Possibilities of Histological Studies in Non-Uremic Rabbit Model of Peritoneal Dialysis

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Abstract: Investigating peritoneal membrane alterations caused by peritoneal dialysis fluid during peritoneal dialysis in humans is still intriguing but limited. Animal models provide important information about peritoneal changes during long term peritoneal dialysis. The aim of the study was to investigate the possibilities of histological (light and transmission electronic microscopy) and morphometric analyses of peritoneal blood vessels using a modified non-uremic infusion model of peritoneal dialysis on rabbits. The study was done on five adult Chincilla rabbits. A part of infusion system Tro-soluset (Troge Medical GMBH, Germany) was used as peritoneal catheter for daily dialysate instillations. The rabbits tolerated surgical and instillation procedure well, increased body weight and no infection signs nor catheter opsttruction were observed during the follow up. Peritoneal tissue samples were obtained during the catheter placement and removal. Morphometric parameters of peritoneal blood vessels (determined with analy SIS 3.1 Soft Imaging System GMBH) showed statistically significant differences before and after peritoneal dialysis. This modified model of peritoneal dialysis on rabbits provided peritoneal tissue samples suitable for histological and morphometric analysis and can be used to study the effects of dialysis solutions on rabbit peritoneal membrane.

Key words: Peritoneal dialysis, experimental model, rabbit, histological studies, morphometric analysis

INTRODUCTION

Peritoneal Dialysis (PD) is a widely applied method for depuration in end stage renal disease patients where peritoneum acts as a living functional barrier for water removal and solute transport between the blood in peritoneal capillaries and the dialysate compartment.

Currently available dialysis solutions are not biocompatible with peritoneal membrane (Coles and Topley, 2000). Long-term PD is associated with the development of structural and functional alterations of peritoneal membrane. The high content of glucose, serving as osmotic agent (Davies et al., 2001) and lactates used to maintain dialysate low pH (Musi et al., 1998) as well as glucose degradation products and advanced glucose degradation products formed during sterilization and preservation of the dialysate (Nakayama et al., 1997, Witowski et al., 2000) cause non-enzymatic glycosylation of tissue proteins, loss of mesothelial layer, thickening of submesothelium due to increased deposition of collagen and hialuron in interstitial, interstitial fibrosis, thickening of mesothelial basement membrane and endothelial basement membrane of small peritoneal blood vessels and neoangiongenesis (Honda et al., 1999, Wieslander et al., 2000). Structural changes of peritoneal tissue increase velocity of low molecular mass solutes transport, increase

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peritoneal microvascular surface and decrease ultrafiltration (Wieczorowska-Tobis et al., 2004). All these alterations affect quality of dialysis (Witowski and Jorres, 2005).

It is still a challenge to investigate the influence of dialysate on human peritoneal membrane due to ethical and technical limitations (Stojimirovic et al., 2001; Smit et al., 2005). Tissue samples of human peritoneum can be obtained solely when the catheter is placed or removed because of peritonitis or obstruction and during surgery in case of other pathological conditions (Di Paolo et al., 1986; Williams et al., 2002). Therefore, in vivo animal models are developed to provide important informations on structural changes in the peritoneum on peritoneal transport pathophysiology and local defense mechanisms (Mortier et al., 2005). In vivo peritoneal dialysis research is hampered by the large variety of available models that make interpretation of results and comparison of studies very difficult. There is no consensus on the ideal experimental model so far and research groups research on animal models that differ substantially according to species and strain of experimental animals, method of peritoneal access, study duration, measurement of solute transport and ultrafiltration and sampling for histological analysis (Topley, 2005; Stojimirovic et al., 2007).

The aim of the study was to investigate a modified infusion non-uremic experimental model of peritoneal dialysis on rabbits which would be easy to perform, reproducible, inexpensive and which would provide peritoneal tissue samples suitable for histological examination as well as for morphometric analyses of peritoneal blood vessels.

