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Benefits of switching from a conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model

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Benefits of switching from a conventional to a low-glucose degradation product (GDP) bicarbonate/lactate-buffered dialysis solution in a rat model.

Background. Long-term exposure to standard peritoneal dialysis fluid (PDF) results in alterations in peritoneal morphology and function. Studies investigating the long-term effects on the peritoneum of a low-glucose degradation product (GDP) bicarbonate/lactate-buffered PDF demonstrated its superior biocompatibility. We examined the potential of the low-GDP bicarbonate/lactate-buffered solution to reverse or reduce standard PDF-induced peritoneal alterations.

Methods. Female Wistar rats received twice daily intraperitoneal infusions with either a lactate-buffered solution with 3.86% glucose at pH 5.5 (Dianeal®, referred to as standard PDF), or a low-GDP bicarbonate/lactate-buffered solution with 3.86% glucose at physiologic pH (Physioneal®, referred to as bicarbonate/lactate PDF) for different periods of time: (*1*) 12 weeks Dianeal® ($N = 9$); (*2*) 12 weeks Physioneal® ($N = 9$); (*3*) 20 weeks Dianeal® ($N = 11$); (*4*) 20 weeks Physioneal® ($N = 10$); (*5*) 12 weeks Dianeal® followed by 8 weeks Physioneal® ($N = 10$).

Results. Chronic standard PDF exposure resulted in loss of ultrafiltration capacity, increased VEGF expression and vascular density, higher advanced glycation end product (AGE) accumulation, up-regulation of TGF-β expression, and development of fibrosis compared to low-GDP bicarbonate/lactate-buffered PDF. The PDF-induced alterations were time-dependent. Crossover from standard PDF to low-GDP bicarbonate/lactate PDF resulted in a less impaired ultrafiltration (UF), less pronounced VEGF expression and neoangiogenesis, and less severe AGE accumulation, TGF-β expression, and fibrosis compared to continuous standard PDF exposure for 20 weeks.

Conclusion. Low-GDP bicarbonate/lactate-buffered PDF has the potential to slow down standard PDF-induced peritoneal membrane damage.

Loss of ultrafiltration capacity is an important cause of treatment failure in peritoneal dialysis (PD), requiring the transfer of patients to hemodialysis or the introduction of an episode of peritoneal rest. Long-term treatment of PD patients with conventional peritoneal dialysis fluid (PDF) is associated with fibrosis and neoangiogenesis, which is especially pronounced in patients with ultrafiltration (UF) failure [1–3].

In vitro studies demonstrated that low-glucose degradation product (GDP), bicarbonate/lactate-buffered PDF better preserved viability and functions of peritoneal leukocytes, mesothelial cells, and fibroblasts than conventional solutions [4–6]. In experimental animal models, the low-GDP bicarbonate/lactate-buffered PDF was associated with less functional and structural alterations of the peritoneal membrane than standard PDF [7, 8]. In clinical trials, alleviation of infusion pain, improved ultrafiltration, increases in CA125 concentrations, and a decrease in hyaluronan levels were observed [9, 10].

While several lines of evidence indicate that these new PDF have the potential to induce less peritoneal membrane damage than standard PDF, their ability to reverse or reduce standard PDF-associated structural and functional alterations of the peritoneum has not been examined. If true, a switch to a more biocompatible PDF could be an alternative to peritoneal resting or transfer to hemodialysis in patients with peritoneal membrane dysfunction. We therefore studied in a standardized rat model of chronic PDF exposure the potential reversibility of standard PDF-induced alterations during treatment with a low-GDP bicarbonate/lactate-buffered PDF.

