Dialysis fluid cytotoxicity and inhibition of host defence in cultured human mesothelial cells are neutralized rapidly with incubation in the peritoneum

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Abstract

Background. A recent survey puts the global dialysis population at 535,100; of those on peritoneal dialysis, 85% are on continuous ambulatory peritoneal dialysis. Current hyperosmolar dialysis fluids are toxic to peritoneal cells and inhibit certain host-defence functions. An alternative preparation, glucose polymer, has recently been developed.

Methods. Mesothelial cell viability, interleukin-6 and prostacyclin synthesis, after exposure to 7.5% glucose polymer, 1.36% glucose or 3.86% glucose peritoneal dialysis effluent solution was assessed.

Results. In its neat form, at an original pH of 5.4, glucose polymer was as toxic as hyperosmolar solutions (P < 0.01). Synthesis of interleukin-6 and prostacyclin were significantly inhibited by neat dialysis fluid, (P < 0.01). However, after an in vivo intraperitoneal incubation of only 15 min the toxicity of all solutions tested in vitro was lost.

Conclusions. Despite rapid in situ neutralization of dialysis fluid toxicity, mesothelial injury and inhibition of host-defence function, early in the dialysis cycle, may affect peritoneal physiology given the complex network of pathways to which these cells contribute. Although recent trials indicate improved ultrafiltration is achievable with glucose polymer, it is not a biocompatible dialysis fluid in its current manufactured form.

Key words: CAPD; cytotoxicity; glucose polymer; host defence; signal transduction

Introduction

A recent editorial in Kidney International [1] predicted that the prevalence of patients on dialysis globally in the year 2000 will be 75% higher than in 1992. Of the global dialysis population, 16% are on PD with 85% of PD patients on continuous ambulatory peritoneal dialysis (CAPD) [2]. Such data underline the importance of CAPD as a treatment modality for end-stage renal failure. Problems associated with CAPD therefore become increasingly important in long-term maintenance of the PD population.

Despite considerable success the long-term viability of the therapy is in question. In order to ensure continuance on PD a functional peritoneum must be maintained. However, during PD instillation of large volumes of unphysiological hyperosmolar dialysis fluid (DF) may damage the peritoneum. Loss of ultrafiltration, increased glucose absorption, protein loss, and impairment of host defence has been implied [3]. With a large increase in patients likely in the near future, more physiological and efficient DFs are required.

Alternatives to unphysiological DFs have been investigated with the focus on two main areas, improved buffer capacity by inclusion of bicarbonate and reduction of osmolarity. The latter has been achieved with the development of an isosmolar glucose polymer (GP) solution; to date this is the only commercially available alternative to hyperosmolar DF. This study was undertaken to assess, in vitro, the effect on human peritoneal mesothelial cell viability and cytokine release of glucose and GP solutions and effluents of varying dwell times.

Subjects and methods

After obtaining ethical approval, omentum was taken from patients undergoing elective vascular procedures and digested by end-over-end rotation in sterile collagenase II (Sigma-Aldrich Company Ltd., Poole, UK) at a concentration of 3 mg/ml in phosphate-buffered saline (PBS, Sigma-Aldrich). All equipment and solutions were sterile and the procedures performed in a class II safety cabinet. Cells were incubated at 37°C in a 5% CO₂ atmosphere in Ham’s F12 (ICN Biomedicals Ltd., Oxon, UK) supplemented with 10% v/v fetal calf serum (Imperial Laboratories Ltd, Andover, UK) L-glutamine 2 mmol, penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin B 1 μg/ml.
Early-dwell glucose and glucose polymer dialysates are toxic to HPMC in vitro

100 µg/ml (Life Technologies Limited, Paisley, UK), insulin 0.5 µg/ml, transferrin 0.5 µg/ml and hydrocortisone 0.4 µg/ml (Sigma-Aldrich.) Large numbers of cells were obtained and developed a classic mesothelial 'cobblestone' morphology at confluence. Positive identification of cells was obtained using indirect immunocytochemistry with a fluorescein isothiocyanate label (DAKO AS, Glostrup, Denmark).

