Understanding and preventing the peritoneal damage caused by conventional dialysis solutions
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Chapter 3

Semiquantitative scoring of peritoneal fibrosis and hydroxyproline content in the rat

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ABSTRACT

BACKGROUND

Semiquantitative methods are generally employed to describe fibrotic changes in peritoneal tissue specimens. In the present study a standardized semiquantitative way of scoring was compared with the hydroxyproline content, which is considered to be the gold standard for quantification of collagen in other tissues than peritoneum.

METHODS

Seventeen rats were studied. Twelve of these had been exposed daily to 3.86% glucose based dialysis solution for 16 weeks. Five untreated (non-exposed) rats from the same weights were used as controls. After sacrifice by cardiac puncture, parietal peritoneum (PP), omentum (O) and mesentery (M) were obtained and stained with picro-sirius red (PSR) or frozen directly in liquid nitrogen and kept until determination of the hydroxyproline content. All tissue specimens were scored for the severity of fibrosis on a 0-3 scale using the PSR stained slides in perivascular (PV), submesothelial (SM) and intersegmental (IS) areas. This scoring was compared to the total amount of hydroxyproline per peritoneal tissue specimen.

RESULTS

The best correlation for both methods in assessing fibrosis was found in all areas of omental tissue (SM: r=0.65, PV: r=0.68, IS: r=0.66, all p<0.05). A correlation was present between hydroxyproline content of all three tissues (PP vs M, r=0.72, PP vs O, r=0.85, O vs M, r=0.81).

CONCLUSIONS

The hydroxyproline assay can be used to measure fibrosis in peritoneal tissue. Furthermore, our results suggest that omental tissue is especially useful both for semiquantitative scoring of fibrosis and for the hydroxyproline determinations.
INTRODUCTION

Peritoneal fibrosis may develop during long-term peritoneal dialysis, probably as a result of the chronic exposure to unphysiologic dialysis fluids [1]. In some patients it may predispose to encapsulating peritoneal sclerosis [2,3] although this is disputed by others [4]. The fibrotic changes are most pronounced in the submesothelial layer of the parietal peritoneum [5,6], but the omentum can also be heavily affected [7]. Peritoneal fibrosis has not only been described in peritoneal dialysis (PD) patients but could also be induced in a chronic peritoneal exposure model in the rat [8]. Scoring of fibrosis in the above-mentioned studies was assessed with semiquantitative methods, either on parietal peritoneum or omentum. Also measurements of the thickness of the submesothelial compact zone of the parietal peritoneum have been performed [6]. These methods are not completely objective and very time consuming. Furthermore, there is no international consensus on the scoring of fibrosis. Apart from fibrotic changes in vessels, fibrosis consists of the deposition of extracellular matrix, especially collagen. Hydroxyproline is a component of collagen. Its quantity in tissue is directly related to the collagen content. Therefore the measurement of hydroxyproline in a tissue specimen has been considered a gold standard for the assessment of the amount of fibrosis [9]. In accordance Margetts et al. found a rough correlation between the thickness of the submesothelial parietal peritoneum and the hydroxyproline content of mesentery in a 4 weeks peritoneal exposure and inflammatory model in rats [10].

The aim of the present study was to compare the semiquantitative scoring of fibrosis in peritoneal tissues of rats, chronically exposed to dialysis solutions, to their peritoneal hydroxyproline content.

