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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 opstruction 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 interstitium, interstitial fibrosis, thickening of mesothelial basement membrane and endothelial basement membrane of small peritoneal blood vessels and neoangiogenesis (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±136.3 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.).

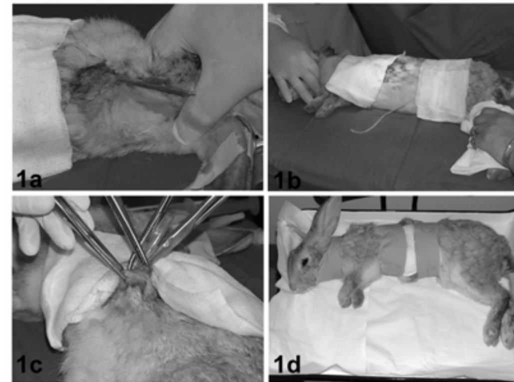

Fig. 1: Surgical procedure of catheter insertion

For catheter insertion and removal, the animals were anesthetized according to existing protocols with Thiopental BP 1G© (Rotexmedica, 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 longitudal 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 than 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-soluset (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. Immidiately 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 Vicril 4-0 and part of the catheter was fixed with the peritoneum. Muscles were then sutured with chromium Cutgut 3-0 and fascias with ongoing suture using Vicril (Dexon) 3-0. Finaly 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 circulary around the animal (Fig. 1d).

Catheter was removed 4 weeks after dialysate instillations were started. New abdominal incision, 2-3 cm long was made in front of the previous one because of the existing scar. The access to the peritoneum was made as previously described. Tissue samples for histological analysis were taken. The catheter was then identified, the end of the catheter covered by rubber cap was cut out and through the tunnel the catheter was pulled out at the neck region. The abdominal wound was closed as previously described.

Infection was prevented using daily injections of cefuroxime (Nilacef©, Hemofarm and GlaxoSmithKline, England). About 3 days prior to catheter placement and 3 days following catheter removal the antibiotic was given intramusculary twice a day at daily dose of 150 mg. After starting dialysate instillation, 75 mg of cefuroxime were administered intraperitoneally through the catheter once a day.

Dialysate instillations were started 7 days after the catheter insertion, beginning with 60 mL of dialysate (Dianeal PD4 Glucose©, Baxter Vertriebs GmbH, Austria) with 3.86% glucose concentration preheated at 37°C. Instillations were then continued once a day, increasing the amount of dialysate used by 10 mL each following day until the full dose of 40 mL kg^{-1} of body mass.

After insertion, the catheter was heparinised with daily injections of 10 i.j. of heparin natricum (Heparin©, Galenika a.d., Serbia) in order to prevent catheter clothing.

Because peritoneal tissue is extremely fragile and susceptible to mechanical irritation and environmental factors, tissue samples were collected strictly obliging the guidelines from literature (Jorres and Witowski, 2005). Immediately after the biopsy, the tissue was fixed for 24 h with 10% formaldehyde with 0.1 M Sorensen's phosphate buffer pH 7.4, dehydrated in 96% ethanol then routinely processed for embedding in paraplast and stained with hematoxylin-eosin and toluidin-blue to be analyzed by light microscopy (Opton Photomikroskop III).

For Transmission Electron Microscopy (TEM) tissue samples were fixed for 24 h in 4% glutaraldehyde with 1% tannic acid to ensure better fixation of membrane structures. Fixatives were diluted in cacodylate or Sorensen's phosphate buffer 0.1 M, pH 7.4. The tissue was then rinsed 3 times for 10 min with the same buffer and then 3 times for 10 min in cacodylate buffer. Samples were postfixed with 1% osmium tetroxide in 0.1 M cacodilate buffer, pH 7.4 and left overnight in 4% uranyl acetate. They were then dehydrated in ethanol and propylene-oxide and embedded in Epon. Fine sections were contrasted with uranyl acetate and lead-citrate and analyzed with transmission electron microscope (Philips M208S).