Peritoneal dialysis effluents (PDE) were collected from patients over the course of the dialysis cycle. The first sample was collected immediately after draining a new bag into the peritoneum and was designated as the zero time point, this sample being neat dialysis fluid. Subsequent samples were collected at 15, 30, 120, 240 and 360 min into the cycle. Samples were collected in sterile universals by qualified personnel using sterile procedure. PDEs were transferred to the laboratory on ice, centrifuged at 1200 r.p.m. to remove cell debris and filtered through a 0.22 µm filter (Sartorius Ltd, Epsom, UK). PDEs were then applied to HPMC cultures in vitro for a 30 min period. Three PDEs were evaluated: 1.36% hyperosmolar glucose (136G), 3.86% hyperosmolar glucose (386G), and 7.5% isosmolar glucose polymer solution (GP); all three DFs were pH 5.4. All fluids were made up with the same electrolyte solution to which glucose or GP was added. Electrolyte solution contained the following (mmol/l): sodium 133, calcium 1.75, magnesium 0.25, chloride 97 and lactate 40.

Effluents were collected from a total of nine CAPD patients. Three patients were dialysing with 136G, three with 386G, and three with GP. The PDEs from individual patients in each respective group were designated a, b, or c. Passage one HPMC from the same cell line were exposed to PDEs for a 30-min period in 24-well plates at a density of 25 000 cells per well. Two milliliters of PDE were added per seeded well, replicates of six wells were treated; a further six wells were treated with culture medium (CM) as controls. Following exposure to PDE conditioned medium was collected for assay. Cells were then washed in PBS and viability assessed in the highly reproducible MTT assay.

IL-6 and prostacyclin (measured as its stable breakdown product 6-keto prostaglandin-F1α, PGF1α) levels in conditioned medium were assayed using the Biotrak® ELISA and EIA systems respectively from Amersham International plc, Buckinghamshire, UK).

Statistics

Data was analysed using one-way analysis of variance (ANOVA) with Dunnett multiple comparisons test.

Results

Cells cultured from human omentum stained highly positively for human cytokeratins 6, 8 and 18 (Sigma-Aldrich), known mesothelial cell markers [4]. Cultures were also completely negative for human CD34 (DAKO A/S), a marker of microvascular endothelium [5] and von Willebrand factor [6], excluding a macrovascular origin for the cell type. The result confirmed the mesothelial origin of the cells isolated.

Correlation regression analysis determined a linear relationship between increase in HPMC number and absorbance in the MTT assay, with a correlation coefficient of 0.985 and a P value of 0.0073, n = 5.

Mesothelial cell viability (mean ± SEM) following exposure to PDE is shown in Figures 1, 2 and 3, n = 6. An almost identical effect on cell viability is apparent with PDE from all patients, regardless of DF used. In all cases 0 PDE caused significant reduction in viability compared with cells exposed to CM for the same period of time (P < 0.01). At 15 min into the CAPD cycle this effect was lost and in two cases, 136G and GPα, exposure to 15 min PDE significantly increased the number of viable cells compared to controls (P < 0.05). HPMC treated with PDE collected after 15-min dwell time did in many cases have significantly increased viability compared to controls.

Interleukin-6 synthesis in HPMC after exposure to PDE is shown in Table 1. No significant difference in IL-6 synthesis compared to control was noted for 386c α PDE. All other α PDE samples caused significant reduction in IL-6 synthesis. The toxicity of current DFs, including GP, is worrying. Denudation of HPMC and subsequent exposure of extracellular matrix may be an initiating event in peritoneal fibrosis [7]. The latter may be further complicated by infection, leading to irreversible damage and dysfunction of the peritoneal membrane. Impairment of HPMC host-defence function is evidenced by the complete inhibition of IL-6 and PGF1α synthesis in cells exposed to neat DF. Although inhibitory effects are lost after only 15 min in vivo, substantial damage to host defence activity may have occurred in this short window of time. It is possible that patient 386c had minor irritation of the peritoneum at the time of sampling and this may explain the steady-state IL-6 and PGF1α levels recorded, compared to the inhibition observed with all other PDEs at α.

