Experimental and clinical studies on peritoneal physiology and morphology during chronic peritoneal dialysis
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Structural and functional changes of the peritoneum in a chronic peritoneal infusion model in the rat

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submitted
Abstract
Loss of ultrafiltration, high mass transfer area coefficients of low molecular weight solutes and also morphological alterations of the peritoneal membrane are associated with long-term peritoneal dialysis. A chronic peritoneal infusion model was developed in the rat to investigate the structural changes in the peritoneal membrane in relation to the changes in peritoneal permeability after chronic exposure to a glucose based dialysis solution.

A vascular access port (Rat-o-Port) with an attached peritoneal catheter was implanted subcutaneously in the neck of male wistar rats. Three groups were investigated: group 1 (n=8), the experimental group, was daily infused with 60 mL/kg body weight (BW) 3.86% glucose containing dialysis solution via the Rat-o-Port; group 2 (n=6) was a control group to investigate the effect of daily infusion with an isotonic non-glucose solution (60 mL/kg BW Ringer's lactate) on peritoneal tissues; group 3 (n=4) was the untreated control group to study the effect of aging on the peritoneum. At time t= 12, 16 and 20 weeks after the start of the infusion, rats of group 1 and 2 were sacrificed. Control group 3 was studied at t = 0 and t=20 weeks after inclusion. All rats underwent a 4-hour standardized peritoneal permeability analysis with 3.86% glucose dialysis solution before sacrifice. Parietal peritoneum and omentum were investigated for the presence and type of fibrous tissue using histochemical stains (pico sirius red: total fibrillary collagen) and immuno histochemical stains (anti-type IV collagen and anti-α-smooth muscle actin immuno-reactivity). Vascularisation was visualized with von Willebrand factor staining in all rats.

In group 1 the vessel density increased with time of exposure to glucose based dialysate, 16-20 weeks: 32.3±5.6 vessels/field (v/f) which was significantly different from the vessel density in both control groups: group 2 (16-20 weeks: 9.1±2.9, p<0.0006) and group 3 (at the start and after 20 weeks: 7.9±2.6, p<0.0001). Moreover, the number of vessels per field increased with the severity of fibrosis: normal fibrous tissue: 6.8±1.6 v/f compared to mild fibrosis: 12.4±0.57 v/f; moderate fibrosis: 32.1±1.6 v/f and severe fibrosis: 32.5±7.8 v/f. Electron microscopy revealed profound lamination of the basal membranes of omental capillaries at 20 weeks in group 1, which was not seen at 20 weeks in group 2 and 3. The glucose absorption was significantly higher in group 1 after 16 and 20 weeks: 66.2±10.8% versus 51.3±3.1% in group 2 after the same period (p<0.014). The time course of the net ultrafiltration at 16-20 weeks was significantly lower in group 1 than in group 2 (p<0.043). The sieving of sodium was significantly less in group 1 than in group 2 (p<0.04).

It can be concluded that the rat model can be used to investigate the effects of chronic exposure to dialysis fluids on structural and functional parameters of the peritoneal membrane. When 3.86% glucose dialysis solution was used the morphological alterations and the changes in the peritoneal permeability show striking resemblance with those observed in long-term PD patients. These findings suggest a major pathophysiological role for glucose (or its degradation products) in the development of the peritoneal alterations in long-term PD, with fibrosis and neoangiogenesis as main features.
Introduction
Ultrafiltration failure is the main functional reason for discontinuation of treatment in long-term peritoneal dialysis [1-5]. Loss of ultrafiltration has often been reported in association with increased mass transfer area coefficients (MTACs) or high dialysate/plasma (D/P) ratios of low molecular weight solutes, suggesting the presence of a large peritoneal vascular surface area [6-10]. Prospective longitudinal studies have shown that this combination of ultrafiltration loss with high MTACs or D/P ratios of low molecular weight solutes develops during long-term peritoneal dialysis [2,4]. Morphological studies in peritoneal dialysis patients have mainly focussed on the loss of mesothelial cells in long-term dialysis treatment [11,12], the development of interstitial fibrosis and on duplication of capillary basement membranes [13-15]. Only recently more attention has been paid to the peritoneal microvasculature. Honda et al. [16] described severe fibrosis and hyalinization of the media of peritoneal venules and deposition of type IV collagen in peritoneal vascular walls in three patients with ultrafiltration failure. These morphological alterations were not detected in a control PD patient without this complication. Recently, we performed a retrospective analysis of the vascularization and fibrous tissue deposition in the peritoneum of peritoneal dialysis patients with the use of morphometric and immunohistochemical methods [17]. We found increased microvascular density, deposition of collagen IV and myofibroblasts in the peritoneal extracellular matrix with time on peritoneal dialysis. Therefore, we hypothesized that high D/P ratios or MTACs of low molecular weight solutes found in long-term peritoneal dialysis could be explained by the increased number and/or dilation of microvessels in the peritoneum of these patients. However, simultaneous analysis of functional and morphological measurements is not possible in human subjects, nor can the time course of the development of the morphological alterations be studied in patients, as serial peritoneal biopsies for investigational purposes are not feasible. Furthermore, some of the morphological alterations in chronic peritoneal dialysis patients may be influenced or caused by episodes of infectious peritonitis [15].