METHODS

Laboratory animals

The studies were performed in 49 female Wistar rats (Iffacredo, Brussels, Belgium) with a mean body weight of 214 ± 1 g, receiving care in accordance with the national guidelines for care and use of laboratory animals. A subcutaneous port (PMINA-CBAS-C30 Soloport; Instech Solomon, Plymouth Meeting, PA, USA) was implanted in the neck under halothane (Fluothane; Zeneca, Destelbergen, Belgium) anesthesia in sterile conditions. The attached polyurethane, heparin-coated

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catheter (Instech Solomon) was tunnelled over the left flank to the peritoneal cavity [11]. After surgery, the animals received an intramuscular injection of buprenorphine (0.1 mL/kg Temgesic; Schering Plough NV/SA, Brussels, Belgium). The first week after implantation, catheters were flushed once daily with 1 mL of Earle's balanced salt solution (EBSS; ICN Biomedicals, Inc., Aurora, OH, USA). Thereafter, 10 mL of PDF was administered twice daily during 12 or 20 weeks. Oxacilline (2.5 mg/day Penstapho; Bristol-Myers Squibb, Brussels, Belgium) and gentamycine (0.04 mg/day Geomycine; Shering-Plough) were added to all solutions [12]. Laboratory technicians wore masks and gloves during manipulations. The area of the port was disinfected with ethanol 97% 20 seconds before puncture.

Study protocol

Five groups of animals were exposed to either a lactate-buffered solution with 3.86% glucose at pH 5.5 (Dianeal®; Baxter, SA, Lessines, Belgium), referred to as standard PDF, or to a low-GDP bicarbonate/lactate-buffered solution with 3.86% glucose at physiologic pH (Physioneal®; Baxter), referred to as bicarbonate/lactate PDF, for different periods of time: (*1*) 12 weeks standard PDF ($N = 9$); (*2*) 12 weeks bicarbonate/lactate PDF ($N = 9$); (*3*) 20 weeks standard PDF ($N = 11$); (*4*) 20 weeks bicarbonate/lactate PDF ($N = 10$); (*5*) 12 weeks standard PDF followed by 8 weeks bicarbonate/lactate PDF ($N = 10$). The weight of the rats was recorded weekly. Catheter patency and the integrity of the skin of the abdomen and around the port were evaluated twice daily. In case of catheter obstruction, an attempt was made to infuse fluids under halothane anesthesia. In case of persistent catheter obstruction, skin lesions, or severe weight loss, dialysate and catheter tip cultures, as well as dialysate white blood cell (WBC) counts were obtained, and the animal was sacrificed. At 4-week intervals, dialysate cultures and WBC counts were performed on 2 mL of fluid obtained through a sterile abdominal puncture with a silicon catheter (Venflon; Becton Dickinson, Erembodegem-Aalst, Belgium) under halothane anesthesia 4 hours after the last dialysate injection. WBC counts were performed in a Bürker chamber. Infection was arbitrarily defined as a positive dialysate culture with a dialysate WBC count higher than 1000/mm^3 [11, 12].

Study of peritoneal function

After the predefined period of dialysate exposure, rats were anaesthetized with thiobutabarbital (100 mg/kg s.c. Inactin; RBI, Natick, MA, USA). The trachea was intubated, and a jugular vein was cannulated for continuous infusion of isotonic saline. After 30 minutes, a silicone catheter was inserted in the abdomen, and 15 mL of 3.86% Dianeal was infused. After 120 minutes, dialysate was recovered through the silicone catheter, and samples were obtained for culture and WBC counts. The abdomen was opened by midline incision to collect the rest of the dialysate for determination of net UF, and to sample tissue. The tunnelled polyurethane catheter was removed in a sterile way, and the tip was cultured.

Study of peritoneal morphology

One sample of visceral and parietal peritoneum was obtained in each experimental animal, fixed in 4% neutral buffered formalin, and embedded in paraffin. Five μm sections were cut for histology and immunohistochemistry.

Immunostaining for endothelial NO synthase (eNOS), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and advanced glycation end products (AGE) were performed. Sections were deparaffinized, rehydrated, incubated in 3% H_2O_2 in phosphate-buffered saline (PBS) for 15 minutes to block endogenous peroxidase, and washed in 10% normal horse serum (Sigma, St. Louis, MO, USA) in PBS for 20 minutes to block nonspecific binding. Subsequently, they were incubated with the primary antibody, mouse antihuman eNOS (Transduction Laboratories, Lexington, KY, USA), mouse antihuman VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antihuman TGF-β1 (Santa Cruz Biotechnology), and mouse antihuman AGE (6D12; Cosmo Bio, Ltd., Tokyo, Japan), respectively. Thereafter, a biotinylated IgG (Vector Laboratories, Burlingame, CA, USA) and streptavidine-peroxidase were applied for 45 minutes each. 3,3′diaminobenzidine (DAB) was used as the chromogenic substrate to visualize immunolabelling, resulting in a brown precipitate.