Interleukin-6 is a multifunctional cytokine and is involved in optimal immune response to injury and infection [8]. The IL-6 receptor (IL-6R) complex is involved in signal transduction pathways via gp130, a class I cytokine receptor. The receptor complex forms associations with at least three of the Janus family of intracellular protein kinases (PK) which catalyse phosphorylation of transcription factors known as Stat proteins 3 (signal transducers and activators). Phosphorylated Stats translocate to the nucleus and...
Fig. 1. HPMC viability following 30-min exposure to 136 PDE collected at 0, 15, 30, 120, 240 or 360-min dwell time from three patients; a, b, c. Results are presented as mean (±SEM) of six experiments. * and ** represent a statistically significant difference of $P<0.05$ and $P<0.01$ respectively compared to controls (CM).

Fig. 2. HPMC viability following 30-min exposure to 386 PDE collected at 0, 15, 30, 120, 240 or 360-min dwell time from three patients; a, b, c. Results are presented as mean (±SEM) of six experiments. * and ** represent a statistically significant difference of $P<0.05$ and $P<0.01$ respectively compared to controls (CM).
Early-dwell glucose and glucose polymer dialysates are toxic to HPMC \textit{in vitro}

![Graph showing HPMC viability following 30-min exposure to GP PDE collected at 0, 15, 30, 120, 240 or 360-min dwell time from three patients; a, b, c. Results are presented as mean (±SEM) of six experiments. * and ** represent a statistically significant difference of \( P<0.05 \) and \( P<0.01 \) respectively compared to controls (CM).

**Fig. 3.** HPMC viability following 30-min exposure to GP PDE collected at 0, 15, 30, 120, 240 or 360-min dwell time from three patients; a, b, c. Results are presented as mean (±SEM) of six experiments. * and ** represent a statistically significant difference of \( P<0.05 \) and \( P<0.01 \) respectively compared to controls (CM).

bind gene response elements inducing gene expression [9]. IL-6 is the activator signal in this system, inhibition of the cytokines synthesis in HPMC may impair optimal immune response to injury or infection.

PGF\(_{1\alpha}\), the stable breakdown product of prostacyclin, is the major prostanoid in PDE. As regards CAPD, prostacyclin has two important properties, the first is vasodilatation. Increased vasodilatation leads to increased glucose absorption and consequently to a decline in ultrafiltration. Elevated protein clearance during peritonitis is also directly related to the dialysate prostaglandin (PG) concentration [10].

PGs also regulate the activity of inflammatory cells. Recently induction of cyclo-oxygenase mRNA, the precursor for prostaglandin synthesis, was effected by stimulation of HPMC with macrophage-derived cytokines. It is thought that during peritonitis, prostaglandins form part of a negative feedback loop via downregulation of cytokine release by macrophages [11]. Inhibition of HPMC prostaglandin synthesis following exposure to DF therefore amounts to an unacceptable compromise of host defence.

Intraperitoneal equilibration of DFs occurs within 15 min but many signal transduction cascades may be activated during this period. The relevance of signal transduction to our understanding of CAPD’s impact is emphasised with the discovery of osmosensing pathways in mammalian cells [12]. Such reports indicate that PKs may mediate mammalian cell responses to osmotic stress. Gargova \textit{et al.} [12] observed marked increase in PK activity in cells exposed to hyperosmolar media within 5 min, with maximal activity between 15 and 30 min. This time frame corresponds with the amount of time required for intraperitoneal equilibration of dialysate.

Another recent report [13] documented protection against osmotic stress by cyclic guanosine \( 3',5' \)-monophosphate-mediated phosphorylation of myosin. This study demonstrated an osmo-induced relocalization of myosin which may provide the physical strength necessary to withstand extensive shrinkage in solutions of high osmolality. This mechanism may provide an ideal sensitive system for future \textit{in vitro} analysis of DF impact on peritoneal cells.

There are at present only limited studies on the biocompatibility of GP compared with conventional DFs. These analyses utilized different cell types and functional parameters and are not directly comparable. De Fijter [14] demonstrated significantly greater PM\( \Phi \) bactericidal activity in GP. Increasing the osmolality of GP to that of the respective glucose-based fluid reduced the effect, suggesting an osmolality-mediated mechanism. However, there was no significant difference in viability with GP compared to conventional DF. Jorres \textit{et al.} [3] and Liberek \textit{et al.} [15] both reported that GP-induced inhibition of host-defence function in leukocytes was reversed by neutralizing the original pH of the solution.

