Oral supplementation with sulodexide inhibits neo-angiogenesis in a rat model of peritoneal perfusion

Anneleen Pletinck¹, Maria Van Landschoot¹, Sonja Steppan², Debby Laukens³, Jutta Passlick-Deetjen⁴, Raymond Vanholder¹ and Wim Van Biesen¹

¹Department of Nephrology, Ghent University, Ghent, Belgium, ²Fresenius Medical Care Germany, Bad Homburg, Germany, ³Department of Gastroenterology, Ghent University, Ghent, Belgium and ⁴Department of Nephrology, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany

Correspondence and offprint requests to: Wim Van Biesen; E-mail: wim.vanbiesen@ugent.be

Abstract

Background. Peritoneal dialysis (PD) is associated with functional and morphological alterations of the peritoneal membrane (PM). It is hypothesized that vascular endothelial growth factor (VEGF) plays a role in this process. Sulodexide is a glycosaminoglycan with effects on vascular biology. Therefore, the impact of oral sulodexide on PM function and morphology in a rat model of peritoneal perfusion was evaluated.

Methods. Rats received 10 mL peritoneal dialysate fluid (PDF) twice daily via a tunnelled PD catheter. The test-PD group (Sul) received 15 mg/kg/day oral sulodexide versus none in the control–PD group (Con). A third group received no PDF (Sham). After 12 weeks, a peritoneal equilibration test was performed and the PM was sampled. Neo-angiogenesis was evaluated using immunostaining with von Willebrand, and epithelial-to-mesenchymal transition (EMT) using co-localization of cytokeratin and α-smooth muscle actin. VEGF was determined in the dialysate by enzyme-linked immunosorbent assay.

Results. PD induced loss of ultrafiltration, also in the sulodexide group. Creatinine and glucose transport were better preserved, and sodium dip was more pronounced in the sulodexide group versus control. Submesothelial thickness, neo-angiogenesis and EMT were more pronounced in the Con versus Sul versus Sham group. VEGF in the dialysate, corrected for diffusion was higher in Con and Sul versus Sham.

Conclusion. Oral sulodexide administration diminishes neo-vascularization, submesothelial thickening and EMT induced by exposure to PDF in a rat model. As there was no difference in VEGF at the protein level in the dialysate, we hypothesize that oral sulodexide inhibits VEGF locally by binding.

Keywords: angiogenesis; fibrosis; peritoneal dialysis; peritoneal membrane; sulodexide

Introduction

Peritoneal dialysis (PD) is an accepted and established renal replacement modality [1, 2]. Whereas it is advocated as a first line technique, the most important limiting factor for its long-term use is the progressive reduction in dialytic efficacy and ultrafiltration. This decrease is due to a progressive degradation of structure and function of the peritoneal membrane (PM) [3–5]. The changes are highly comparable to those observed in diabetes and mainly consist of two distinct but concurrent processes: fibrosis with submesothelial thickening and neo-angiogenesis [6], leading to increased small solute clearance and ultrafiltration loss, and finally to technique efficacy failure. Conceivably, these pathophysiologic similarities have been related to the presence of high concentrations of glucose and its degradation products [7, 8]. The non-physiology of dialysis solutions and the subsequent peritoneal failure have prompted the search for preventive options, one of these being the use of glycosaminoglycans (GAGs). Heparin is a GAG with a well-known anticoagulant activity [9], a property used in animal models of PD to maintain catheter patency and to prevent the development of peritoneal adhesions [10]. Heparin has a host of biologic actions beyond its role as an anticoagulant, including immunomodulating effects, antiproliferative effects, effects on the extracellular matrix, effects on angiogenesis and anti-inflammatory effects [9, 11, 12]. Low-molecular weight heparins can inhibit fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) activity [13]. The PM is specifically subjected to inflammation, fibrosis and angiogenesis during the course of PD therapy, and some of these processes have been linked to VEGF [14–16]. All this provides ample rationale for the study of GAGs in PD. Heparin use is accompanied by serious side effects, such as bleeding tendency and can only be administered intraperitoneally or subcutaneously (s.c.), limiting its everyday use [17–19]. Therefore, alternative GAGs should be evaluated.
Sulodexide is a highly purified mixture of GAG containing 80% low-molecular weight heparin and 20% dermatan sulphate. [18–20] The physiological behaviour of sulodexide differs substantially from unfractionated heparin and is mainly characterized by a prolonged half-life and a reduced effect on global coagulation and bleeding parameters. Sulodexide has been approved for human use and has been related to improvement of proteinuria and cardiovascular disease in diabetes. [21–23] Experiments using an intraperitoneal formulation in humans [24] or an s.c. formulation in rats [25] demonstrated a beneficial impact of sulodexide on the, respectively, functional and morphological properties of the PM. Sulodexide administration can thus potentially have a beneficial impact on PD patients through conservation of the integrity of the PM. 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and buffer [Tris-buffered solution (TBS) + Tween 0.05%), sections were incubated with UltraSense Block (Immunologic kit, Klinipath, Olen, Belgium) for 10 min to avoid cross reactions. Subsequently, they were incubated with the primary antibody anti-FVIII (1/250, A0082, Dako) for 1 h, followed by incubation with the secondary antibody (Immunologic kit) for 10 min and streptavidin peroxidase (Immunologic kit) for 10 min. 3,3’-diaminobenzidine (Dako) was used as the chromogenic substrate to visualize immunolabeling resulting in a brown precipitate. Finally, counterstaining was performed with Haematoxyline Mayer (Mallinkrodt Baker, Deventer, The Netherlands). Between every step, slides were rinsed in buffer solution.