The aim of the present study was to develop a chronic peritoneal infusion model in rats, in which the development of structural alterations can be investigated prospectively, without interference of peritonitis, and in which these alterations can be related to changes in peritoneal transport characteristics, using a standardized methodology.

Methods
Animals
Eighteen male wistar rats (Harlan CBP, Zeist, The Netherlands) with a mean body weight of 297 g (standard deviation, SD 21 g) were randomly divided into 3 groups: 8 rats in group 1, 6 rats in group 2 and 4 rats in group 3. Eventually 16 rats were included in the analysis (see results). The rats were housed solitarily with standard chow and water at libitum. Group 1 and 2 were anesthetized by intra muscular administration of a mixture of ketamine, xylazine
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and atropine (8 mg, 4 mg, 5μg per 100 g body weight) when implanted with a Rat-o-Port (Access Technologies, Norfolk Medical, Skokie, IL USA), group 3 was the untreated control group. The Rat-o-Port was implanted subcutaneously in the neck and the attached silicone catheter (lumen 1.1 mm, length was adjusted per rat) with 1 dacron cuff, was tunneled subcutaneously over the left flank after which it was inserted into the peritoneal cavity proximal of the umbilicus. Recovery occurred during one week following the catheter implantation with daily infusion of 1 mL heparinized saline (5 UI/mL 0.9% NaCl). The intervention period started directly after recovery.

Group 1, the experimental group, was infused daily with heparinized (5 IU/mL) 3.86% glucose dialysate (Baxter Healthcare S.A., Castlebar, Ireland) 60 mL/kg body weight per day, preheated to 37°C. At the time points 16 and 20 weeks after the start of the daily intraperitoneal infusions with the glucose based dialysis solution, 3 rats were investigated with a standardized peritoneal permeability analysis (SPARa) and sacrificed thereafter. Two rats were investigated 12 weeks after inclusion. Peritoneal specimens of the omentum and the parietal peritoneum were obtained from each rat. Group 2 was infused daily with heparinized (5 IU/mL) Ringer's lactate (hospital pharmacy) 60 mL/kg body weight per day, also preheated to 37°C before peritoneal administration. This group was included as a control group to investigate the effect on peritoneal tissues, of daily infusion with an isotonic non-glucose, lactate containing solution. At time 12, 16 and 20 weeks after the start of the daily infusions with Ringer's lactate 2 rats underwent a SPARa and they were subsequently sacrificed to obtain the peritoneal tissues for morphological investigation. When the body weight of a rat exceeded 420 g, the rat was assumed to be fully grown and the infused volume was set at 20 mL/day for both infusion groups. Group 3 was included to investigate the effect of aging on the peritoneum morphology. Two of these rats were investigated at the start of the experimental period (t=0) and 2 rats after 20 weeks. Specimens of omental tissue and parietal peritoneum were obtained in each rat for light and electron microscopy.

Morphological analysis

Omental and parietal peritoneal tissues were obtained from every rat. Each specimen was prepared for light and electron microscopy. For light microscopy the tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections of 5 μm were obtained for (immuno)-histochemistry. All specimens were stained with pico-sirius red (Gurr, BDH, England) which provides a brick red staining of all fibrillary collagen. A semi-quantitative assessment of the staining behavior was performed using a light microscope (Leitz Dialux 20, Leica, Rijswijk, The Netherlands) with a 25x flat field objective (x 10 ocular).
Figure 1. Omental sections stained with pico sirius red (x132) as representative example of the semiquantitative score of fibrous tissue deposition: 0 = normal fibrous skeleton Figure 1a, 1 = mild depositions Figure 1b, 2 = moderate Figure 1c, 3 = severe Figure 1d.