More recently Jorres \textit{et al.} [16] noted that conventional DF downregulated tumour necrosis factor \( \alpha \)
### Table 1. HPMC IL-6 synthesis after 30-min exposure to 136G, 386G, or GP PDE collected at 0, 15, 30, 120, 240 or 360-min dwell time from three patients; a, b, c

<table>
<thead>
<tr>
<th>PDE</th>
<th>136Ga</th>
<th>136Gb</th>
<th>136Gc</th>
<th>386Ga</th>
<th>386Gb</th>
<th>386Gc</th>
<th>GPA</th>
<th>GPb</th>
<th>GPc</th>
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<td>3287 (73)</td>
<td>1017 (85)</td>
<td>312 (16)</td>
<td>1205 (96)</td>
<td>1538 (47)</td>
<td>1133 (99)</td>
<td>2300 (111)</td>
<td>364 (15)</td>
<td>1136 (30)</td>
</tr>
<tr>
<td>0</td>
<td>28 (3)</td>
<td>** 5.4 (0.85)**</td>
<td>** 6.15 (1.8)**</td>
<td>** 29 (8)**</td>
<td>** 200 (28)**</td>
<td>** 976 (88)**</td>
<td>ns</td>
<td>16.4 (1.4)**</td>
<td>5 (0.18)**</td>
</tr>
<tr>
<td>12</td>
<td>3621 (75)</td>
<td>ns 1441 (104)</td>
<td>* 425 (21)</td>
<td>* 1318 (42)</td>
<td>ns 1826 (122)</td>
<td>* 2364 (63)**</td>
<td>** 3194 (94)**</td>
<td>** 398 (17) ns 1461 (78)**</td>
<td>**</td>
</tr>
<tr>
<td>30</td>
<td>3438 (480)</td>
<td>ns 1599 (129)</td>
<td>** 441 (39)</td>
<td>* 1499 (94)</td>
<td>* 1566 (103)</td>
<td>ns 2160 (145)**</td>
<td>** 3509 (98)**</td>
<td>** 602 (11) ns 1456 (56)**</td>
<td>**</td>
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<tr>
<td>120</td>
<td>3548 (110)</td>
<td>ns 2467 (137)</td>
<td>** 572 (46)</td>
<td>** 1342 (38)</td>
<td>ns 1211 (41)</td>
<td>* 2387 (107)**</td>
<td>** 2980 (128)**</td>
<td>** 505 (22) ns 1076 (48) ns 2047 (78)**</td>
<td>**</td>
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<tr>
<td>240</td>
<td>3869 (195)</td>
<td>ns 2651 (99)</td>
<td>** 426 (44)</td>
<td>ns 1248 (133)</td>
<td>ns 1254 (39)</td>
<td>* 1985 (66)**</td>
<td>** 2929 (24)**</td>
<td>** 436 (36) ns 1092 (51) ns 995 (58) ns 2047 (78)**</td>
<td>**</td>
</tr>
<tr>
<td>360</td>
<td>2733 (208)</td>
<td>ns 1784 (78)</td>
<td>** 440 (28)</td>
<td>* 1099 (91)</td>
<td>ns 1146 (55)**</td>
<td>** 1188 (78) ns 2719 (73)**</td>
<td>** 344 (26) ns 995 (58) ns 2047 (78)**</td>
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</table>

Results are presented as mean (SEM) of six experiments; ns, not significantly different from CM; *significant difference from CM (P<0.05); **significant difference from CM (P<0.01).

### Table 2. HPMC PGI₂ synthesis (measured as its stable breakdown product 6-keto prostaglandin-F₁α) after 30-min exposure to 136G, 386G or GP PDE collected at 0, 15, 30, 120, 240 or 360 min dwell time from three patients; a, b, c