The degree of fibrosis was evaluated using a Picro Sirius Red staining F3B (Klinipath, Geel, Belgium). Sections were deparaffinized, rehydrated, and stained briefly with Giemsa. Subsequently, sections were washed and stained with the Sirius Red solution, resulting in a brick red staining of all fibrillary collagen.

Morphometric analysis

Morphometric measurements of the eNOS, VEGF, TGF-β, AGE, and Picro Sirius Red staining were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification ×200. For each sample of peritoneum, 2 sections were analyzed quantitatively with a computerized image analysis system (Zeiss). A camera sampled the image of the stained sections, and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analyzed with KS400 Software (Zeiss). Each pixel in a color image was divided into 3 color components (hue, saturation, and intensity). The threshold

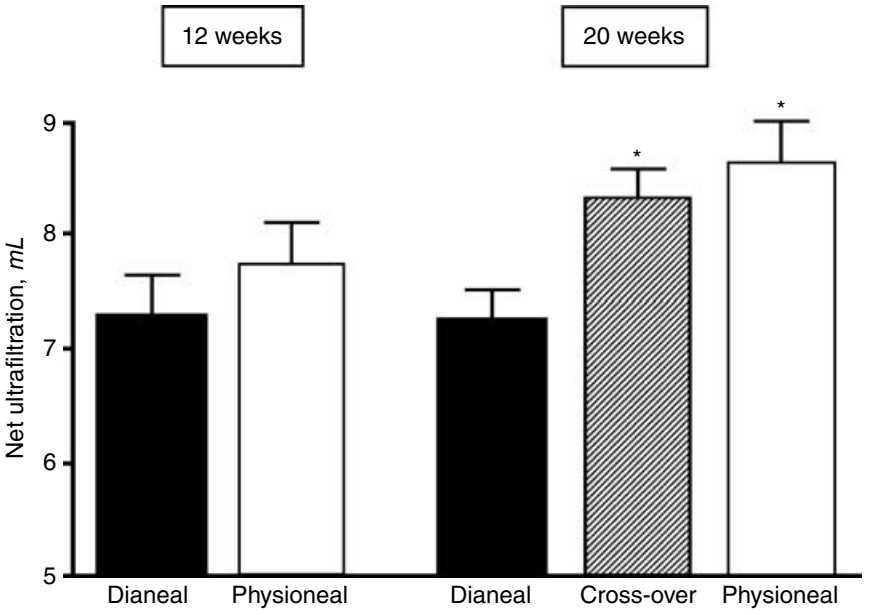

Fig. 1. Net ultrafiltration after a 120-minute dwell of 15 mL of 3.86% glucose dialysate in experimental animals exposed to standard lactate-buffered peritoneal dialysis fluid (PDF) for 12 or 20 weeks ($N = 9$ or $N = 11$, closed bars), low-glucose degradation product (GDP) bicarbonate/lactate-buffered PDF ($N = 9$ or $N = 10$, open bars) for 12 or 20 weeks, and standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks ($N = 10$, hatched bar). $^*P < 0.01$ vs. Dianeal (20 weeks).

for each color component of the staining was defined and kept constant throughout the analysis. In a predefined area, eNOS, VEGF, TGF-β, AGE, and Picro Sirius Red staining were measured and expressed as a percentage. In addition, eNOS-labeled blood vessels were counted as N/field.

Statistical analyses

The results are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) and, where appropiate, the Tukey test was used as multiple comparison *t* test. The significance level was set at $P \leq 0.05$.

RESULTS

Technique survival and infection rate of laboratory animals

Body weight was similar in the different experimental groups at all time points (data not shown). Technique survival was 100% in all groups. Except for one transient episode of infection, defined as a positive dialysate culture and a dialysate WBC count >1000/mm^3, in an animal exposed to the standard PDF, no infection was diagnosed (data not shown).