Morphometric analysis

All slides were coded and blindly analysed by the same operator with an Olympus BX41 microscope (Olympus, Aartselaar, Belgium). From each experimental animal, three peritoneal samples were analysed. For each sample of peritoneum, three sections were digitalized and quantified with a computerized image analysis system (CellD software; Olympus). The submesothelial thickness (micrometers) was measured using the Masson’s Trichome staining. Microvascular density of the vWF immunostainings was determined by counting the number of blood vessels (number per square millimeters) and by calculating the percentage of the stained area per section.

The double α-SMA/cytokeratin staining was viewed with a fluorescence microscope (Axioscoop, Zeiss, Germany) and pictures were taken using CellF Software (Olympus Soft Imaging Solutions, Münster, Germany). A semiquantitative assessment was performed independently and blindly by two operators. Each section was screened to estimate the extent and distribution of colocalization of α-SMA and cytokeratin. Staining results were classified using a visual score: 0 = no co-localization at all, 1 = suggestive for co-localization, 2 = evidence of co-localization and 3 = clear colocalization and migration of mesothelial cells into the interstitium. The results were calculated as the mean of the individual scores of the two operators for each animal.

Statistical analysis

Data analysis was performed with SPPS version 15.0 (SPSS Inc, Chicago, IL).

For all analyses, one-way analysis of variance for the three groups and post-hoc testing with Least Significant Difference was used. The results are expressed as mean ± SD. The significance level was set at P ≤ 0.05.

Results

Technique survival

During the experiment, the well-being of the animals was monitored daily and no apparent abnormalities were observed. Body weight was similar in the different experimental groups at all time points (data not shown). Technique survival was 100% in all groups. Two animals of group Sul were excluded because of positive dialysate culture (one streptococcus viridans, one with Bacteroides species), positive tip culture (Paecilomyces sp.) and increased dialysate leukocyte count. In the Con group, one dialysate culture yielded Corynebacterium species but without increased dialysate leukocyte count.

Peritoneal transport studies

Exposure to PDF (Sul and Con) reduced net ultrafiltration significantly after 240 min of dwell time as compared to Sham, but sulodexide did not have a protective effect (Figure 1A).

Fig. 1. Peritoneal transport parameters. †P < 0.05 versus Sham, *P < 0.05 Con versus Sham; **P < 0.01 Con versus Sham, ***P < 0.01 Con versus Sul, #P < 0.05: Con versus Sul. Bars and line graphs represent mean ± SD.
In addition to the decrease of net ultrafiltration capacity, PDF in the Con group induced a significantly enhanced creatinine transport (Figure 1B) compared to Sham at every time point and compared to Sul group after 60 and 240 min. Addition of sulodexide partially prevented this enhancement of diffusive creatinine transport, because no significant differences in D/Pcreatinine were found between the Sul and Sham group. Glucose absorption was significantly increased in the Con compared to Sham group after 30 and 60 min (Figure 1C). Sodium dip was less pronounced and earlier in the Con group versus the Sul and the Sham group (Figure 1D). Protein loss was significantly higher in the Con group versus the Sham and Sul group after 240 min (Figure 1E).

