ORIGINAL ARTICLES

HYPERBRANCHED POLYGLYCEROL IS AN EFFICACIOUS AND BIOCOMPATIBLE NOVEL OSMOTIC AGENT IN A RODENT MODEL OF PERITONEAL DIALYSIS

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♦ **Objectives:** To enhance the effectiveness of peritoneal dialysis (PD), new biocompatible PD solutions may be needed. The present study was designed to test the efficacy and biocompatibility of hyperbranched polyglycerol (HPG)—a nontoxic, nonimmunogenic water-soluble polyether polymer—in PD.

♦ **Methods:** Adult Sprague–Dawley rats were instilled with 30 mL HPG solution (molecular weight 3 kDa; 2.5%–15%) or control glucose PD solution (2.5% Dianeal; Baxter Healthcare Corporation, Deerfield, IL, USA), and intraperitoneal fluid was recovered after 4 hours. Peritoneal injury and cellular infiltration were determined by histologic and flow cytometric analysis. Human peritoneal mesothelial cells were assessed for viability *in vitro* after 3 hours of PD fluid exposure.

♦ **Results:** The 15% HPG solution achieved a 4-hour dose-related ultrafiltration up to 43.33 ± 5.24 mL and a dose-related urea clearance up to 39.17 ± 5.21 mL, results that were superior to those with control PD solution ($p < 0.05$). The dialysate-to-plasma (D/P) ratios of urea with 7.5% and 15% HPG solution were not statistically different from those with control PD solution. Compared with fluid recovered

from the control group, fluid recovered from the HPG group contained proportionally fewer neutrophils ($3.63\% \pm 0.87\%$ vs $9.31\% \pm 2.89\%$, $p < 0.0001$). Detachment of mesothelial cells positive for human bone marrow endothelial protein 1 did not increase in the HPG group compared with the stain control ($p = 0.1832$), but it was elevated in the control PD solution group ($1.62\% \pm 0.68\%$ vs $0.41\% \pm 0.31\%$, $p = 0.0031$). Peritoneal biopsies from animals in the HPG PD group, compared with those from control PD animals, demonstrated less neutrophilic infiltration and reduced thickness. Human peritoneal mesothelial cell survival after HPG exposure was superior *in vitro* ($p < 0.0001$, 7.5% HPG vs control; $p < 0.01$, 15% HPG vs control). Exposure to glucose PD solution induced cytoplasmic vacuolation and caspase 3-independent necrotic cell death that was not seen with HPG solution.

♦ **Conclusions:** Our novel HPG PD solution demonstrated effective ultrafiltration and waste removal with reduced peritoneal injury in a rodent model of PD.

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Peritoneal dialysis (PD) is a simple and safe renal replacement therapy (1–4) that currently constitutes 0%–70% of national dialysis programs worldwide (5). To

date, glucose remains the most common osmotic agent in commercial PD solutions, although other osmotic agents such as amino acids and glucose polymer (icodextrin) are available. A high level of glucose in PD solution has been documented to be associated with many systemic and locoregional health complications for PD patients. Daily exposure to glucose can cause hyperglycemia, hyperinsulinemia, obesity, and exacerbation of diabetes mellitus (6). Moreover, long-term exposure to glucose and glucose degradation products has been shown to directly damage the peritoneal membrane, leading to abnormal mesothelial transformation, maladaptive angiogenesis, and ultrafiltration (UF) failure (7–9). Thus, PD may become less effective over time or lead to metabolic complications that are not favorable over the long term, thereby adversely affecting both PD duration and mortality. New biocompatible PD solutions that address some of these unwanted complications are needed.