Ultrafiltration

After 12 weeks of PDF exposure, net UF did not differ significantly between animals exposed to standard PDF and to bicarbonate/lactate PDF, although a trend toward a better UF in the bicarbonate/lactate PDF group was present (Fig. 1). At 20 weeks, net UF was significantly lower in the animals exposed to standard PDF than in the crossover group and in bicarbonate/lactate PDF-treated group. Net UF was not different between the crossover and the bicarbonate/lactate PDF group (Fig. 1).

Peritoneal morphology

Vascular density was higher in the standard PDF than in the bicarbonate/lactate PDF group, both after 12 weeks and 20 weeks of PDF exposure (Table 1, Fig. 2). In the crossover group, vascular density was lower than in the standard PDF group, and did not differ significantly from that of the bicarbonate/lactate PDF group (Table 1, Fig. 2). After 12 weeks of PDF exposure, eNOS expression was not different in the standard PDF and bicarbonate/lactate PDF group (Table 1, Fig. 2). At 20 weeks, eNOS was up-regulated in the standard PDF group compared with the bicarbonate/lactate PDF group. Intermediary values were measured in the crossover group (Table 1, Fig. 2). VEGF expression was more pronounced in animals exposed to standard PDF compared to those exposed to bicarbonate/lactate PDF, both at 12 and 20 weeks (Table 1, Fig. 3). At 20 weeks, VEGF expression in the crossover group did not differ significantly from that in other experimental groups (Table 1, Fig. 3). Vascular density correlated positively with both eNOS (Pearson $r = 0.6646$, $P < 0.0001$) and VEGF expression (Pearson $r = 0.6004$, $P < 0.0001$).

AGE accumulation was more pronounced in the standard PDF group than in the other experimental groups at 12 and 20 weeks (Table 1, Fig. 4). No difference in AGE accumulation was found between the crossover group and the bicarbonate/lactate PDF-treated group (Table 1, Fig. 4). After 12 weeks of PDF exposure, TGF-β expression was not different in the standard PDF and bicarbonate/lactate PDF group (Table 1, Fig. 5). At 20 weeks,

Table 1. Histologic and immunohistochemical analysis of the peritoneum

	Dianeal 12 weeks	Physioneal 12 weeks	Dianeal 20 weeks	Crossover	Physioneal 20 weeks
Blood vessels N/mm^2	183.93 ± 16.84	120.75 ± 6.34[a]	299.72 ± 9.10	255.46 ± 16.06[b]	219.53 ± 11.70[b]
eNOS staining%	0.36 ± 0.05	0.36 ± 0.06	0.88 ± 0.10	0.58 ± 0.04[c,d]	0.39 ± 0.05[c]
VEGF staining%	1.04 ± 0.12	0.62 ± 0.09[e]	1.40 ± 0.12	1.13 ± 0.09	0.89 ± 0.10[f]
Picro Sirius Red staining%	2.46 ± 0.28	1.80 ± 0.14[e]	3.76 ± 0.28	3.24 ± 0.16	2.95 ± 0.21[b]
AGE staining%	3.41 ± 0.88	1.06 ± 0.12[e]	3.88 ± 0.21	1.22 ± 0.28[g]	1.41 ± 0.19[g]
TGF-β staining%	0.47 ± 0.04	0.58 ± 0.13	1.55 ± 0.30	1.23 ± 0.14[d]	0.77 ± 0.15[b]

[a] $P < 0.005$ vs. Dianeal (12 weeks); [b] $P < 0.05$ vs. Dianeal (20 weeks); [c] $P < 0.01$ vs. Dianeal (20 weeks); [d] $P < 0.05$ vs. Physioneal (20 weeks); [e] $P < 0.05$ vs. Dianeal (12 weeks); [f] $P < 0.005$ vs. Dianeal (20 weeks); [g] $P < 0.0001$ vs. Dianeal (20 weeks).