MATERIALS AND METHODS

Animals

Seventeen male Wistar rats (HSD, Harlan, Zeist, The Netherlands) were included in this study. Twelve rats of 7–8 weeks old, were acclimatized for one week and handled and at day 7 antibiotic prophylaxis (enrofloxacin 0.02 mL/100 mg BW s.c.) was applied. At day 8 a peritoneal catheter (7 French Intisil catheter, lumen 1.1 mm, with a Dacron felt cuff glued at 2 cm) was introduced proximal of the umbilicus and tunneled subcutaneously to the neck via the left flank under anesthesia (0.05 mL/100 gram BW of a mixture of ketamine/xylazine/atropine, 4:2:1). The length of the catheter was adjusted for each rat and a titanium/silicone device (Rat-o-Port, MTINC, 71S, Vascular Access technologies) was attached to the catheter and implanted subcutaneously. Sufficient pain sedation was applied (buprenorfin 0.3 mg/ml). From one day post-operatively, intraperitoneal infusion was achieved by daily puncture of the subcutaneous device implanted in the neck of the rat, using a 0.6 mm wing-end infusion set (microperfusor, Vygon, France). Before puncture the skin was cleaned with gausses and a small amount of chlorhexidine in
alcohol. The solution was armed to 37°C prior to infusion. Peritoneal healing was allowed for one week after the catheter insertion by daily infusion of 1 ml of heparinized saline (5 IU/1 mL NaCl 0.9%), after which the experimental period started. These twelve rats were infused with standard, heat-sterilized, 3.86% glucose containing, lactate buffered dialysate (Baxter, S.A., Castlebar, Ireland). Four of them were only given daily infusions for 8 weeks and 8 rats received lisinopril (150 mg/ml in drinking water) next to the infusions for 16 weeks. These rats were selected from different studies that took place at the same time at our laboratory to investigate small amounts of fibrosis as well as more pronounced. All rats had completed the experimental period, no rats had catheter dysfunction or other signs of peritonitis as assessed by peritoneal histology. Control rats (n=5) were included for comparison. These rats had similar weights, but had no catheter implanted and received no peritoneal infusions. After the experimental period the animals were sacrificed and peritoneal tissue was obtained. The study was performed in accordance with the regulations required by the local ethics committee for animal experimentation.

HISTOPATHOLOGY

Omentum, parietal peritoneum and mesentery were obtained in each rat. A part of each was frozen directly in liquid nitrogen and kept at -20°C until the hydroxyproline content was determined, and the other part was fixed in 4% buffered formaldehyde and embedded in paraffin. Paraffin-embedded tissue was serially sectioned at 5 micrometer thickness and stained with picro-sirus red, providing a brick red staining of all fibrillary collagen. Fibrosis was assessed using a light microscope (Leitz Dialux 20, Leica, Rijswijk, The Netherlands) with a 25x flat field objective (x10 ocular) by two blinded observers (SA and RVW). Inter-observer variability was less than 10%. The semiquantitative score was performed in omental, parietal and mesentery sections. The amount of fibrosis was assessed in three areas: submesothelial (SM), perivascular (PV) and intersegmental or interstitial (IS). This latter applies in parietal peritoneum to the compartments between the muscle fibers. Scoring was as follows: 0=absence of fibrous tissue, 1=mild presence, 2=moderate, 3=severe fibrosis. These visual assessments were done before the results of the hydroxyproline determinations were available.

HYDROXYPROLINE ASSAY

For the measurement of hydroxyproline an adaptation of the Woessner method was used [11,12]. In short: tissues were weighed (about 0.02 g of tissue was used per sample), homogenized and centrifuged (3000 rpm for 10 min, 4°C). Vacuum suction and TCA precipitation were performed, followed by another centrifugation step (3000 rpm 10 min 4°C). Samples were left overnight to hydrolyze (HCl 85°C) and the next day quantified by HPLC, after incubation first with o-phtalaldehyde and than with 4-chloro-7-nitrobenzofurazan. For detection a fluorescence detector was used with the excitation wavelength set at 470 nm and the emission wavelength set at 530 nm. A good correlation was present between the original colorimetric quantification and this HPLC method (r=0.88, p=0.01) although the values found with this latter method were on
average 19% higher than those obtained with the original colorometric method. Hydroxyproline levels were assessed in parietal peritoneum (n=12), omentum (n=8) and mesentery (n=8) from the infused animals and also in these tissues from 5 untreated rats. All tissues were assayed after pretreatment. Omentum and mesentery were hydrolyzed and then assayed in duplicate. For parietal peritoneum it appeared that the hydroxyproline determinations showed variable results when redetermined. Therefore we used two pieces of parietal peritoneum and performed the hydroxyproline assay in triplicate.