MATERIALS AND METHODS

The study was performed on five adult healthy Chinchilla rabbits both sexes (3 males and 2 females), weighing 2699.0±36.3 g at the beginning of the follow up. The rabbits were kept in the individual cages under conventional laboratory conditions and they were allowed free access to food (standard rabbit pellets, Veterinary Institute, Serbia) and water. The animals were prevented from taking food and water 1 day before the surgery and they were allowed to take water and food ad libitum the following day. They were acclimatized for 5 days before catheter insertion and they were allowed to recover for 1 week following catheter placement before starting intraperitoneal dialysate infusions. During the whole follow up a diary of animal behavior was kept including data concerning body weight, body temperature, food intake, diuresis, defecation, antibiotics administration, other therapy and interventions if necessary (wound toilette, catheter suturing, etc.).

Fig. 1: Surgical procedure of catheter insertion

For catheter insertion and removal, the animals were anesthetized according to existing protocols with Thiopenal BP 1G⁰ (Rotenmedica, Germany), 0.5 mg kg⁻¹ body mass, via ear vein.

Surgical procedure of catheter placement was a modified version of the procedure described in the literature (Zweers et al., 1999). Anesthetized animals were standardly prepared for catheter placement (including shaving and surgical field preparation). A longitudinal incision, 3-4 cm long was made with a scalpel, starting 2-3 cm laterally from the left costal arch edge and at 4-5 cm distance from median line and parallel with it. A cut was made through the skin and subcutaneous space was entered, partly sharply partly bluntly and tunneled with mandren from thoracic drain No 16, using rotating and rectangular movements. The exit site of the mandren was made at the dorsal part of the neck between the ears (Fig. 1a). A part of infusion system Tro-solusset (Troge Medical GmbH, Germany) was used as peritoneal catheter for daily dialysate instillations. The catheter was pulled over on mandren and carefully pulled back through the tunnel to the abdomen (Fig. 1b). Muscles were sharply or bluntly moved apart to access the peritoneum. Immediately after opening the peritoneal cavity, biopsies of parietal peritoneal tissue were taken from diagonal edges and a catheter was placed at the bottom of peritoneal cavity (Fig. 1c). Intraperitoneal end of catheter (sharply cut to adjust to animal size) was previously protected with 1 cm soft rubber cap, cut from the same infusion set and proximal to rubber cap four holes (2-3 mm each) were made with surgical scissors. Peritoneum was sutured with ongoing suture using Vieril 4-0 and part of the catheter was fixed with the peritoneum. Muscles were then sutured with chromium Catgut 3-0 and fascia with ongoing suture using Vieril (Dexon) 3-0. Finally the skin was closed with single sutures. The catheter was fixed in the tissue at both entering and exit site. At both sites a sterile gauze was placed and fixed with bandage wrapped circularly around the animal (Fig. 1d).
Researchers conducted a double-blind histological investigation. Two researchers independently studied and described the samples not being aware of their origin.

Morphometric parameters of peritoneal blood vessels were determined by analy SIS 3.1 Soft Imaging System GmbH by direct measuring of histological structures on the image projected from Opton Photomikroskop III on computer screen using digital camera (Olympus C3030). Transversal outer and luminal surface, outer and luminal diameter, wall thickness, peritoneal tissue surface covered with blood vessels (in%) and blood vessels numerical density (number of blood vessels/100000 μm² of tissue) were analysed. It is only tock in account the vessels present on tissue sample as a whole. All experimental procedures were performed in accordance with the European Council Directive (86/609/EEC) and were approved by the Animal Care Committee of the University of Belgrade. Statistical analysis of the data was made in Microsoft Excel 2002. Results were presented as mean±standard deviation.

RESULTS AND DISCUSSION

The dose of anesthetic used was sufficient for adequate catheter implantation and removal and showed no adverse effects on the animals that recovered completely just after the surgery.

Rabbits body weight was constantly increasing during the follow up (Zunic-Bozinovski et al., 2008). No wound infection was observed after surgery. Dialysate instillations were started with about half the total estimated dose of dialysate. The dose was daily increased by 10 mL to prevent possible complications and respiratory distress by administering higher quantities of dialysate. The rabbits tolerated the instillations well.