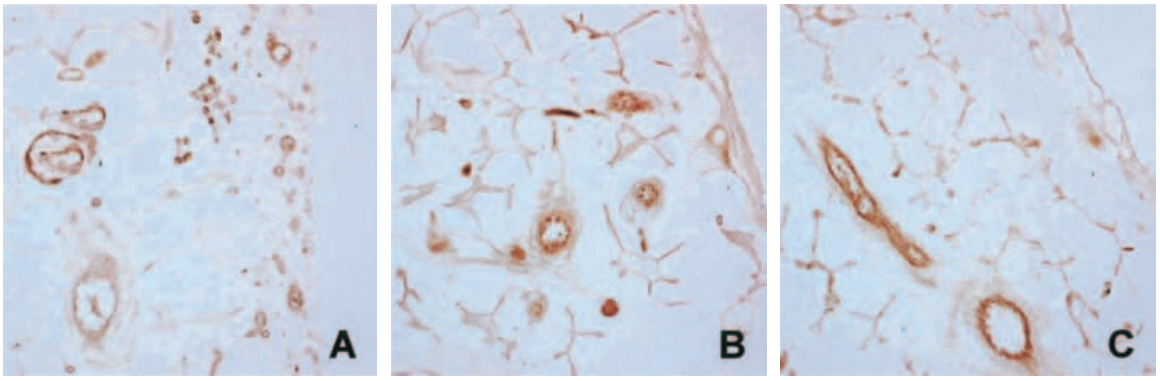

Fig. 2. Immunostaining for eNOS of the visceral peritoneum (×200) exposed to standard lactate-buffered PDF for 20 weeks (*A*, *N* = 11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (*B*, *N* = 10), and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (*C*, *N* = 10).

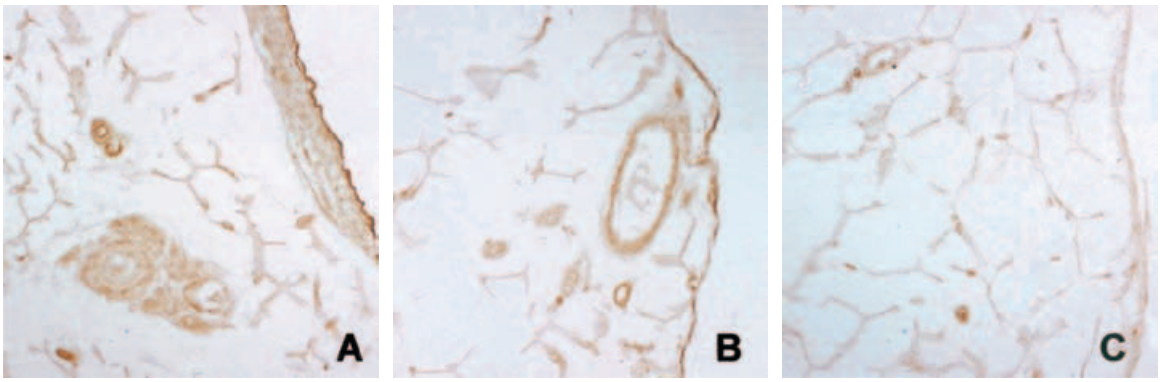

Fig. 3. Vascular endothelial growth factor (VEGF) expression, expressed as percentage staining, was evaluated by a VEGF immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (*A*, *N* = 11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (*B*, *N* = 10), and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (*C*, *N* = 10).

TGF-β was up-regulated in the standard PDF group compared with the bicarbonate/lactate PDF group. Intermediary values were measured in the crossover group (Table 1, Fig. 5). Fibrosis was more pronounced after 12 and 20 weeks exposure to standard PDF compared to bicarbonate/lactate PDF (Table 1, Fig. 6). The degree of fibrosis in the crossover group did not differ significantly from both the standard PDF and the bicarbonate/lactate PDF treated groups (Table 1, Fig. 6). Fibrosis correlated positively with TGF-β expression (Pearson $r = 0.3129$, $P < 0.05$). Furthermore, AGE accumulation and VEGF expression showed a positive correlation (Pearson $r = 0.3229$, $P < 0.05$).