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 μm^2 of tissue) were analysed. It is only took 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

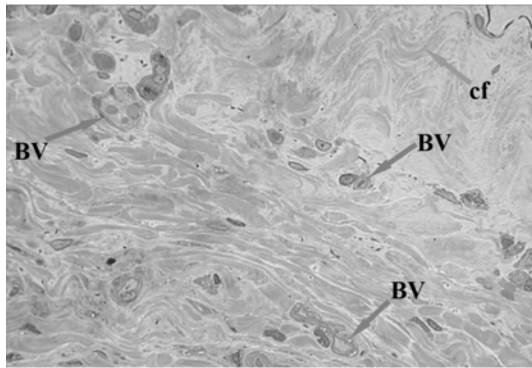

Fig. 2: Rabbit peritoneum after exposure to peritoneal dialysate (LM×250); (BV-Blood Vessel, cf-collagen fiber)

Table 1: Morphometric parameters of peritoneal tissue blood vessels before and after PD

Parameters	Before PD (mean±SD)	After PD (mean±SD)	t-test (p)
Outer surface (μm²)	578.15±132.99	174.79±159.91	<0.05
Outer diameter (μm)	97.25±11.090	49.92±24.620	<0.05
Lumen surface (μm²)	401.24±107.92	105.31±99.350	<0.05
Lumen diameter (μm)	83.75±11.750	41.58±23.800	<0.05
Wall thickness (μm)	6.75±1.0700	4.17±2.1100	>0.05
LD/WT	12.72±3.4500	12.88±13.170	>0.05

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

outlines of blood vessels transversal sections were digitally marked for morhometric determination (Fig. 2). Morphometric analysis showed statistically significant differences in blood vessels parameters before and after PD (Table 1).

Outer and lumen surface of observed blood vessels transversal sections were significantly larger before PD ($p<0.05$) and the same was true for outer and lumen blood vessels diameter ($p<0.05$). Following exposure to PD solution observed blood vessels with thinner walls then prior to PD but the difference was not statistically significant. Lumen diameter-wall thickness ratio was not significantly different before and after PD. Blood vessels numerical density was higher after PD (24.27 blood vessels/100 000 μm²) then before the treatment (9.05 blood vessels/100 000 μm²). Percent of peritoneal tissue surface covered with blood vessels was smaller after dialysis (2.70 vs. 5.23) although, researchers observed more blood vessels on the sample after PD because these vessels had smaller sections then the ones before the treatment.

When observed with Transmission Electron Microscope (TEM), blood vessels walls showed single layer of endothelial cells on a continuous, single basement membrane (Fig. 3). Endothelial cells were elongated with large, predominantly euchromatic and centrally positioned nuclei. Mitochondria, ribosomes and

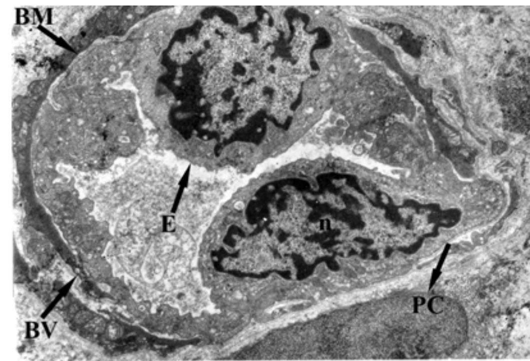

Fig. 3: Blood vessel in rabbit peritoneum (TEM×30000); (BV: Blood Vessel, E: Endothelial cell, BM: Basement Membrane; PC: Pericyte; n-nucleus

pinocytotic vesicles, prominent rough endoplasmatic 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 morhological alterations were observed on peritoneal tissue samples from animal models of PD and humans undegoring 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).

Mices, 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 extremelly 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, particulary due to the fact that rabbits are very sensitive animals, difficult to keep (Schambye *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 (Schambye *et al*., 1992).

Different researchers used different methods for fluid instilation in PD models. In some studies the test fluid was directly injected into peritoneal cavity using a 22-gauge niddle (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 (Troge 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 neoangiogenesis. 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 occured 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 profilactic administration of antibiotics during entire study period. Such regimen successfully prevented intraperitoneal infection (Peng *et al*., 2000). Prophylactic administration of cefuroxime 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 neoangiogenesis 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

with thin walls following PD treatment. The changes we observed could be attributed to the process of neoangiogenesis which represents the initial reaction of the tissue to non-physiological conditions elicited by exposure to PD solution.

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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