**Statistical Analysis**

The results are presented as medians and ranges. For semiquantitative measurements between the three different tissues analysis of variance was performed using Kruskall-Wallis. Wilcoxon rank sum tests were used to investigate differences between exposed and non-exposed groups. The t-test was used for analysis of the hydroxyproline concentrations, because a normal distribution was present. Correlation coefficients were determined with Spearman's rank correlation. All tests were performed using SPSS 10.0.7.

**Results**

The results of the semiquantitative scoring are given in Table 1. An example is given in Figure 1. Higher scores were found in the exposed animals than in the non-exposed as reported by our group previously [8]. The highest scores were found in the omental tissues. Also, the hydroxyproline content of all tissue specimens was higher in the exposed than non-exposed animals (Table 2). In both groups the highest levels were found in parietal peritoneum. A correlation was present between the hydroxyproline content of all three tissues (parietal peritoneum vs mesentery, r=0.72, parietal peritoneum vs omentum, r=0.85, omentum vs mesentery, r=0.81). Table 3 shows the correlation coefficient between the semiquantitative scoring and the hydroxyproline content. The best correlation was found for omental tissues. This is shown in Figure 2. The semiquantitative scoring in the mesentery was not correlated with its hydroxyproline content. The intra-assay variability was 9.5%, the interassay variability 7.2%.
**Table 1:** The results of the semiquantitative scoring method are given as median and range per tissue (PP: parietal peritoneum, O: omentum, M: mesentery) in three different areas (SM: submesothelial, PV: perivascular, IS: intersegmental).

<table>
<thead>
<tr>
<th></th>
<th>SM score (MM)</th>
<th>PV score (PV)</th>
<th>IS score (IS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed animals (n=8)</td>
<td>1.5 (1-3)</td>
<td>2.0 (1-3)</td>
<td>1.4 (0.5-2)</td>
</tr>
<tr>
<td>SM score</td>
<td>1.0 (0.5-2)</td>
<td>1.5 (1-2)</td>
<td>1.4 (0.5-2)</td>
</tr>
<tr>
<td>PV score</td>
<td>1.0 (0.5-2)</td>
<td>1.5 (1-2)</td>
<td>0.9 (0.5-1.5)</td>
</tr>
<tr>
<td>Non-exposed animals (n=5)</td>
<td>0.5 (0-0.5)</td>
<td>0.5 (0-0.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>SM score</td>
<td>0.5 (0-0.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PV score</td>
<td>0.5 (0-0.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IS score</td>
<td>0 (0)</td>
<td>0.5 (0.5-1.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*p*<0.05 between PP and O. *p*=0.01; *p*=0.08 all between exposed and non-exposed animals.

**Table 2:** Hydroxyproline content (means and standard deviations) in the various peritoneal tissues in mg/g. See Table 1 for abbreviations.

<table>
<thead>
<tr>
<th></th>
<th>Exposed animals</th>
<th>Non-exposed animals (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM score</td>
<td>2.0 ± 1.0 (n=12)*</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>PV score</td>
<td>1.3 ± 0.7 (n=8)*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>IS score</td>
<td>0.7 ± 0.3 (n=8)</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

*a* *p*<0.05 exposed vs non-exposed

**Table 3:** The correlation coefficients between the hydroxyproline content and the semiquantitative scoring.

<table>
<thead>
<tr>
<th></th>
<th>SM score</th>
<th>PV score</th>
<th>IS score</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM score</td>
<td>0.56*</td>
<td>0.65a</td>
<td>0.11</td>
</tr>
<tr>
<td>PV score</td>
<td>0.58*</td>
<td>0.68a</td>
<td>0.11</td>
</tr>
<tr>
<td>IS score</td>
<td>0.44</td>
<td>0.66a</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*a* *p*≤0.017 (see methods)
DISCUSSION

The present study has shown that the hydroxyproline content of omental tissue can be considered as the standard method for assessment of peritoneal fibrosis, induced by chronic exposure to peritoneal dialysis solutions. This is based on the good correlations with the semiquantitative scorings in omental tissues, artificially high concentrations in parietal peritoneum probably due to contamination with anterior abdominal wall muscle, and the good correlations with the hydroxyproline content in mesentery and parietal peritoneum. The amino acid hydroxyproline is especially present in collagen and therefore the determination of hydroxyproline in various tissues and cells has been used to quantify the amount of collagenous proteins. Hydroxyproline represents not only the quantity of collagen in a given tissue, but also the rate with which collagen is synthesized or degraded in such tissues. Hydroxyproline can be present in two isoforms: 3-hydroxy-L-proline and 4-hydroxy-L-proline of which the 3-isoform is less abundant and has been identified only in collagen. These isoforms can be present in both trans- and cis epimers.

