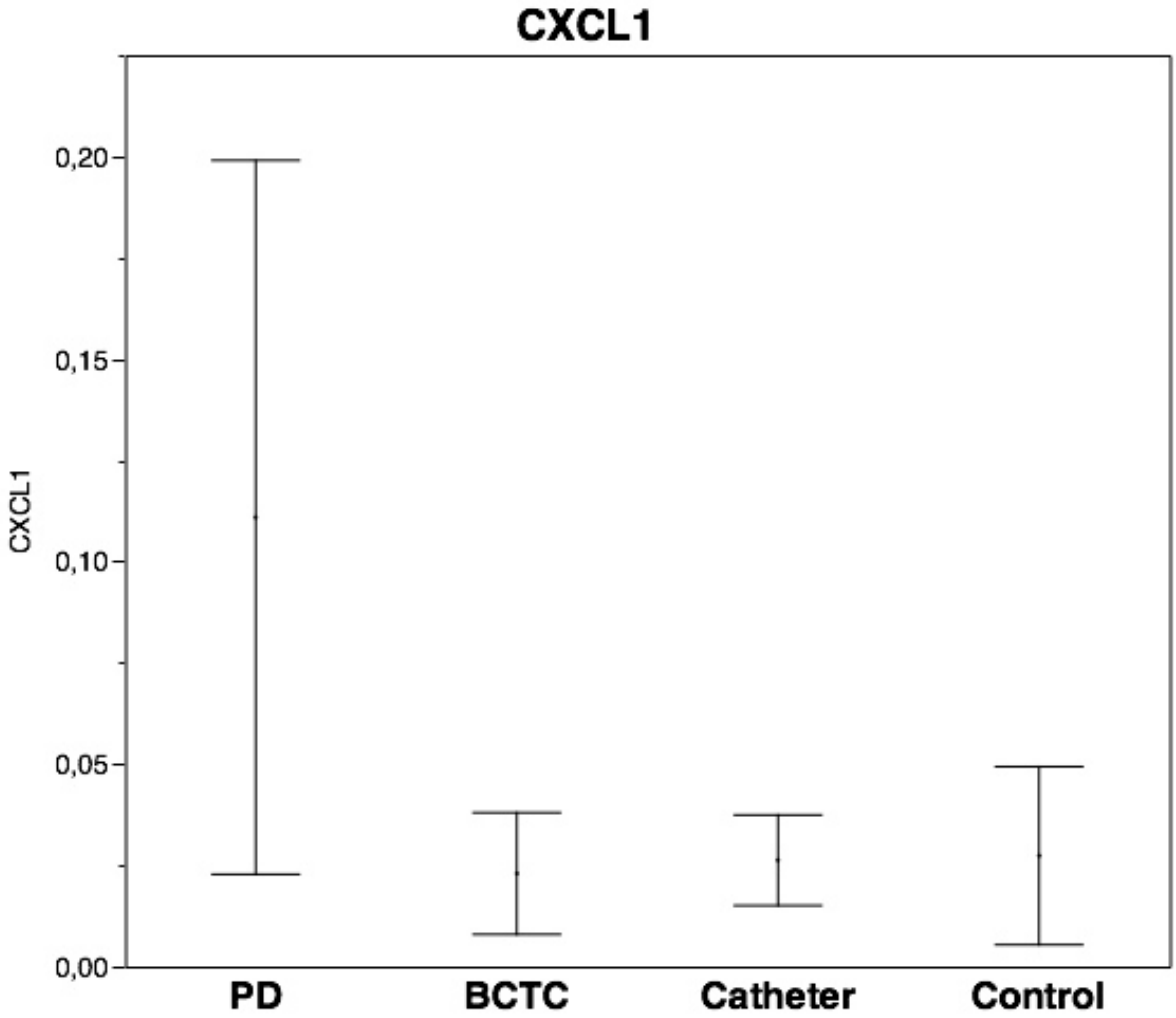

fig 1.1

Cycles to threshold (Ct) values were linearized ($0{,}5^{Ct}$).
Graph shows $0{,}5^{Ct}$ for CXCL-1 divided with $0{,}5^{Ct}$ for reference gene RPLP0. Values presented as mean +/- 1 standard error of the mean.

All treatment groups align except the PD group, which shows a wider scatter with a higher mean value. The mean of the BCTC group is slightly lower than in other groups.

BCTC = Implanted catheter, peritoneal dialysis and i.v. treatment with TRPV1-antagonist. Catheter = Implanted catheter, no peritoneal dialysis. Control = no treatment. PD = Implanted catheter, peritoneal dialysis.