**Peritoneal morphology**

The Masson’s Trichrome staining showed a significant thickening of the submesothelial layer of the PP in the groups on peritoneal perfusion (Table 1 and Figure 2), with significant lower values in the Sul group compared to the Con group.

Neo-angiogenesis was more expressed in the Con group as compared to the Sul group and the Sham group, without a difference between the latter two (Table 1 and Figure 3) and this both for the number of vessels per square millimeters as for the percentage surface stained with vWF. There was a higher score for epithelial-to-mesenchymal transition (EMT) in the Con versus Sul versus Sham group (Table 1 and Figure 4).

**VEGF pathway**

VEGF levels in the dialysate were, after correction for diffusion, higher in the Con and Sul group as compared to the Sham group (9.3 ± 6.1 versus 7.8 ± 4.9 versus 1.5 ± 1.2, P = 0.02), but the difference between Con and Sul animals did not reach significance.

**Relationships**

Significant correlations were observed between the number of blood vessels and the functional PM parameters after 30 min of the PET: D/Pcreatinine (r = 0.575, P < 0.01), D/Dglucose (r = −0.525, P < 0.01) and D/P protein (r = 0.598, P < 0.01).

No correlation was found between the vessel count and VEGF levels in the dialysate (r = 0.381, P = 0.060), but after excluding the Sul group, a significant correlation was observed (r = 0.635, P < 0.05).

VEGF correlated positively with the high transport rate of D/Pcreatinine (r = 0.551, P < 0.01) and correlated negatively with D/Dglucose (r = −0.420, P < 0.05) after 240 min of PET.

There was a weak correlation between the number of blood vessels and submesothelial thickness (r = 0.344, P < 0.05). EMT was not correlated with the number of blood vessels, but there was a correlation with submesothelial thickness (r = 0.405, P < 0.01).

A weak negative correlation was detected between ultrafiltration and submesothelial thickness (r = −0.393, P < 0.05).

**Discussion**

In this study, an oral formulation of sulodexide was tested in a rat model for PD, based on previous observations of a beneficial impact of this GAG on the PM during PD, and in the hypothesis that sulodexide could inhibit the activity of VEGF in the PM [24, 25, 29, 37].

We found a significantly worse peritoneal performance in the Con group by 12 weeks of exposure to PDFs, as evidenced by the increase of transport rate for creatinine, loss of ultrafiltration capacity and peritoneal tissue remodelling. Although no differences in net ultrafiltration were seen between the group with and without oral sulodexide administration, transport rates for creatinine and glucose and sodium sieving were better preserved in the sulodexide group. This functional improvement induced by sulodexide was associated with less neo-angiogenesis and reduced submesothelial thickening but not with a reduction of VEGF up-regulation in the dialysate.

As we observed higher VEGF levels in the dialysate of animals treated with peritoneal perfusion versus Sham but no differences between the animals on peritoneal perfusion treated versus not treated with sulodexide, we hypothesize that sulodexide inhibits VEGF activity either by binding it or by inhibiting the interaction with its receptor.

The role of VEGF in neo-angiogenesis during PD has been well established [34, 38]. Also in the current experiments, VEGF levels were higher in the animals submitted to peritoneal perfusion as compared to Sham animals. Structure–function relationship studies have shown that, both in rat models as in patients on long-term PD, loss of ultrafiltration capacity is associated with submesothelial thickening and the presence of neo-angiogenesis in the PM [39, 40]. For most people, exposure to glucose and especially glucose degradation products are the driving factors of the changes observed. However, also other factors can play a role [41]. Many additives and substances have been studied to counteract these alterations.