Hyperbranched polyglycerol (HPG) is a compact, branched, and highly water-soluble polyether polymer (Figure 1) synthesized by single-step, multi-branching, ring-opening polymerization of glycidol under slow monomer addition (10). Hyperbranched polyglycerol can be precisely synthesized over a wide range of molecular weights and is chemically stable as a narrow distributed polymer in aqueous solution (11–13). It is highly hydrophilic and water-soluble (>400 mg/mL), with a very low intrinsic viscosity ($4 - 7 \text{ mL g}^{-1}$), and therefore large amounts of the polymer can easily be dissolved in water (13,14). It also has multiple hydroxyl groups per molecule of polymer and exists at physiologic pH in aqueous solution (14). In experimental studies, we demonstrated that HPG and its derivative molecules

are highly blood-compatible, nonimmunogenic, and nontoxic (14–17). Unlike other polymers, HPG has been shown to have very limited organ accumulation after intravenous injection (14,18). It does not activate the platelet, coagulation, or complement systems (14). Taken together, these properties make HPG a promising candidate for use as an osmotic agent in PD.

METHODS

As proof of concept, novel osmotic agents must fulfill three criteria to be considered for use in PD:

- They must produce net fluid movement into the peritoneal cavity (UF).
- They must facilitate removal of waste molecules from the bloodstream into dialysis fluid (clearance).
- They must cause the least amount of mesothelial injury or inflammation of the peritoneal membrane (biocompatibility).

To evaluate the foregoing criteria, solutions of 3 kDa HPG at various concentrations (Table 1) were compared directly with conventional glucose-based PD solution (Dianeal PD4 2.5%: Baxter Healthcare Corporation, Deerfield, IL, USA), herein denoted as “PDS.”

ANIMALS AND CELL CULTURES

Inbred male Sprague–Dawley rats (approximately 300 g body weight, 10 – 12 weeks old) were purchased from Charles River Laboratories International (Wilmington, MA, USA), and maintained in the animal facility of the Jack Bell Research Centre of the University

Figure 1 — Chemical structure of hyperbranched polyglycerol.

TABLE 1
Osmolality, Density, and pH of Hyperbranched Polyglycerol (HPG) Solutions

HPG concentrations (% w/v)	Osmolality (mOsm/kg) ^a	Density (g/mL) ^a	pH [median (range)] ^b
2.5	279	1.0240	6.62 (6.60–6.67)
5	294	1.0264	6.79 (6.63–6.90)
7.5	324	1.0290	7.19 (7.04–7.30)
15	424	1.0482	7.27 (7.19–7.32)

^a Measured in duplicate at the Chemistry Laboratory of the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada).

^b Recorded during a period of 10 minutes using a laboratory pH meter (Accumet B15 Basic: Fisher Scientific, Toronto, ON, Canada).

of British Columbia (Vancouver, BC, Canada). Animal experiments were performed in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at the University of British Columbia.

Human peritoneal mesothelial cells (HPMCs) were isolated from PD effluent donated by anonymous PD patients at our clinic, using the protocol approved by the Clinical Research Ethics Board at the University of British Columbia. The HPMC's were immortalized with origin-deficient SV40 DNA. Primary and SV40-immortalized HPMC's were both grown in complete K1 medium as previously described (19).

PREPARATION OF HPG SOLUTIONS

Hyperbranched polyglycerol {number of average molecular weight (M_n): 3000 g/mol; polydispersity index [weight of average molecular weight (M_w) / M_n]: 1.1} was synthesized and characterized as previously described (10,11). The absolute molecular weight and polydispersity index of the HPG was determined by gel permeation chromatography using multi-angle laser light scattering (DAWN-EOS laser photometer: Wyatt Technology, Santa Barbara, CA, USA) and Optilab RI detectors (Wyatt Technology) in aqueous 0.1 N NaNO_3 solution. The experimentally determined dn/dc value (0.120 mL/g) was used for the molecular weight determination. Ultrahydrogel columns (Waters Corporation, Milford, MA, USA) were used for the analysis.

The HPG solutions (2.5% – 15%) were prepared by dissolving HPG (2.5 – 15 g polymer) in 100 mL sterile electrolyte solution containing NaCl (53.8 mg/L), $\text{NaC}_3\text{H}_5\text{O}_3$ (44.8 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.83 mg/L), and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.508 mg/L)—that is, the same electrolyte composition used in the control PDS. The osmolality of each solution was measured using an Advanced Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, MA, USA) at the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada). The pH of each HPG solution was recorded using a laboratory pH meter within a period of 10 minutes.