The whole tissue section was screened to estimate the extent and distribution pattern of fibrosis in each slide. Staining results were classified from 0 to 3: 0 = normal fibrous skeleton of the peritoneum (Figure 1a), 1 = mild presence of fibrosis in the submesothelial and perivascular tissue (Figure 1b), 2 = moderate deposition of a discontinuous type of perivascular, submesothelial and interstitial fibrosis (Figure 1c), 3 = severe, extensive confluent fibrosis at intersegmental, submesothelial, perivascular and interstitial localizations (Figure 1d). In case of
interstitial, submesothelial and perivascular deposition of fibrous tissue, the
presence of type IV collagen and myofibroblasts was also scored with a similar
semiquantitative score. Immunohistochemistry was performed using a
streptavidin-biotin-peroxidase method [18]. The sections were deparaffinized in
xylene and rehydrated in ethanol, followed by incubation with hydrogen
peroxide 0.3% in methanol to block endogenous peroxidase activity. Non-
specific binding sites were blocked by incubation with 10% normal goat serum.
The applied primary antibodies were reactive with von Willebrand factor (vWF),
to investigate the number of vessels per field (polyclonal, DAKO, Denmark), α-
smooth muscle actin (SMA) to determine the presence of activated fibroblasts
present as myofibroblasts (monoclonal, DAKO, Denmark) and type IV collagen
(polyclonal, Organon Teknika, The Netherlands), to study the extent of collagen
IV deposition in the interstitium and vessel walls. After the incubation with the
primary antibodies, second step biotinylated rabbit anti-mouse F(ab')2 antibodies
against the monoclonal antibody and biotinylated swine anti-rabbit antibodies
against the polyclonal antibodies (both DAKO, Denmark) in PBS-NHS containing
5% normal rat serum were applied, followed by horse radish peroxidase-
conjugated streptABCcomplex (DAKO, Denmark) as conjugate. The peroxidase
activity was detected with 1 mg/mL 3.3-diaminobenzidine tetrahydrochloride
(Sigma, St Louis, MO, USA) and 0.015% H2O2 in 50 mM Tris-HCl buffer, pH
7.6, yielding the brown coloration. All sections were counterstained with Mayer's
hematoxylin, dehydrated through a graded series of ethanol and mounted with
Pertex mounting medium. The sections stained with anti-vWF and collagen type
IV required enzymatic pretreatment with 0.25% pepsin (Sigma, St Louis, MO,
USA) in 0.1 M HCl at 37°C.

The number of vessels in a peritoneal tissue section was counted in the
anti-vWF stain in 5 non-overlapping fields, using a light microscope (25x flat
field objective, x 10 ocular, Leitz Dialux 20, Leica, Rijswijk, The Netherlands).
The mean vessel count of the 5 fields was used as the number of vessels in that
specimen. Both the semiquantitative analysis of the fibrous tissue depositions,
and the quantitation of the vessels were done by two independent observers
(AvdW and MMZ).

The ultrastructure of basal lamina of peritoneal capillaries in all groups
was investigated. For this purpose the collected tissue was fixed in 4%
paraformaldehyde, followed by: postfixation in 1% osmiumtetroxide, block-
staining with 1% uranyl acetate, one-step dehydration in dimetoxyp propane
and embedding in epoxyresin LX-112. EM sections were stained with tannic acid
[19], uranyl acetate and lead citrate and studied with a Philips CM 10
(Eindhoven, The Netherlands).