In the present study, oral administration of sulodexide resulted in lower peritoneal transport rate of creatinine and glucose and more sodium sieving as compared to the
control group, suggesting better preservation of the PM. This was in line with a lower microvascular density in the sulodexide group. Vessel count and functional membrane parameters were significantly correlated, which was also demonstrated by Vrtovsnik et al. [42]. Low molecular weight heparin can inhibit the binding of different growth factors to their receptors, and hyaluronan, another GAG, is a known important regulator of growth and migration of vascular endothelial cells and neo-angiogenesis. Heparin fragments of <18 saccharides inhibit the activity of FGF and VEGF [13, 43, 44]. Pyda et al. [45] reported complexation of VEGF by heparin in patients presenting with acute myocardial infarction. In a rat model of oral administration of labelled sulodexide, fluorescent material was observed in the endothelium of veins and arteries [26, 46]. It can thus be hypothesized that oral sulodexide inhibits neo-angiogenesis by locally blocking the enhanced VEGF activity produced during PDF exposure. We observed that oral administration of sulodexide resulted in significantly lower neo-angiogenesis, but not in lower up-regulation of VEGF at the protein level. These findings are compatible with the hypothesis that sulodexide interacts with the vascular effects of VEGF by either blocking the binding of VEGF to its receptor or by binding the VEGF molecule itself. This hypothesis is supported by our observation that VEGF levels and vessel count appeared to be significantly correlated, but only after excluding the sulodexide group. The data of the sulodexide group blur the correlation between VEGF and neo-angiogenesis, as the VEGF is present, but not active. Further experiments to explore the exact nature of this inhibition mechanism are warranted.

Several authors have already tried to modify outcome of PD using GAGs, with conflicting results [24, 47–50]. Using a similar rat model of peritoneal perfusion as ours, Schilte et al. [51] failed to demonstrate a beneficial impact of heparin. However, Schilte et al. administered heparin intraperitoneally, while systemic activity of GAGs might be crucial to obtain the described pleiotropic effects. Indeed, the beneficial effects on PM damage after acute peritonitis were only observed when sulodexide was administered systemically by intramuscular injection and not when administered in the PDF [52]. Finally, the half-life of heparin is much shorter than that of sulodexide, so a once daily

Fig. 2. Submesothelial thickness of PP. (A) Significant differences were found between groups on peritoneal perfusion (Con: 19.9 ± 7.26 and Sul: 15.5 ± 4.86) and control group (Sham: 10.0 ± 1.57). *P < 0.01 versus Sham, **P < 0.05 versus Sham and ***P < 0.05 versus Sul. Bars represent mean ± SD. (B) Histological images of the Masson’s Trichome staining. (magnification ×400) The submesothelial thickness of the PP was significantly higher in the groups on peritoneal perfusion (Con and Sul), but with significantly lower values for the Sul group, compared to the Con group.
injection of heparin might be ineffective [19]. Sjoland et al. [53] demonstrated a reduction in D/P creatinine, with an improvement in ultrafiltration capacity in a small human study of intraperitoneal heparin administration during 3 months. There was, however, a very high peritonitis rate in this study, making the results difficult to interpret.

Oral administration of sulodexide did not result in differences in ultrafiltration capacity in the present study, despite a (beneficial) difference in angiogenesis and D/P creatinine. Breborowicz et al. [54] also found changes in transperitoneal permeability after intraperitoneal administration of hyaluronan, however, with an opposite pattern. Wang et al. [55] reported that intraperitoneal administration of hyaluronan reduced peritoneal fluid absorption without changing transperitoneal fluid transport in a rat model. All these observations raise the suspicion that also changes in the physicochemical properties of the peritoneal interstitium take place when GAGs are administered and that the obtained effect depends upon the physicochemical properties of the substance used. Sulodexide is a negatively charged molecule, interstitial accumulation might hamper transport of water differently from that of non-polarized small solutes. It is, however, extremely difficult to visualize the presence of sulodexide in peritoneal tissue, as it is cleansed out during the normal processing of the tissue, so it was impossible to confirm this accumulation in our experiments.

Breborowicz et al. demonstrated that hyaluronan had cytoprotective effects in an in vitro model of peritoneal mesothelial cells [54, 55]. Sulodexide also has anti-inflammatory properties. It is well-known that in animal models for PD, the presence of an indwelling catheter induces microtraumatization and inflammation [56]. It could thus be that sulodexide only inhibits this mechanical stress on the PM induced by the presence of the catheter. As we have no negative control group with an indwelling catheter, but without peritoneal perfusion, we cannot make any conclusion from our current study in that regard, however.