DISCUSSION

Chronic exposure of the rat peritoneal membrane to standard PDF is characterized by progressive fibrosis and

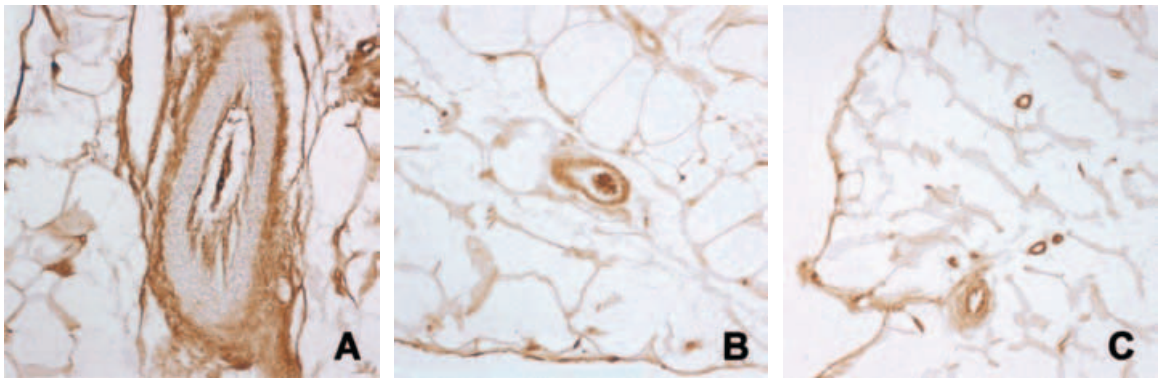

Fig. 4. Advanced glycation end product (AGE) accumulation, expressed as percentage staining, was evaluated by an AGE immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (*A*, *N* = 11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (*B*, *N* = 10), and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (*C*, *N* = 10).

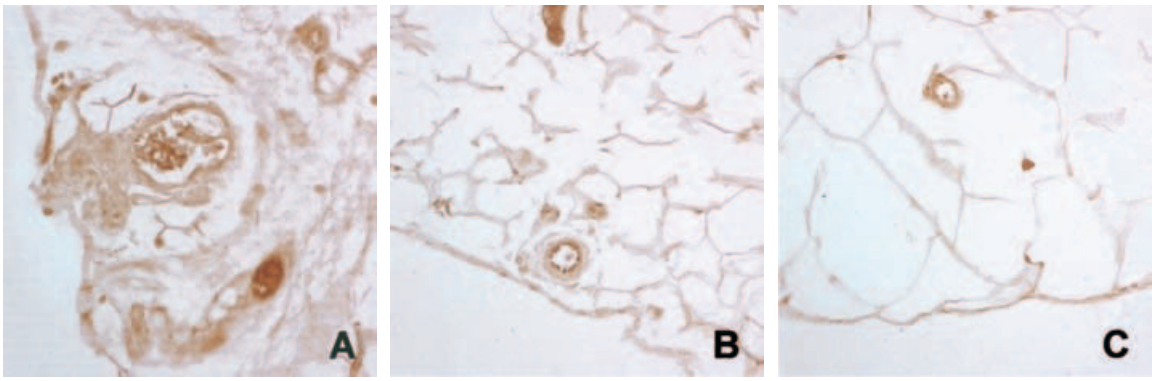

Fig. 5. TGF-β expression, expressed as percentage staining, was evaluated by a TGF-β immunostaining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (*A*, *N* = 11), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 week (*B*, *N* = 10), and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (*C*, *N* = 10).

neoangiogenesis associated with up-regulation of TGF-β and VEGF, respectively. In addition, a pronounced AGE accumulation was observed. Functionally, the membrane is characterized by a loss of ultrafiltration capacity. Although the present observations are descriptive and do not prove causal relations, they support a key pathogenic role for TGF-β and VEGF in the development of fibrosis and neoangiogenesis. The importance of these growth factors has previously been demonstrated in animal models [13–16], but direct evidence in PD patients is presently lacking. Furthermore, AGE accumulation is involved in the development of peritoneal alterations, as their capacity to promote TGF-β expression through interaction with RAGE has been evidenced [16], and they are known to up-regulate VEGF [17, 18]. The peritoneal changes tend to progress with time, confirming the results obtained in cross-sectional and longitudinal studies in PD patients [2, 3, 19].