Figure 1.2

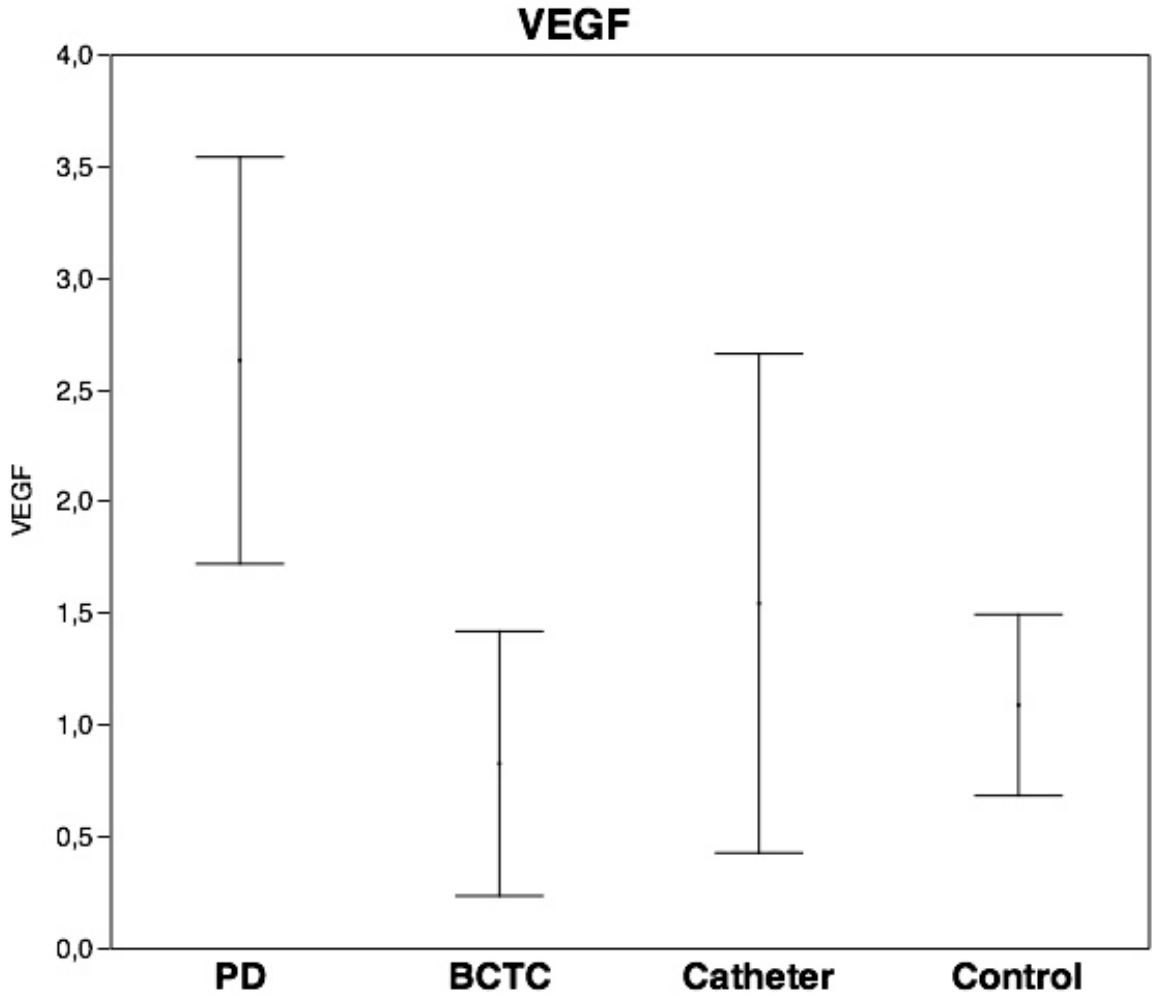

fig 1.2

Cycles to threshold (Ct) values were linearized ($0{,}5^{Ct}$).
Graph shows $0{,}5^{Ct}$ for VEGF divided with $0{,}5^{Ct}$ for reference gene RPLP0. Values presented as mean +/- 1 standard error of the mean.

All groups except the PD group aligns. The Catheter group shows a wide scatter. The BCTC group shows a lower mean value than other treatments.

BCTC = Implanted catheter, peritoneal dialysis and i.v. treatment with TRPV1-antagonist. Catheter = Implanted catheter, no peritoneal dialysis. Control = no treatment. PD = Implanted catheter, peritoneal dialysis.