RAT MODEL OF PD

The efficacy and biocompatibility of the HPG solutions compared with control PDS was examined using Sprague–Dawley rats in a model that simulates a single PD dwell. Under general anesthesia, 30 mL of pre-warmed HPG solution or PDS was slowly injected into the peritoneal cavity. Immediately (basal control at 0 hours) or 4 hours after intraperitoneal injection, the animals were

humanely euthanized, and peritoneal effluent and serum were collected. Parietal peritoneum was collected in 3 randomly selected rats from each group. A single intraperitoneal injection was used to avoid the confounding factor of catheter insertion, which might exacerbate peritoneal injury.

EVALUATION OF UF AND WASTE REMOVAL

As a marker of UF capacity, the volume of peritoneal effluent recovered 4 hours after intraperitoneal injection was compared with the volume recovered at 0 hours (20). Urea nitrogen was used as a marker of waste removal because the creatinine level in peritoneal effluent was below the minimal level for measurement in the laboratory (data not shown). Absolute urea removal was calculated as the urea concentration in recovered dialysate (D) multiplied by its volume (V). Small-solute transport was evaluated by calculating the dialysate-to-plasma (D/P) ratio of urea. Urea clearance was calculated by multiplying the D/P ratio by the volume of dialysate ($D/P \times V$). As in previous studies, uremia was not considered necessary to accurately assess fluid and waste transport in our rodents over a single dwell (21).

HISTOLOGIC EXAMINATION

Peritoneal tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were cut at 4- μm thickness and stained with hematoxylin and eosin. In each peritoneal specimen, two separate sections of each strip were examined under microscopy (400 $\times$ magnification) in a blinded fashion. Submesothelial thickness was measured, and polymorphonuclear infiltration was used as a marker of peritoneal inflammation.

FLOW CYTOMETRIC ANALYSIS

Fluid biocompatibility profile was assessed *in vivo* after PD exposure by flow cytometric analysis of the cell populations in recovered peritoneal effluent. Flow cytometric analysis was performed using a BD FACSCanto II (BD Biosciences, Mississauga, ON, Canada). At least 10 000 events were counted for each sample, and data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Neutrophils/granulocytes, lymphocytes, and monocytes in PD effluent were identified based on size and granularity in the dot plot of forward scatter versus side scatter as previously described (22) and were counted in the gated area as the percentage of the total cell count in peritoneal effluent. Cell populations

were not statistically different between the various HPG concentration groups, and those data were therefore pooled for comparison with the PDS group.

Detachment of mesothelial cells (MCs) into PD effluent is a reliable marker for peritoneal membrane injury (23–26). Mesothelial cells express a unique cell-surface protein, human bone marrow endothelial cell (HBME) 1 (27,28), which was identified using fluorescence-activated cell-sorting with rabbit polyclonal anti-HBME-1 antibody conjugated fluorescein isothiocyanate (FITC) (Biorbyt, Riverside, UK) versus rabbit polyclonal anti-mouse immunoglobulin A-FITC (Cayman Chemical, Ann Arbor, MI, USA) as a stain control. The cells without antibody stain were used as a negative background for FITC positivity. The data were not statistically different between the HPG concentration groups, and the HPG data were therefore pooled for analysis.

To simulate acute osmotic shock, immortalized HPMCs were exposed to PD solutions (7.5% and 15% HPG or PDS) for 1 hour and were then allowed to recover in K1 culture medium for 6 hours. Apoptosis and necrosis in HPMCs were then measured by fluorescence-activated cell-sorting analysis using an annexin V conjugated with phycoerythrin and with 7-amino-actinomycin D (7-AAD) kit according to the manufacturer's protocol (BD Biosciences). In graphs, viable cells appear in the lower left quadrant (double negative); necrotic cells, in the upper left quadrant (7-AAD-positive only); late apoptotic cells, in the upper right quadrant (both annexin V- and 7-AAD-positive); and early apoptotic cells, in the lower right quadrant (annexin V-positive only). The intensity of fluorescence for apoptotic or necrotic cells was measured compared with background controls.