Long-term treatment of the rat peritoneal membrane with the low-GDP bicarbonate/lactate-buffered PDF induced less neoangiogenesis with a concomitant lower eNOS and VEGF expression, and was associated with less AGE accumulation, TGF-β expression, and fibrosis compared to standard PDF. In addition, ultrafiltration capacity was better preserved. These findings confirm previous observations in chronic rat models of peritoneal exposure [7, 8]. Clinical studies found increased ultrafiltration [10], elevated CA125, and decreased hyaluronan levels [9] in patients treated with bicarbonate/lactate-buffered PDF, suggestive of a better preservation of peritoneal membrane homeostasis. Unfortunately, but for obvious reasons, no direct comparisons of peritoneal membrane morphology are available. In our previous work, peritoneal membrane function and structure was similar after 12 weeks exposure to bicarbonate/lactate PDF and a buffer solution, suggesting that high glucose concentrations in the absence of other bioincompatible factors were not harmful to the peritoneum at that point. However, the present results at 20 weeks of study demonstrate that fibrosis and neoangiogenesis progress with time, also

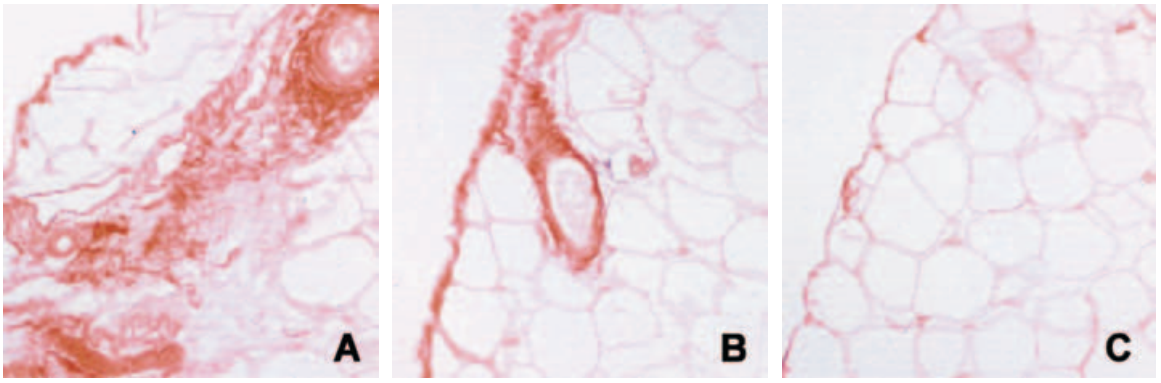

Fig. 6. Fibrosis was evaluated with a Picro Sirius Red staining of the visceral peritoneum exposed to standard lactate-buffered PDF for 20 weeks (*A*, $N = 11$), standard lactate-buffered PDF for 12 weeks followed by exposure to low-GDP bicarbonate/lactate-buffered PDF for 8 weeks (*B*, $N = 10$), and low-GDP bicarbonate/lactate-buffered PDF for 20 weeks (*C*, $N = 10$).

in the low-GDP bicarbonate/lactate-buffered treated animals, suggesting that glucose by itself is capable of inducing these changes. The present study thus indicates that the PDF-induced changes are time-dependent, and underlines the importance of sufficiently long exposure periods in experimental animal models in order to reveal long-term PDF-induced effects.

The salient observation of the present study, however, is that crossover from the standard PDF to low-GDP bicarbonate/lactate-buffered PDF was associated with less peritoneal membrane deterioration than continuous exposure to standard PDF. In PD patients, crossover from a conventional PDF to a glucose-free regimen [20] or low-GDP lactate-buffered PDF [21] was associated with improvement of different markers of peritoneal integrity: CA125 levels increased, whereas hyaluronan levels, local VEGF production, and circulating AGE levels decreased. If the present observations are confirmed in clinical trials, a switch to low-GDP bicarbonate/lactate-buffered PDF may be an alternative to peritoneal resting or transfer to hemodialysis for the management of patients with peritoneal membrane dysfunction.

CONCLUSION

The present study confirms the superior biocompatibility of the low-GDP bicarbonate/lactate-buffered PDF during a long-term treatment period. Furthermore, this new low-GDP, bicarbonate/lactate-buffered PDF with physiologic pH has the potential to slow down peritoneal damage induced by the standard PDF.

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