Experimental and clinical studies on peritoneal physiology and morphology during chronic peritoneal dialysis
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Substrate and inhibitor for nitric oxide synthase during peritoneal dialysis in rabbits

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Abstract
The objective of this study was to investigate the possible influence of nitric oxide (NO) on peritoneal transport during non infected peritoneal dialysis.

A chronic peritoneal dialysis model in New Zealand White rabbits (2624 g, range 2251-3034 g) was used. In 13 rabbits 250 mg/L L-arginine, a substrate for NO synthesis, was added to 3.86% glucose dialysate. L-NMMA 25 mg/L, an inhibitor of NO synthase, was added to the dialysate in 10 rabbits. Standard peritoneal permeability analyses in rabbits (SPAR) were performed to analyze the effects of these interventions on solute and fluid transport during 1-hour dwells. The addition of 4.5 mg/L nitroprusside to the dialysate in 5 separate experiments was used for validation of this model.

For the transport of urea and creatinine mass transfer area coefficients (MTAC) were calculated. Furthermore, the glucose absorption, the peritoneal albumine clearance, peritoneal fluid kinetics and the dialysate/plasma (D/P) ratio of nitrate were calculated.

Nitroprusside caused an 86% (48%-233%) increase in albumin clearance, which is similar to the nitroprusside induced increase found in humans. Contrary to human studies, no effect was found on the clearances of urea and creatinine, or on peritoneal fluid kinetics. This suggests a lower sensitivity of the rabbit peritoneal membrane for the effect of NO on small solute transport. L-arginine affected neither the MTACs of urea and creatinine, nor the absorption of glucose. Also peritoneal fluid kinetics were similar. Peritoneal albumin clearance increased 18% (-24% to 609%). This resembles the NO mediated effects of nitroprusside. Administration of L-NMMA caused no change in the transport rate of small solutes, albumin clearance or fluid profile. This suggests that NO synthase is not induced during non infected peritoneal dialysis, which is in accordance with previous studies.

This rabbit dialysis model can be used for analyzing the effects of interventions on peritoneal permeability characteristics, although the rabbit peritoneal membrane is probably less sensitive to NO compared to humans. L-arginine induced effects are similar to those of nitroprusside, which suggests that these effects are possibly mediated by NO. Because L-NMMA did not affect peritoneal transport, it is unlikely that NO is involved in the regulation of peritoneal permeability during stable CAPD.

Introduction
Transport across the peritoneal membrane during peritoneal dialysis can be influenced by many endogenous and exogenous factors, like cytokines, prostaglandins and pharmacological interventions [1-3]. One of these substances is the vasoactive nitric oxide. Intra-peritoneal administration of the nitric oxide donor nitroprusside increases peritoneal solute transport [4-8]. In the acute phase of peritonitis, a situation with increased peritoneal surface area and increased intrinsic permeability [9-12], high dialysate/plasma (D/P) ratios of nitrite and nitrate have been found, sometimes even exceeding 1.0 [13-15]. This suggests local generation of nitric oxide during peritonitis. The role of nitric oxide during uninfected peritoneal dialysis is unclear. Davenport et al. reported D/P ratios of nitrite and nitrate above 1.0 during stable CAPD [15]. We previously found that, when no infection was present, the nitrate concentrations in dialysate were in accordance
with diffusive transport, without evidence for local generation [8,13,16].

In the present study the possible influence of nitric oxide on peritoneal transport during non infected peritoneal dialysis was investigated in a chronic dialysis model in rabbits [17]. Therefore, L-arginine, a substrate for nitric oxide synthesis [18,19], or L-NMMA, an inhibitor of nitric oxide synthase [20], was added to 3.86% glucose dialysate during 1-hour dwells. Standard peritoneal permeability analyses in rabbits were used to analyze the effects of these interventions on solute and fluid transport. Furthermore, nitrite and nitrate were measured in plasma and dialysate, because nitrite and nitrate are formed by oxidation of nitric oxide [21]. Nitroprusside was added to the dialysate in separate experiments for validation of this peritoneal dialysis model.