MAY-GRÜNWARD-GIEMSA CYTOCHEMICAL STAIN

The presence of MCs in peritoneal effluent was confirmed using May-Grünwald-Giemsa cytochemical stain as previously described (25,29). In brief, cells in peritoneal effluents were spun down by centrifugation at 6000 rpm for 10 minutes and were smeared over glass microscopy slides. After being fully air-dried, cell smears were fixed in methanol. After rehydration with phosphate-buffered saline (PBS), the cell smears were stained with May-Grünwald solution [Sigma-Aldrich Canada, Oakville, ON, Canada (1:5 dilution with PBS)] for 10–15 minutes, washed with PBS, and then stained again with Giemsa stain solution [Sigma-Aldrich Canada (1:5 dilution with PBS)] for 30 minutes. The colors of the various cell types were differentiated by a further wash with PBS.

SURVIVAL OF HPMC CULTURES

Confluent monolayers of HPMCs were exposed to hyperosmotic solutions (HPG solutions vs PDS) at 37°C in a 5% CO₂ atmosphere for various periods of time up to 3 hours, after which viable cells were counted in a TC10 automated cell counter (Bio-Rad Laboratories Canada, Mississauga, ON, Canada) with trypan blue stain exclusion. The percentage of surviving cells was calculated as $(T_x / T_0) \times 100$, where T_x represents the total number of viable cells at the selected time point, and T_0 indicates the total number of viable cells in the untreated cell monolayer (0 hours). The number of viable cells at each time point is presented as the average of at least 3 determinations.

WESTERN BLOT

Apoptosis of HPMCs after osmotic shock was also examined by Western blot analysis of the active form of caspase 3. Protein extracts (50–100 µg per sample) were fractionated using 10% SDS-PAGE, and then transferred onto nitrocellulose membrane. Active caspase 3 proteins (17 kDa and 19 kDa) were identified using rabbit polyclonal anti-activated caspase 3 antibody (Cell Signaling Technology, Danvers, MA, USA), and visualized using an enhanced chemiluminescence assay (ECL: Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were re-probed using anti-beta-actin immunoglobulin G (Sigma-Aldrich Canada) for confirmation of loaded protein in each sample.

STATISTICAL ANALYSIS

Data are presented as mean ± standard derivation of each group. A two-tailed Student t-test was used for comparing groups, and analysis of variance was used for comparing groups at different concentrations or different time points. A *p* value of 0.05 or less was considered significant.

RESULTS

HPG SOLUTIONS INDUCE SIGNIFICANT UF

Figure 2 describes the UF capacity of various concentrations of 3 kDa HPG and PDS in rodents 4 hours after intraperitoneal injection. One-way analysis of variance analysis with a Tukey multiple comparison test showed that HPG induced a concentration- or osmolality-dependent increase in UF or volume of recovered peritoneal effluent ($p < 0.0001$). Compared with the 0-hour control,

UF was 40 ± 1.24 mL for 7.5% HPG ($p < 0.0001$) and 43.33 ± 5.24 mL for 15% HPG ($p < 0.0001$). The volume of peritoneal effluent for 5% HPG solution (27.88 ± 1.65 mL) after a 4-hour peritoneal dwell was not statistically different from the 0-hour control ($p = 0.7371$). Ultrafiltration for a 7.5% HPG solution was similar to the 37.23 ± 4.72 mL for PDS after a 4-hour dwell ($p = 0.1879$), but 15% HPG achieved more effective UF than PDS did ($p = 0.0268$). In aggregate, these data demonstrate the efficacy of the HPG polymer as an osmotic agent in PD solution.

Figure 2 — Ultrafiltration profiles of hyperbranched polyglycerol (HPG) and glucose-based peritoneal dialysis solution (PDS). Each rat received an intraperitoneal injection of 30 mL HPG solution or PDS. After 0 or 4 hours, the fluid was recovered from the peritoneal cavity, and its volume was measured. Groups: 0-hour control ($n = 10$), 2.5% HPG ($n = 11$), 5% HPG ($n = 4$), 7.5% HPG ($n = 4$), 15% HPG ($n = 6$), and PDS ($n = 11$). $p = 0.7371$, 5% HPG after 4 hours vs 0-hour control; $p = 0.1879$, PDS vs 7.5% HPG.