Four techniques have been described to determine hydroxyproline: (1) Gaschromotography [12], (2) Electrophoresis [13], (3) Aa colorimetric reaction [14], (4) HPLC [11,15]. We chose to use the latter method because of its ease and its low detection limit after derivatizing with a fluorescent label [16]. Fibrosis consists of deposition of extracellular matrix material like collagen. Collagen fibers constitute the largest component of the space between the cells and it has been described that the amount of collagen measured as hydroxyproline can range from 5 mg/g in the lung, to 50–100 mg/g in skeletal muscle and 170 mg/g in the skin [17,18]. The submesothelial peritoneal interstitial tissues normally contain substantial amounts of quite compact bundles of collagen that are usually interposed between the blood microvessels and the mesothelial layer [19]. Nevertheless we found much lower values in all peritoneal tissue specimens than in the above mentioned tissues. This is most likely explained by the large variation in collagen content among different tissues, as described by Chavpil [20]. Chronic peritoneal dialysis results in fibrotic changes in peritoneal tissues [21-23]. These changes can be induced in a chronic peritoneal exposure model in rats [8]. The present study shows that the hydroxyproline content of dialysate-exposed peritoneal tissues tended to be higher than that of non-exposed animals, although it was still low compared to e.g. the lung. The highest values were always present in parietal peritoneum, probably due to small amounts of muscle tissue, present in the specimens. Consequently the same tissue should always be used for the assessment of the peritoneal collagen content. The omentum is likely to be the most useful tissue because there is no muscle contamination and the correlations with the semiquantitative scoring system were good. Interestingly, we found no correlation between both methods for the mesentery. Also, the mesentery was the only tissue in which we could not find increases in hydroxyproline content when we compared exposed tissue to non-exposed tissue. The values we found in mesentery were somewhat lower than those mentioned by Margetts et al. (1.8-2.9 mg/g vs 0.5-0.7 mg/g in the present study), but these authors investigated adenovirus-treated animals that underwent one peritoneal equilibration test and not daily infusions [24]. Total hydroxyproline was measured per gram of frozen tissue. The weight of the tissue is dependent on
the fluid content that may be increased in animals exposed to PD fluid due to tissue absorption. Therefore, one could argue that the difference in hydroxyproline content of exposed and non-exposed animals could be due to a difference in fluid content. However, the semiquantitative scoring showed normal to mild (0–0.5) fibrosis in untreated vs mild-moderate (1.5–2) fibrosis in treated animals (p=0.01). The good correlation between both methods for omental tissue makes it likely that there indeed is a difference in fibrosis between exposed and non-exposed animals. Semiquantitative scoring of fibrosis in tissue specimens has the disadvantage of subjectivity. Also, such a score is discontinuous, which makes it impossible to establish an over-all score from the sum of the various regions where the individual scores were made. For instance a score of 2 does not necessarily mean that the fibrosis is twice as severe as a score of 1. Another difficulty is that the relationship between score 1 and score 2 may vary in different parts of the peritoneal membrane. The correlations that were nevertheless found between the fibrosis score in the perivascular regions of parietal peritoneum and all regions of omentum on one hand and the hydroxyproline content of these tissues on the other, suggest that the hydroxyproline content of omentum can be considered the reference method for assessment of peritoneal fibrosis, induced by chronic exposure to peritoneal dialysis solutions.

Acknowledgements

We thank Dr. Margetts for supplying a modified version of the Woessner method for the hydroxyproline determination.
We adapted this method for HPLC.

References

11. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small portions of this imino acid. *Archives of Biochemistry and Biophysics* 1961;93:440-447.