**Materials and methods**

**Study protocol**

Peritoneal dialysis was performed in unanesthetized female New Zealand White rabbits with a median body weight on the study days of 2624 g (2251 to 3034 g). A coiled silastic catheter (Coil-Cath™, Accurate Surgical Instruments Corporation, Toronto, Canada) was implanted. Following this procedure, the catheter was flushed everyday with 2.5 ml heparin (5 IU/ml in 0.9% NaCl). After seven days, peritoneal dialysis was performed once daily with 1.36% glucose dialysate (Dianeal®, Baxter BV, Utrecht, The Netherlands). A rapid exchange with 40 mL/kg bodyweight was followed by instillation of the same volume minus the residual volume of the exchange. One week after the start of peritoneal dialysis, standard peritoneal permeability analyses in rabbits (SPAR) were performed with 3.86% glucose dialysate [17]. The SPAR is a modification of the standard peritoneal permeability analysis (SPA) in humans [22], adapted for 1-hour dwells in rabbits. Dextran 70 (Macrodex®, NPBI, Emmer-Compascuum, The Netherlands or Hyskon®, Medisan Pharmaceuticals AB, Uppsala, Sweden) 1 g/L was added for the measurement of peritoneal fluid kinetics. In 13 rabbits a control SPAR without intervention was performed and a SPAR with the addition of 250 mg/L L-arginine to the dialysate fluid (L-arginine HCl, 40 mg/mL, hospital pharmacy). A control SPAR and a SPAR after the addition of 25 mg/L L-NMMA was done in 10 rabbits. L-NMMA acetate salt (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) was dissolved in 3.86% glucose dialysate in the hospital pharmacy under aseptic conditions. Nine rabbits participated in both intervention studies. On the intervention days, L-arginine or L-NMMA was added both to the first rinsing bag and the test bag. The interval between two SPARs ranged between one and five days. Two times the intervention SPAR was performed first and the other times the control SPAR was done first. In five other rabbits SPARs were performed after the addition of 4.5 mg/L nitroprusside and these were compared with control SPARs in the same rabbits. The protocol was approved by the Committee of Animal Ethics of the University Hospital of Amsterdam.

**Laboratory methods**

Urea was determined with an enzymatic method on an automated analyzer (Hitachi 747, Boehringer Mannheim, Mannheim, Germany). Creatinine was measured by an enzymatic PAP* method on another automated analyzer (Hitachi 911, Boehringer Mannheim, Mannheim, Germany). Albumin was determined by the bromocresol
Green (BCG) method and glucose by the glucose oxidase-peroxidase method (SMA-II, Technicon, Terrytown, USA). Determination of creatinine, also using enzymatic methods, is influenced by high glucose concentrations \([G]\) \([23,24]\). Therefore, the measured creatinine concentrations were corrected with a correction factor (CF) that was calculated for the method used in our laboratory:

\[
CF = -3.10 \cdot 10^{-4} [G]^2 + 0.11 [G] + 104.7
\]

Total dextran 70 was measured by means of high-performance liquid chromatography \([25]\). Nitrite and nitrate were determined by ion-pair high performance liquid chromatography (HPLC), with a lower detection limit of 2.5 \(\mu\text{mol/L}\).

**Calculations**

For the transport of urea and creatinine mass transfer area coefficients (MTAC) were calculated according to the model of Wanieński et al. \([26]\), adapted for 1-hour dwells in this rabbit model. The MTAC calculation according to Wanieński, corrects for the ratio between diffusion and convection (F=0.5). The solute concentration in plasma was expressed per volume of plasma water \([27]\). The MTACs were calculated according to the equation:

\[
MTAC(\text{mL/min}) = \frac{V_m}{t} \ln \frac{V_0^{0.5}(P \cdot D_{10})}{V_t^{0.5}(P \cdot D_t)}
\]

in which \(P\) is the plasma concentration of the solute. \(V_0\) represents the intraperitoneal volume and \(D_{10}\) the dialysate concentration at \(t=10\), whereas \(V_t\) and \(D_t\) are these parameters at the end of the dwell. \(V_m\) is the mean intraperitoneal volume. The mean intraperitoneal volume is calculated as the area under the intraperitoneal volume versus time curve, divided by the dwell time. This area under the curve is calculated by the trapezium rule \([28]\). Using this method, the area under the curve between two dialysate samples is calculated as the mean of the intraperitoneal volume at the two sample times, multiplied by the time interval between the samples.