Standard peritoneal permeability analysis in the rat: SPARa

The SPARa is a modification of the human standard peritoneal permeability
analysis (SPA) described by Pannekeet et al. [20]. The SPARa is performed
during a 4-hour dwell with 30 mL 3.86% glucose based dialysate solution,
preheated to 37°C. Dextran 70, 5 g/L (Hyskon®, Medisan Pharmaceuticals AB,
Uppsala, Sweden), was added to the test solution as a volume marker for the
calculation of solute kinetics [21]. The procedure included a rinsing step of the peritoneal cavity with 30 mL 3.86% glucose dialysis solution prior to the test to avoid possible effects of a residual volume present before the onset of the analysis. Directly after drainage of the dialysate used for the test, another rinsing step was performed with 20 mL 1.36% glucose based dialysis solution, to calculate the residual volume after the experiment. Both rinsing solutions were preheated to 37°C. Dialysate samples were taken before instillation of the test solution and 10, 30, 60, 120, 180 and 240 minutes after completion of inflow. Inflow, sampling and outflow of the dialysate during the SPARa occurred via an intravenous infusion needle with pvc-sheet, which was inserted intraperitononealy, lateral in the left lower quadrant of the abdomen. Outflow was accomplished by gravity. Blood was obtained by heart puncture at the end of the experiment; the rats were sacrificed thereafter. The animals were anesthetized during the SPARa procedure by intra muscular administration of a mixture of ketamine, xylazine and atropine (8 mg, 4 mg, 5µg per 100 g body weight). The protocol was approved by the Committee of Animal Experiments of the University of Amsterdam.

Assays
Total dextran 70 was measured in all dialysate samples by high performance liquid chromatography [22]. Both in plasma and effluent, urea (Hitachi H747, Boehringer Mannheim, Mannheim, Germany) and creatinine (Hitachi H911, Boehringer Mannheim, Mannheim, Germany) were measured with enzymatic methods. The glucose concentration was assessed by glucose oxidase-peroxidase assay (SMA II, Technicon, Terrytown, NJ, USA). Enzymatic methods for the determination of creatinine are influenced by high glucose concentrations as are present in glucose based dialysis solutions [23-25]. A correction factor (CF) was determined for the measurement of creatinine which is used in our laboratory: 
\[ CF = -3.10^{4}\cdot (\text{gluc})^2 + 0.11 \cdot (\text{gluc}) + 105 \]
where (gluc) is the glucose concentration in the effluent. Sodium was measured by an ion selective electrode (Hitachi H747, Boehringer Mannheim, Mannheim, Germany). Albumin concentrations were determined by the bromocresol green method. IgG was measured with a peroxidase sandwich enzyme-linked immuno assay (ELISA). ELISA plates (Maxisorp immunoplate, NUNC, Roskilde, Denmark) were coated with goat anti-rat IgG (Nordic Immunology, Tilburg, The Netherlands). Horseradish peroxidase labeled goat anti-rat IgG (H+L) (Nordic Immunology, Tilburg, The Netherlands) was applied as conjugate and o-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) as substrate. Absorbance was read at 490 nm against a buffer blanc, and ion exchange and gelfxfication chromatography purified rat IgG (Sigma Albrich Chemie BV, Zwijndrecht, The Netherlands) was used as a standard.
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Figure 2a. Omental tissue from a rat after 20 weeks daily infusion with Ringer's lactate (PSR x132): fibrous tissue deposition was normal in the submesothelial and mild in the perivascular layer. Omental sections from rats in group 1 after 12 and 16 weeks of daily infusion with glucose containing dialysis solution (PSR x 132). Mild to moderate deposition of fibrous tissue was present in the submesothelial, perivascular and intersegmental areas after 12 weeks Figure 2b. which became moderate to severe after 16 weeks of daily infusion Figure 2c.

Figure 3a. Omentum obtained from an untreated control rat in group 3, 20 weeks after inclusion (α SMA x 132): few small vessels are present. Omental tissue from rats in group 1 after 12 and 16 weeks of daily infusion with glucose containing dialysis solution. A greater number of vessels per field was observed after 12 weeks infusion with glucose dialysate Figure 3b. in comparison with the untreated control; the number of vessels increased after 16 weeks of daily infusion Figure 3c.

Figure 4a. Omentum from a rat in group 2 after 20 weeks infusion with Ringer's lactate (x132). Type IV collagen staining was weekly positive in the wall of the small vessels. Figure 4b-c. Omental tissue obtained in rats from group 1 after 16 and 20 weeks combined infusion with glucose containing dialysis solutions. Moderate to very strong stainings of type IV collagen in the vascular walls and the interstitium were present in both specimens (x 132).