<table>
<thead>
<tr>
<th>PDE</th>
<th>136Ga</th>
<th>136Gb</th>
<th>136Gc</th>
<th>386Ga</th>
<th>386Gb</th>
<th>386Gc</th>
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<td>51 (2)</td>
<td>56 (3)</td>
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<td>14 (1)</td>
<td>** 15 (2)</td>
<td>** 4 (2)</td>
<td>** 12 (2)</td>
<td>** 28 (2)</td>
<td>** 124 (9)</td>
<td>ns 42 (1) ** 14 (1)</td>
<td>** 35 (2) **</td>
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<tr>
<td>15</td>
<td>91 (5)</td>
<td>ns 65 (2)</td>
<td>ns 50 (11)</td>
<td>ns 67 (2)</td>
<td>ns 93 (9)</td>
<td>ns 197 (20)</td>
<td>ns 115 (4)</td>
<td>ns 43 (6) ns 137 (10) ns 55 (7)</td>
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</tr>
<tr>
<td>30</td>
<td>93 (7)</td>
<td>* 63 (3)</td>
<td>ns 62 (7)</td>
<td>ns 71 (6)</td>
<td>* 91 (5)</td>
<td>ns 205 (10)</td>
<td>ns 121 (7)</td>
<td>ns 54 (7) ns 125 (5) ns 55 (7)</td>
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<tr>
<td>120</td>
<td>86 (5)</td>
<td>ns 76 (5)</td>
<td>** 59 (8)</td>
<td>ns 73 (3)</td>
<td>** 56 (7)</td>
<td>ns 223 (24)</td>
<td>ns 110 (11)</td>
<td>ns 61 (9) ns 98 (5) ns 55 (7)</td>
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<tr>
<td>240</td>
<td>73 (5)</td>
<td>ns 72 (3)</td>
<td>** 45 (4)</td>
<td>ns 71 (2)</td>
<td>* 52 (7)</td>
<td>ns 261 (76)**</td>
<td>** 93 (9)</td>
<td>* 51 (2) ns 104 (6) ns 55 (7)</td>
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<tr>
<td>360</td>
<td>74 (3)</td>
<td>ns 57 (5)</td>
<td>ns 47 (7)</td>
<td>ns 65 (3)</td>
<td>ns 64 (8)</td>
<td>ns 142 (9)</td>
<td>ns 94 (13)</td>
<td>* 53 (3) ns 95 (6)</td>
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</table>

Results are presented as mean (SEM) of six experiments; ns, not significantly different from CM; *significant difference from CM (P<0.05); **significant difference from CM (P<0.01).
Early-dwell glucose and glucose polymer dialysates are toxic to HPMC in vitro

(TNFα) and IL-6 message, as did bicarbonate-buffered solution of high glucose concentration. However, low glucose concentration bicarbonate-buffered solution and GP at pH 5.5 and pH 7.5 did not cause any inhibition of IL-6 or TNFα message. Cytokine synthesis in GP was inhibited at low pH, as was the case with HPMC in our study. Since at low pH GP does not affect cytokine mRNA expression, the inhibitory mechanism must occur at the post-transcription stage in the cytosol, or at the translation level.

How peritoneal cells interact in host defence is largely unknown, as is the interaction between the mediators they produce. Recently models of the host-defence network have been described [17]. The peritoneal mesothelial cell is considered a major contributor to this network. Not only do HPMC secrete IL-6, IL-8, IL-1α, and IL-1β, but they also secrete PGE₂ and PGI₂, which are upregulated in response to inflammatory cytokines. As well as proinflammatory functions, IL-6 and PGI₂ may function as control elements by their inhibition of macrophage cytokine synthesis.

There seems little doubt that current DFs are toxic at a cellular level, but what mechanisms are involved? One question to be answered is what occurs at the intracellular level? Are signal transduction cascades activated in HPMCs following exposure to DF? Is there a long-term toxic effect at the level of gene transcription, as recently proposed by Robertson [18], following exposure to hyperosmolar solutions?

The biocompatibility of dialysis fluid has recently been described as ‘the biological effect that a peritoneal dialysis solution exerts on the normal function of the tissues and cells of the peritoneum during both uninfected and infected states’ [19]. On the basis of this definition and with respect to our in vitro study and others thus far reported, GP is not a biocompatible solution.

Despite the negative aspects of such studies, recent trials have shown improved ultrafiltration and satisfactory biochemical profiles in patients using GP [20]. When all data are considered, GP remains a realistic alternative for long dwell peritoneal dialysis. However, conventional DF, GP, and any new experimental solutions must be made more physiological to avoid damaging the peritoneum in the first minutes of the CAPD cycle. By failing to make solutions biocompatible we may impede optimum host defence and activate pathways leading to overexpression of inflammatory mediators.

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References


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