The MTACs of urea and creatinine are expressed per 1.73 \(\text{m}^2\) body surface area. Therefore, the MTACs were divided by 0.23, the mean body surface area of rabbits \([29]\), and multiplied by 1.73, the mean body surface area of humans. The glucose absorption was estimated as the difference between the instilled and the recovered amount of glucose, relative to the amount of glucose instilled. Nitrite concentrations in plasma and dialysate were below the lower detection limit. Dialysate/plasma (D/P) ratios were calculated for nitrate, with a correction for plasma water. Because in some rabbits the nitrate concentrations in dialysate exceeded the plasma concentrations, it was not possible to calculate MTACs. Albumin transport was expressed as its peritoneal clearance. Peritoneal fluid transport was calculated as described previously in humans \([30]\), modified for 1-hour dwells \([17]\).

Results are given as median values and ranges because not all data were symmetrically distributed. Wilcoxon matched pairs rank sum test was used for distribution free testing.
Results

Nitroprusside

Figure 1 depicts the MTACs of urea and creatinine, the glucose absorption and the peritoneal albumin clearance in five rabbits during control SPARs and after the addition of nitroprusside. No effect was found on the transport of small solutes. However, the albumin clearance increased 86% (48% to 233%) with nitroprusside. Peritoneal fluid kinetics were not influenced by nitroprusside: the effective lymphatic absorption rate was 0.52 mL/min, range 0.37 to 0.68 mL/min in the control experiments and 0.42 mL/min, range 0.23 to 0.54 mL/min during nitroprusside; transcapillary ultrafiltration rate was 0.73 mL/min, range 0.41 to 0.91 mL/min (control) and 0.71 mL/min, range 0.50 to 0.87 mL/min (nitroprusside). The net ultrafiltration rate, the difference between these, was similar during the control dwells (0.21 mL/min, range -0.11 to 0.54 mL/min) and the nitroprusside experiments (0.30 mL/min, range 0.1 to 0.41 mL/min). The D/P ratio of nitrate was 0.59 (range 0.29 to 0.99) during the control dwells and 0.54 (range 0.32 to 1.04) with nitroprusside.

![Figure 1. MTACs of urea and creatinine, glucose absorption and the clearance of albumin with 3.86% glucose dialysate and after the addition of nitroprusside. The transport rate of small solutes was not influenced by nitroprusside. The albumin clearance increased in all five rabbits.](image)

L-arginine and L-NMMA

The addition of L-arginine affected neither the MTACs of urea and creatinine nor the absorption of glucose (Table 1). The peritoneal albumin clearance increased 18% (range -24% to 609%). Figure 2 shows the fluid profiles during the 1-hour dwells. The time course of the fluid profiles did not change after the addition of L-arginine. No differences were found in the D/P ratios of nitrate between the control dwells (0.84, range 0.31 to 1.11) and the experiments with L-arginine (0.74, range 0.44 to 1.12). L-NMMA did not affect the transport of small solutes (Table 1). Furthermore, no effect on the albumin clearance was found. Peritoneal fluid kinetics were also not influenced by the addition of L-NMMA, as shown in Figure 2. The D/P ratio of nitrate was 0.84 (range 0.31 to 1.11) in the control experiments and 0.74 (range 0.41 to 0.90) during the L-NMMA dwells.
Table 1. Effect of L-arginine and L-NMMA on solute transport