We also observed a reduction of EMT indirectly, using a score for co-localization of α-SMA and cytokeratin and directly, by the lower degree of submesothelial thickening, itself a result of EMT. Although, for technical reasons, we were not able to further elaborate this, our observations fit

Fig. 3. Vascularization of the VP. Significantly fewer blood vessels (23.6 ± 9.3 versus 31.8 ± 12.6 versus 48.1 ± 16.248.1 ± 16.2) and percentage of surface positive for vWF staining (0.28 ± 0.17 versus 0.48 ± 0.26 versus 1.05 ± 0.64) were observed in the Sham versus Sul versus Con group. *P < 0.01 versus Sham; **P < 0.01 versus Sul and ***P < 0.01 versus Sham. Bars represent mean ± SD. Pictures show representative von Willebrand immunostainings of VP (magnification ×200).
with those of Breborowicz et al., who demonstrated a dose-dependent protective effect on healing of cultured monolayer cells after mechanical trauma, when sulodexide was added to the culture medium [57]. Others have demonstrated that certain GAGs inhibit Transforming Growth Factor (TGF)-β (over)expression at the transcription level, and it has been well established that up-regulation of TGF-β is one of the driving mechanisms of EMT and fibrosis [33, 58].

Although it can be seen as a limitation, we did not include a uraemic rat model in the current study. As uraemia per se can induce changes in peritoneal morphology [42, 51, 59] and, at least in our hands, a nephrectomy model can create large differences in states of uraemia, additional bias in the interpretation of the results would have resulted, which we wanted to avoid. It would be interesting to repeat the experiments in a uraemic model as described by Vrtovsnik et al.

In conclusion, we have confirmed that PD induces significant damage to peritoneal tissues within 12 weeks, and in animals that received orally administered sulodexide, these changes were less present. Neo-angiogenesis, submesothelial fibrosis and EMT were less pronounced in the group treated with oral sulodexide. Our data are compatible with the hypothesis that sulodexide inhibits VEGF activity at

![Fig. 4. α-SMA, cytokeratin and a double staining for α-SMA and cytokeratin (+DAPI nuclear staining) of the VP. Representative pictures are shown (magnification ×400). Serial sections of the VP from Sham-rats (A–C), Sul-rats (D–F) and Con-rats (G–L) were stained for cytokeratin (A, D, G and J), α-SMA (B, E, H and K) and double stained for α-SMA and cytokeratin (C, F, I and L). In the Sham-animals, only mesothelial cells stain for cytokeratin (A), only vascular smooth muscle cells stain for α-SMA (B) and virtually no α-SMA/cytokeratin co-localization (C), occurs. In the PM of the Sul and the Con animals, α-SMA staining is found not only in the vascular smooth muscle layer of blood vessels, but also in submesothelial tissue (E, F, H and I, respectively). In the Con rats, cytokeratin staining is additionally found in the interstitial tissue (J and L). Co-localization of α-SMA and cytokeratin is pronounced in submesothelial tissue (I) and in the interstitial tissue (L). Arrows, co-localization.]
Sulodexide and PD

the local level and support further investigation of the long-term administration of sulodexide as a potential protective agent during chronic PD treatment.

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Conflict of interest statement. None declared.

References

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**Abstract**

**Background.** The number of patients with chronic kidney disease (CKD) is continuously growing worldwide. Treatment with traditional Chinese medicine might slow the progression of CKD.

**Methods.** In this study, we evaluated the renal protective effects of the Chinese herb *Cordyceps sinensis* in rats with 5/6 nephrectomy. Male Sprague–Dawley mice (weighing 150–200 g) were subjected to 5/6 nephrectomy. The rats were divided into three groups: (i) untreated nephrectomized group (OP group, n = 16), (ii) oral administration of *C. sinensis*-treated (4 mg/kg/day) nephrectomized group (CS group, n = 16) and (iii) sham-operated group (SO group, n = 16). The rats were sacrificed at 4 and 8 weeks after 5/6 nephrectomy, and the kidneys, serum and urine were collected for $^1$H nuclear magnetic resonance spectral analysis. Multivariate statistical techniques and statistical metabolic correlation...