Figure 1.3

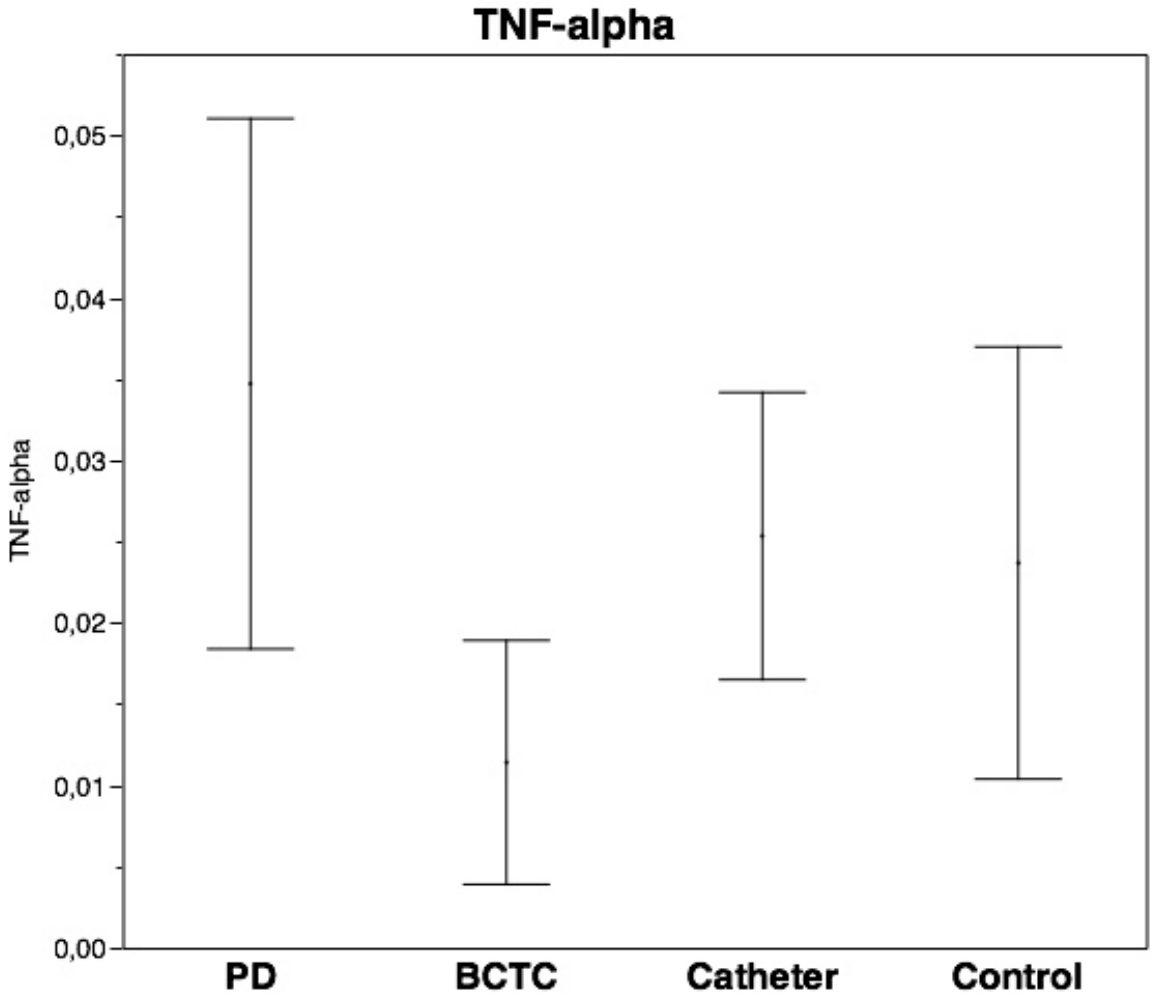

fig 1.3

Cycles to threshold (Ct) values were linearized ($0{,}5^{Ct}$).
Graph shows $0{,}5^{Ct}$ for TNF-α divided with $0{,}5^{Ct}$ for reference gene RPLP0. Values presented as mean +/- 1 standard error of the mean.

The BCTC group shows the lowest mean value. Both the Catheter- and the Control group shows higher mean values and the PD group shows the highest mean value.

BCTC = Implanted catheter, peritoneal dialysis and i.v. treatment with TRPV1-antagonist. Catheter = Implanted catheter, no peritoneal dialysis. Control = no treatment. PD = Implanted catheter, peritoneal dialysis.

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