<table>
<thead>
<tr>
<th></th>
<th>L-arginine</th>
<th>L-NMMA</th>
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<tbody>
<tr>
<td>MTAC urea</td>
<td>18.1 (9.9-28.9)</td>
<td>21.5 (15.6-24.9)</td>
</tr>
<tr>
<td>MTAC creatinine</td>
<td>12.9 (8.9-20.5)</td>
<td>16.1 (7.4-21.6)</td>
</tr>
<tr>
<td>glucose absorption</td>
<td>60 (37-66)</td>
<td>60 (53-67)</td>
</tr>
<tr>
<td>clearance albumin</td>
<td>55 (13-156)</td>
<td>52 (13-130)</td>
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</table>

MTACs of urea and creatinine (mL/min/1.73 m²), glucose absorption (%) and peritoneal albumin clearances (µL/min) during 1-hour control dwells with 3.86% glucose dialysate and after the addition of L-arginine or L-NMMA. Values are given as medians with ranges.

Discussion

A chronic peritoneal dialysis model was applied in the present study to examine the effects of a substrate for nitric oxide synthesis and an inhibitor of nitric oxide synthase on peritoneal permeability. After the addition of L-arginine no effect was found on the transport of small solutes and fluid kinetics, but the peritoneal albumin clearance increased 18%. This was not accompanied by changes in the D/P ratio of nitrate. L-NMMA influenced neither the transport of small solutes nor the albumin clearance or the fluid profile. Administration of nitroprusside caused an increase in the transport of albumin.

The effect of intraperitoneal nitroprusside in CAPD patients is most pronounced on the large pore system [8]. The effect of nitroprusside on the peritoneal membrane of rabbits was similar: the increase in albumin clearance was 86% in rabbits and 70% in humans [8]. Although increases in small solute transport of 13% to 43% were found with nitroprusside in humans, no change in the MTACs of urea and creatinine was observed in rabbits. Using kinetic modeling, the nitroprusside induced increase in the transport of low molecular weight solutes appeared to be due to both an enlargement of the vascular surface area and to a lesser extent a larger small pore radius [8]. The effect on the surface area also caused higher transcapillary ultrafiltration and net ultrafiltration rates during the initial phase of the dwell. The absence of an effect of nitroprusside in rabbits on low molecular weight solute transport and fluid kinetics suggests that the small pore system in rabbits is less sensitive to nitric oxide than in humans. However, the effect on the large pore radius was not different in the two species, as judged from the albumin clearances. Some transport studies with nitroprusside in rabbits have been published previously [31-34]. The nitroprusside dosages used in these studies were about three times higher than the concentration of 4.5 mg/L, that has been shown to be effective in humans [35]. With these high dosages, an increase in the transport rate of small solutes was reported, but the effects on clearances of high molecular weight solutes were not studied. Using lower nitroprusside dosages, similar to the dosages of 4.5 mg/L used in our study, no significant effects were found on the clearances of creatinine and urea [31]. This has been confirmed by Schneider et al. [34], who administered 3-6 mg/L nitroprusside intraperitoneal in rabbits. They found no effect on the clearances of creatinine and inulin. Moreover, also the clearance of total protein was not influenced by nitroprusside in that study. This lower sensitivity of the rabbit peritoneal membrane for the effect of nitric oxide on the transport of low molecular weight solutes should be taken into account, for analyzing the effects of interventions.
L-arginine is a substrate for nitric oxide production [18,19]. Various cell types in the peritoneal cavity might be capable to produce nitric oxide, like mesothelial cells, fibroblast and possibly macrophages [36,37]. Peritoneal macrophages of mice were able to produce nitric oxide [38-40]. Cultured human peritoneal macrophages produced only low levels of nitric oxide, despite stimulation with several different combinations of cytokines, growth factors and microbial stimulants [41]. To our knowledge, it is unknown whether macrophages obtained from rabbits are capable to produce nitric oxide.