Rabbits body temperature didn’t change significantly during the follow up (Zunic-Bozinovski et al., 2008). No peritonitis was suspected during the period of follow up proving that the animals were efficiently protected by antibiotics administered. No catheter obstruction occurred during the follow up thanking to heparinization.

Peritoneal tissue samples were first analyzed by Light Microscopy (LM). The sample taken before exposure to PD solution showed large blood vessels with large lumen and thin walls. The sample taken following exposure to PD solution had numerous small blood vessels with thin walls (Fig. 2).

Peritoneal lamina propria of dialyzed rabbits shows scattered collagen fibers, occasional fibroblasts, mononuclear phagocytes and adipocytes. Outer and inner
Table 1: Morphometric parameters of peritoneal tissue blood vessels before and after PD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before PD (mean±SD)</th>
<th>After PD (mean±SD)</th>
<th>t-test (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer surface (μm²)</td>
<td>578.1±132.99</td>
<td>174.79±159.91</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Outer diameter (μm)</td>
<td>97.2±11.090</td>
<td>49.92±21.620</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lumen surface (μm²)</td>
<td>401.24±107.92</td>
<td>105.31±99.350</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lumen diameter (μm)</td>
<td>83.75±11.759</td>
<td>41.38±23.800</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>6.75±1.070</td>
<td>4.17±2.1100</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LD/WT</td>
<td>12.72±3.4500</td>
<td>12.88±13.170</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

PD = Peritoneal Dialysis; SD = Standard Deviation; LD = Lumen Diameter; WT = Wall Thickness

Pinocytotic vesicles, prominent rough endoplasmic reticulum, well-developed Golgi apparatus and nuclei were observed in endothelial cytoplasm. Pericytes with long, foot-like processes were also observed.

Conventional peritoneal dialysis fluids alter the morphology and function of the peritoneal membrane during long-term use. Similar morphological alterations were observed on peritoneal tissue samples from animal models of PD and humans undergoing PD. However, these changes appeared in animals after much shorter time of exposure to bioincompatible dialysis solutions (Margetts et al., 2001a, b, 2002). Large variety of animal models of PD is present with no consensus in methodological approach (Mortier et al., 2005, Topley, 2005).

Mice, rats and rabbits are commonly used for experimental models of PD. Rats are cheap and easy to handle and therefore, preferred by most investigators while the miniature size of mice make manipulations extremely difficult (Mortier et al., 2005). Researchers chose rabbit for the study because this animal was more suitable for housing and feeding conditions then because of surgical treatment (surgical instruments, improvised peritoneal catheter, biopsies) available to us and finally because the ratio of peritoneal surface area and exchange volume in rabbits and humans is similar (Zweers et al., 1999, 2001). The rabbit model however has limitations, particularly due to the fact that rabbits are very sensitive animals, difficult to keep (Schamby et al., 1992, Di Paolo et al., 1995).

Although, the animals in this procedure have normal kidney function and no fluid exchanges were performed, this procedure is accepted as a valuable tool for evaluating the responses of peritoneal tissue upon exposure to peritoneal dialysis fluids (Zweers et al., 1999). A healthy animal model is easier to manage because survival rates are higher than in uremic models (Schamby et al., 1992).
Different researchers used different methods for fluid instillation in PD models. In some studies the test fluid was directly injected into peritoneal cavity using a 22-gauge needle (Wieczorowska-Tobis et al., 1997). In others, development of custom made miniature peritoneal catheters allowed the so-called open PD system with possible instillation and removal of PD fluid usually passively by gravity or occasionally by gentle abdominal massage (Peng et al., 2000). In another, the so-called closed system, a permanent catheter was tunneled from the peritoneal cavity to the neck of the animal but drainage of the dialysate was not possible (Flessner et al., 2006).

Since we could not provide an original peritoneal catheter for animals, we decided to try to use a part of infusion system Tro-soluset (Troger Medical GMBH, Germany) as a peritoneal catheter. The animals endured it well and dialysate instillations were easily performed.