Figure 5a. Parietal peritoneum obtained in a rat from group 2 after 20 weeks infusion with Ringer's lactate. Few small vessels with very week staining of type IV collagen were present and mild deposition of fibrous tissue was found in the submesothelial tissue (x 132). Figure 5b-c. Parietal peritoneum obtained in a group 1 rat after 20 weeks daily infusion with glucose containing dialysis solution. An increase in the number of vessels per field with deposition of type IV collagen was found after 20 weeks daily infusion with glucose based dialysis solution and a collagenous rind was seen in the submesothelial layer (x132).
Calculations

Peritoneal fluid and solute kinetics were calculated as described previously [20,21]. In brief, the transcapillary ultrafiltration increases the intraperitoneal volume, and fluid loss from the peritoneal cavity is assumed to occur by transcapillary back filtration and uptake into the lymphatic system. The resultant of these is the net ultrafiltration. The transcapillary ultrafiltration was calculated from the dilution of the volume marker, dextran 70. The convective disappearance of the volume marker from the peritoneal cavity can be used as an indirect method to estimate the contribution of the peritoneal lymphatics in the absorption of fluid from the peritoneal cavity [26]. These calculations of the effective lymphatic absorption include all pathways of uptake into the lymphatic system, both interstitial and subdiaphragmatic. The change in intraperitoneal volume during the dwell can be calculated from the dilution of the volume marker after correction for incomplete recovery. The net ultrafiltration is the difference between the transcapillary ultrafiltration and the effective lymphatic absorption.

The MTACs of urea and creatinine were calculated according to the model of Waniewski et al. [27], in which the solute concentration was expressed per volume of plasma water [28]. Glucose absorption was estimated as the difference between the instilled and the recovered amount of glucose, relative to the instilled quantity of glucose. The peritoneal clearances of albumin and IgG were determined according to the equation: Cl(mL/min) = (D*V)/(P*t), in which Cl represents the clearance, D the dialysate concentration at the end of the dwell, V is the dialysate volume at the end of the dwell, P is the plasma concentration and t is for the duration of the dwell. Aquaporin-mediated water transport was estimated by the sieving of sodium, expressed as the lowest D/P ratio of sodium during the dwell [29,30]. A diffusion correction was made when the initial dialysate concentration differed more than 5 mmol/L from the plasma concentration, because the concentration difference causes Na⁺ diffusion from the circulation to the dialysate, which leads to an underestimation of the actual sodium sieving [31].

Statistical analysis

Means and standard deviations are presented unless stated otherwise. One way analysis of variance (ANOVA) was performed to compare the severity of the deposition of fibrous tissue and the number of vessels per field within the groups of investigation. When ANOVA showed a significant difference among the groups, a modified t-test with a Bonferroni correction for multiple comparisons was used to investigate the differences between the groups. The peritoneal permeability parameters of the two infusion groups and the untreated control were investigated with Mann-Whitney tests for distribution free testing. The difference in the time courses of the net ultrafiltration, obtained in both infusion groups, was investigated with repeated measurement analysis of variance.
Results

Peritoneal fibrosis

Histological screening the peritoneal tissue sections, signs of peritoneal inflammation were observed in two rats, one in group 1 at 16 weeks daily infusion with the glucose based dialysis solution and one in group 2 at 16 weeks daily infusion with Ringer's lactate. These rats were excluded for further analysis. Therefore, 16 rats were included in the histochemical investigation.

Semiquantitative assessment of peritoneal fibrosis in pico sirius red stained sections is shown in Figure 2-5. Fibrous tissue deposition was normal in group 2 after 12, 16 and 20 weeks of daily infusion with Ringer's lactate (Figure 2a). This was also found in group 3 at the onset of the experiment and after 20 weeks of inclusion. Mild to moderate deposition of fibrous tissue was found in group 1 after 12 weeks of daily infusion with the glucose based dialysis solution, located in the submesothelial, intersegmental and perivascular interstitial tissue (Figure 2b). Moderate (discontinuous type of fibrosis) to severe (extensive confluent fibrosis) depositions were observed, both in the omentum and parietal peritoneum after 16 (Figure 2c) and 20 weeks (Figure 5c). Because no substantial differences were seen after 16 weeks and 20 weeks infusion with glucose dialysis solution, these two groups were combined for further analysis.