Previously, we have attempted to stimulate nitric oxide synthesis in CAPD patients [16]. Therefore, amino acid based dialysate (Nutrineal, Baxter), containing L-arginine, was used to increase the substrate for nitric oxide production. Amino acid based dialysis solution affected the peritoneal permeability characteristics, but these effects were different from the effects found with nitroprusside [42]. This implies that the amino acid induced differences were probably not mediated by nitric oxide. In the present study, the used L-arginine concentrations were about six times greater compared to the L-arginine concentration in the amino acid based dialysate. The volume increase due to the addition of L-arginine to the dialysis fluid was about 0.6 ml per 100 mL dialysate. This led to a decrease in osmolarity of the dialysis solution from 486 mosmol/L to 485.8 mosmol/L. Therefore, it is unlikely that this addition will have influenced the results. The effect of the addition of L-arginine to the dialysate was most pronounced on the albumin clearance. This suggests that L-arginine modifies especially the large pores in the peritoneal membrane. This resembles the nitric oxide mediated effects of nitroprusside. When L-arginine stimulates nitric oxide production, nitric oxide will be oxidated to nitrite and nitrate within a few seconds [21]. If this occurs in the peritoneal cavity, increased D/P ratios of nitrite and nitrate should be expected. However, nitrite was not detectable in plasma and dialysate and the D/P ratios of nitrate were not influenced by the addition of L-arginine. This is similar to the situation in humans, where also no effect on nitrite and nitrate was found after the addition of nitroprusside. Possibly, the amount of formed nitrite and nitrate is too small to detect with the methods used.

Production of nitric oxide by nitric oxide synthase can be inhibited by L-arginine analogues such as N\(^\text{G}\)G-monomethyl-L-arginine (L-NMMA) and N\(^\text{G}\)G-nitro-L-arginine methyl-ester (L-NAME) [20]. Three isoforms of nitric oxide synthase have been described, two constitutive and one inducible isofrom [43]. Expression of the inducible nitric oxide synthase can be induced in macrophages and many other cells with bacterial lipopolysaccharide and cytokines. In the present study the effect of L-NMMA was studied, because L-NMMA is able to inhibit both constitutive and inducible nitric oxide syntheses, while L-NAME is a relatively poor inhibitor of inducible nitric oxide synthase [44]. The use of dialysate with dissolved L-NMMA did not affect the peritoneal permeability in the present study. Two explanations are possible for the absence of an effect of L-NMMA. First, the used dosage was too low. However, the same dosage of L-NMMA (about 1.0 mg/kg) intravenously administered in patients with septic shock caused a dosage dependent increase in blood pressure [45]. It could therefore be expected that this same dosage applicated locally might inhibit nitric oxide synthesis in the peritoneal cavity, although the sensitivity of the rabbit peritoneal membrane to L-NMMA is unknown. The second possibility would be that nitric oxide synthase is not induced during non infected peritoneal dialysis. This possibility is in accordance with our previous results.
Figure 2. The time course of the transcapillary ultrafiltration (■), lymphatic absorption (▼) and the resulting change in intraperitoneal volume (▲) during 1-hour dwells with 3.86% glucose dialysate. Paired control experiments for L-arginine (upper left); after the addition of L-arginine (upper right); paired control experiments for L-NMMA (lower left); after the addition of L-NMMA (lower right). With both interventions no significant differences in fluid kinetics were found.

[8,13,16]. It can be concluded that the peritoneal dialysis model in rabbits can be used for analyzing the effects of interventions on peritoneal transport. However, for nitric oxide mediated intervention studies it should be kept in mind that the rabbit peritoneal membrane is probably less sensitive to nitric oxide compared to the peritoneal membrane of humans. Addition of high dosages L-arginine to the dialysate resulted in effects, similar to those of nitroprusside. This suggests that the L-arginine induced effects are possibly mediated by nitric oxide. L-NMMA did not affect peritoneal transport. Therefore, it is unlikely that nitric oxide is involved in the regulation of peritoneal permeability during stable CAPD.

References