One of the most important technical problem in animal models of chronic PD is frequent obstruction of peritoneal access. To avoid mechanical catheter obstruction, several researchers perform omentectomy before catheter is implanted. The implications of omentectomy on the immune status of the animal should not be underestimated as the omentum is an important source of mesothelial cells and macrophages and its absence impairs antibacterial defense. Furthermore, omentectomy does not always prevent catheter obstruction (De Vriese et al., 2002). The group did not perform omentectomy previous to catheter placement but choose heparinization as prevention of catheter obstruction. Use of heparin, besides desirable anticoagulant effects is followed with undesirable effects such as modulation of inflammatory cells activity, proliferation of the cells, synthesis of extracellular matrix and neangiogenesis. Despite these side effects of heparin use, researchers still preferred to use heparinization then to perform omentectomy. Heparin usage on animal models actually mimics real-life situations also because heparin is used in clinical practice when problems with catheter functioning occur. The use of heparin-coated catheters when available seems to be the method of choice of peritoneal access (De Vriese et al., 2002). There would like to emphasize that no catheter obstruction occurred in the animals during the follow up.

One of the most important clinical problems in animal models of chronic PD is the development of infection. Definition of peritonitis in animal models is still arbitrary. Most often used criteria are positive dialysate culture and dialysate WBC count >1000 cells mm⁻³ while in the chronic peritoneal dialysate exposure model peritonitis is suspected on the following clinical signs: fever over 40°C, loss of body weight over 5% and diarrhea.

Different strategies are in use to prevent peritonitis. Many studies support prophylactic administration of antibiotics during entire study period. Such regimen successfully prevented intraperitoneal infection (Peng et al., 2000). Prophylactic administration of cephalosporine in the study successfully prevented intraperitoneal infection in the animal (Mortier et al., 2003). There were no signs of wound infection nor signs of peritonitis in the rabbits during the follow up.

There is a variety of the frequency of instillations among investigators ranging from once to twice or even three times daily (Mortier et al., 2005). Duration of peritoneal dialysate exposure varies also among investigators between <4 weeks to 12 or more weeks. Peritoneal fibrosis and neangiogenesis as well as alterations in peritoneal permeability were found within a 4 weeks follow up in an experimental infusion model of peritoneal dialysis in rats (Margetts et al., 2001a, b) and in rabbits (Zweers et al., 2001). Researchers chose the daily instillations protocol and a 4 weeks follow up in order to study morphological changes of peritoneal membrane in the experimental model.

During the last years, the presence of hypervascularization in the peritoneal membrane of patients on long-term peritoneal dialysis is mentioned. Neovascularization and capillary dilatation were reported in biopsies of long-term peritoneal dialysis patients and the number of microvessels per area increased with treatment duration and correlated with the degree of interstitial fibrosis and with an upregulation of endothelial nitric oxide synthase (De Vriese et al., 2001; Devuyst, 2001; Hekking et al., 2001; Trbojevic-Stankovic et al., 2007).

Vascular density in experimental models of peritoneal dialysis is examined on light and electron microscopy (Hekking et al., 2001) by intravital microscopy (De Vriese et al., 2001) by analysis of VEGF (De Vriese et al., 2001) and eNOS expression (Hekking et al., 2001) by histological analysis on imprints of mesothelial monolayer (Hekking et al., 2001) or using the antiCD31 as the endothelial marker (Zareie et al., 2003). Neovascularization shown by these methods, occurred after some period of exposure to different types of dialysate solution and was most severe following exposure to conventional dialysate fluid with high glucose concentration and low pH due to lactate presence (De Vriese et al., 2001; Hekking et al., 2001; Zareie et al., 2003). The obtained samples of peritoneal tissue in the experimental model of PD were adequate for histological studying.

In the study morphometric analysis showed statistically significant differences in blood vessels parameters before and after exposure to PD solution. Researchers generally observed more small blood vessels...
CONCLUSION

The presented chronic infusion rabbit model of peritoneal dialysis is well tolerated by the animals is relatively inexpensive and does not require sophisticated technology. The special advantage of this procedure is that animals were unrestrained and awake have free access to food and water and did not loose weight. The model provided peritoneal tissue samples suitable for histological examination and for morphometric analysis of peritoneal blood vessels and can be used to analyze the effects of different dialysis solutions on rabbit peritoneal membrane.

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