Infusion with Ringer's lactate did not alter the peritoneal morphology, independent from the duration of infusion. However, to sustain the parallel in the infusion periods between both infusion groups, the results obtained after 16 and 20 weeks infusion with Ringer's lactate were also combined for further analysis. The results from group 3 obtained at the start and after 20 weeks inclusion were also combined as aging did not alter the peritoneal morphology. The severity of peritoneal fibrosis was significantly greater in group 1 at 16-20 weeks infusion in comparison with group 2 at 16-20 weeks, p<0.018, and greater than in group 3, p<0.008. The anti-collagen IV immuno-reactivity was very weak in the vascular wall of small capillaries in group 2 (Figure 3a and Figure 5a), and was not different from the presentation in group 3. The intensity of the staining was markedly greater in group 1 at 16 (Figure 3b) and 20 weeks (Figure 3c and Figure 5b) of daily infusion with glucose dialysate in comparison with group 2 after the same infusion period (group 1 16-20 weeks versus group 2 16-20 weeks p<0.018) and in comparison with the untreated controls (group 3 p<0.008). There was no difference in type IV collagen deposition in the rats between the time interval 16 to 20 weeks. The intensity of the immunohistochemical staining of the myofibroblasts was not different between group 2 and 3 independent from the duration of inclusion in the experiment, nor did the location of the presentation differ (Fig 4a). The presence of α-SMA was detected in the vascular walls of peritoneal vessels in all rats. However, deposition of myofibroblasts in the interstitium became evident in group 1 with duration of infusion (Figure 4b and Figure 4c). The mesothelial cell-lining of the peritoneal cavity was found intact in most tissue specimens in a similar form and presentation, independent from the time point, the presence of a catheter or the administered solution.
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Figure 6. The scores of the severity of fibrous tissue deposition and the number of vessels per field in the respective groups. Symbols are group 1: (Δ) 12 weeks, (▼) 16 weeks and (▲) 20 weeks; group 2: (□) 12 weeks, (◆) 16 weeks and (■) 20 weeks; group 3: (○) at the start of the experiment and (●) 20 weeks after inclusion.

Peritoneal vascularization

Few vessels were found in the peritoneal interstitium in group 2 after 12 weeks: 8.2 ± 4.1 vessels per field (v/f), 16 weeks 12 ± 3.8 v/f and 20 weeks 7.7 ± 1.8 v/f (Figure 3a and 5a). In group 3 a similar number of vessels was found at the start 8.8 ± 5.7 v/f and after 20 weeks inclusion 6.9 ± 0.4 v/f (Figure 4a). The number of vessels per field in group 1 increased with the duration of the infusion from 22.7 ± 5.0 v/f (12 weeks, Figure 4b) to 28.3 ± 3.8 v/f (16 weeks, Figure 4c), and to 35.1 ± 5.3 v/f (20 weeks, Figure 3c). This increase in number of vessels with the duration of exposure to the glucose based dialysis solution did not reach significance. However, the number of vessels per field was significantly greater in group 1, combining the 16 and 20 weeks results (32.3 ± 5.6 v/f, Figure 3b and 3c) in comparison with control group 2 (9.1 ± 2.9 v/f, p < 0.0006, Figure 3a) and compared to group 3 (7.9 ± 2.6 v/f, p < 0.0001, Figure 4a). The scores of the fibrous and collagenous tissue deposition in relation to the number of vessels per field found in the individual animals are shown in Figure 6.

Investigation with transmission electron microscopy of the basal membranes of omental capillaries of group 1 revealed extensive lamination and reduplication after 20 weeks daily infusion (Figure 7a). This was in contrast to groups 2 and 3, where clear single basement laminas were observed at the onset of the experiment (Figure 7b) and after 20 weeks daily infusion with Ringer's lactate (Figure 7c).

Peritoneal permeability

The parameters of peritoneal permeability obtained in the two infusion groups are shown in Table 1. The peritoneal permeability characteristics obtained within the groups at 16 weeks and 20 weeks, were not different. Therefore,
rat were excluded due to signs of inflammation found in the peritoneal tissue specimens (see Results).

Data are expressed as means ± SD. Abbreviations: TAC, transcapillary ultrafiltration; NUF, net ultrafiltration; ETA, effective ultrafiltration; ELA, effective lymphatic absorption; MTC mass transfer area coefficient. Data of one group could not be calculated due to blood stained effluents. Data of one group could not be calculated due to blood stained effluents.

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<th>Group</th>
<th>Fluid kinetics</th>
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Table 1. Peritoneal permeability characteristics
analogue to the morphological investigations the results of both infusion groups obtained after 16 and 20 weeks were combined for the analysis of these parameters.

The transcapillary ultrafiltration after the 4-hour dwell was not different between group 1 and 2. However, the time course of the change in intraperitoneal volume during the dwell was significantly different between the two infusion groups, p<0.043 (Figure 8). The intraperitoneal volume in group 1 reached its maximum at 120 minutes, which was sooner than in group 2, where the maximum was reached after 180 minutes (p<0.014, Figure 8). The aquaporin mediated water transport estimated as the maximum sieving of sodium and expressed as a percentage of the initial D/P Na⁺, was significantly less in group 1 compared to group (p<0.043, Table 1). The MTACs of urea and creatinine were not significantly different between the two infusion groups. However, the glucose absorption was greater in the glucose infusion group compared to the Ringer's lactate group (group 1: 66.2±10.8% and 51.3±3.1% in group 2, p<0.014). The clearances of the macromolecules albumin and IgG were similar in the two infusion groups.

Discussion
The daily intraperitoneal infusion model in the rat proved to be a good reflection of morphological and functional alterations in the peritoneal membrane and have a close resemblance to those described in patients treated with long-term peritoneal dialysis. The model is similar to chronic peritoneal dialysis in humans.
because it provides continuous exposure to dialysis fluids over a long treatment period, but it also differs from the patient situation in some aspects. These include: (1) no drainage of the instilled volume, (2) the absence of uremia, and (3) the omission of animals with peritonitis from further investigation. These similarities and dissimilarities will be discussed below.

The life time of a rat is approximately 2.5 years. Twelve to 20 weeks of daily infusion therefore represents 9-15% of its life time. Extrapolating this to the human situation, assuming an average maximum age of 75 years, 9-15% represents a period of 7-11 years. Such duration of peritoneal dialysis is long-term treatment in a patient. Therefore, the experimental infusion period up to 20 weeks was not unlikely to be an accurate representation of clinical long-term peritoneal dialysis treatment. Growth or aging of the rats during this period did not influence the membrane morphology. Pinto et al. [32] found no effect of aging on peritoneal transport kinetics in Sprague Dawley rats investigated several times during a follow-up of 39 weeks. These animals were not on chronic PD.

Unlike in peritoneal dialysis, the dialysis effluent was not drained in our model, but left in the peritoneal cavity to be absorbed overnight. This was done to avoid blockage of the catheter. Although obstruction can often be prevented by omentectomy, this operation was not performed because its effect on long-term peritoneal dialysis is unclear [33,34]. Also very high protein losses have been described after omentectomy [35,36]. Uremia may influence peritoneal permeability [37]. This was also found in uremic rats [38]. In that study uremic rats had higher peritoneal glucose absorption than non-uremic animals. However, in both groups of animals very high values were found, much higher than in the present study. The poor survival of dialysed uremic rats in the study of Lameire et al. [38] together with our interest in effects of dialysis solutions on the peritoneal membrane and the necessary capacity of the animals to absorb an intra-peritoneal fluid load without getting overhydrated, were the reasons that a non-uremic model was used in the present study. A modifying effect of uremia on our results cannot be excluded. However the similarity of our results with the abnormalities described in chronic peritoneal dialysis patients, makes it unlikely that the presence or absence of uremia is a major factor in the reactions of the peritoneal membrane to dialysis solutions.

Peritonitis influences peritoneal permeability [39-41] and may induce some of the morphological alterations in long-term CAPD patients [11]. We have therefore excluded animals with peritonitis, either during the study or after sacrifice, from further analysis.

We used Ringer's lactate as the control fluid. This isotonic solution contains the same constituents as the glucose based dialysis solution in similar concentrations, but without the high glucose concentration. Continuous exposure of the peritoneal membrane to this solution up to 20 weeks did not affect the peritoneal morphology or permeability. Therefore, the daily infusion into the peritoneal cavity was not the trigger to induce alterations in the peritoneum. In contrast, the 3.86% glucose based dialysis solution caused marked morphological and functional abnormalities in the peritoneal membrane.
The time dependent morphological alterations were similar in the visceral and parietal peritoneum, and in the omentum; independent of the location. They also changed to a same extent in all tissue specimens with the duration of treatment.

The interstitial changes induced by the 3.86% glucose based dialysis solution were similar to those described in PD patients [13-15,17,42]. These include the presence of a submesothelial collagenous 'rind' [12] and the presence of type IV collagen [17]. Vascular abnormalities in long-term PD have received relatively little attention so far. We found an increase in the number of peritoneal vessels that was already present after 12 weeks, but reached its maximum at 20 weeks. This is in accordance with the high number of peritoneal vessels that we found recently in long-term PD patients and in patients with peritoneal sclerosis [17]. At the ultrastructural level marked reduplications of the capillary basement membranes were found, as has also been described in CAPD patients [13,14].

Changes in mass transfer area coefficients of low molecular weight solutes or their D/P ratios are likely to be caused by alterations in the number of perfused peritoneal capillaries [7,46]. These parameters are therefore likely to represent the peritoneal vascular surface area. In the present model we found a higher peritoneal absorption of glucose in the glucose dialysate exposed animals than in those exposed to Ringer's lactate. This functional representation of the peritoneal vascular surface area fits with the higher number of vessels found on morphological examination. Also the mean values of the MTACs of urea and creatinine were higher in the glucose dialysate group, although not significant. The most likely explanation for the lack of statistical significance is the absence of uremia with concomitant low plasma concentrations of these solutes. In the situation of near equilibration of these low molecular weight solutes between
plasma and dialysate, the absolute difference between plasma and dialysate concentration is so low that an accurate determination of small changes is not always possible.

No differences between the groups were found for the peritoneal clearances of albumin and IgG. This is in accordance with results of a longitudinal prospective study in new PD patients with a follow-up of 2 years [10] and with those in a case-control study comparing CAPD patients treated for more than 4 years with matched controls treated for less than 1 year [9]. The clearances of macromolecules are not only dependent on the vascular surface area but also on the intrinsic permeability (size selectivity) of the peritoneal membrane [7,44]. It is speculative whether the effects of an enlargement of the peritoneal vascular surface area on peritoneal protein clearances, is counteracted by a decreased intrinsic permeability, for instance caused by the interstitial alterations.

The development of a large peritoneal vascular surface area causes a more rapid dissipation of the glucose gradient leading to a decreased ultrafiltration. Therefore the presence of a large peritoneal vascular surface area has been suggested as one of the major causes of impaired ultrafiltration in long-term peritoneal dialysis [6-10,29]. This morphological/functional relationship was confirmed in our model because the glucose group had the lowest number of vessels, the highest glucose absorption and the least change in intraperitoneal volume during the SPARa. The profile of the intraperitoneal volume in the glucose treated animals was very similar to that observed in CAPD patients during peritonitis: a maximum value that was lower than after recovery, and was reached earlier during the dwell [40]. This is in accordance with the presence of an enlarged vascular surface area as this allows rapid transport of water, but this effect is counteracted by the accompanying high absorption rate of glucose. Not just the ultrafiltration decreased but also a significant decrease in the sieving of sodium was found. In some long-term peritoneal dialysis patients impaired aquaporin-mediated water transport has been reported as additional cause of ultrafiltration failure [29,30]. Furthermore, we previously found evidence that aquaporin-mediated water transport decreased with time on peritoneal dialysis [29,30,45]. This suggests that besides the induction of neoangiogenesis by continuous exposure to the glucose containing dialysis solution, aquaporin mediated water transport was also affected.

Recurrent peritonitis has often been claimed to be responsible for the morphological and functional alterations in the peritoneal membrane of long-term PD patients. The present model shows that these changes can be induced by continuous exposure to glucose based dialysis solutions, in the absence of peritonitis. Our study was not designed to elucidate which components of the commercially available dialysis solutions are most important in the induction of the described abnormalities. However, the results suggest a pathogenetic role of glucose and/or its degradation products.

The present study shows that simultaneous analysis of functional and morphological measurements of the peritoneal membrane is possible in our chronic peritoneal infusion model. We could induce similar abnormalities as are
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found in long-term peritoneal dialysis patients. Furthermore, the time course of the development of morphological alterations could be studied. Therefore this model may be used to investigate biocompatibility of other PD solutions in vivo during a long-term follow-up.

References
15. Dobbie JW, Lloyd JK, Gall CA. Categorization of ultrastructural changes in peritoneal mesothelium, stroma and blood vessels in uremia and CAPD patients.
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