HPG SOLUTION IS EFFECTIVE AT CLEARING NITROGENOUS WASTE

Table 2 describes the urea concentration, urea removal, D/P urea ratio, and urea clearance by dialysate for all PD solutions. One-way analysis of variance with the Tukey multiple comparison test indicated that the concentration-dependent increase in urea concentration or total urea in dialysate (urea clearance) was statistically significant ($p < 0.0001$). Compared with PDS solution, the 7.5% and 15% HPG solutions both achieved better urea clearances, higher urea concentrations in dialysate, and more total urea removal after 4 hours of dwell time. The D/P ratios of urea for 5%, 7.5%, and 15% HPG were not statistically different from those for PDS ($p > 0.05$), indicating similar small-solute transport characteristics for those solutions. The D/P ratio with 2.5% HPG solution was higher than that with PDS ($p = 0.0248$) in the context of negative UF from the peritoneal cavity into the bloodstream. Net urea removal and urea clearance were higher with 7.5% HPG than with PDS despite similar rates of UF and solute transport.

HPG SOLUTION INDUCES LESS NEUTROPHIL INFILTRATION AND PERITONEAL MEMBRANE INJURY

Histology examination revealed that peritoneal membrane thickness increased after 4 hours, compared with 0 hours, for all treatment groups after exposure to hyperosmotic solution (7.5% or 15% HPG and PDS solution), and that the thickness of the peritoneal membrane in animals of the HPG groups was only half that in animals of the PDS group (Figure 3). Moreover, when polymorphonuclear infiltration (that is, neutrophils) was

TABLE 2
Urea Measurements in Dialysate (D) and Plasma (P)^a

Experimental group	Urea in dialysate		Urea clearance (mL/4 h) ^b	D/P ratio of urea
	Concentration (mmol/L)	Total amount (mmol)		
2.5% HPG	4.388±0.383	0.101±0.015	22.44±3.75	0.97±0.06
5% HPG	5.575±0.427	0.156±0.02	23.13±3.71	0.94±0.06
7.5% HPG	7.3±1.08	0.3±0.047	36.08±2.05	0.95±0.06
15% HPG	7.85±0.74	0.337±0.042	39.17±5.21	0.91±0.04
PDS	5.356±0.993	0.198±0.043	32.01±2.67	0.88±0.08
15% HPG vs PDS	$p=0.0002$	$p=0.0001$	$p=0.0037$	$p=0.4384$
7.5% HPG vs PDS	$p=0.0088$	$p=0.0027$	$p=0.0209$	$p=0.3075$

HPG = hyperbranched polyglycerol; PDS = glucose-based peritoneal dialysis solution.

^a Measured at the Chemistry Laboratory of the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada).

^b Calculated as described in the Methods section.

examined in HE-stained tissue sections, fewer neutrophils were seen in the peritoneal membrane of animals from the HPG groups than in those from the PDS group, particularly in the swelling submesothelial zone. This histologic observation was further supported by flow cytometry data (Figure 4). The recovered effluent from the HPG groups, compared with that from the PDS group, had proportionally fewer neutrophils ($3.63\% \pm 0.87\%$ vs $9.31\% \pm 2.89\%$, $p < 0.0001$).

Figure 3 — Peritoneal membrane sections taken from 0-hour control rats and from rats after 4 hours of peritoneal dialysis exposure (hematoxylin and eosin stain). On histology examination, hyperbranched polyglycerol (HPG) solutions demonstrated less peritoneal damage and neutrophilic infiltration. The thickness of the peritoneal membrane is indicated by the distance between the arrows. Cellular infiltrates (polymorphonuclear leukocytes, including neutrophils) are stained dark blue. PDS = glucose-based peritoneal dialysis solution.

As shown in Figure 5(A,B), detachment of mesothelial cells positive for HBME-1 did not increase in control stains of effluent from the HPG group ($0.70\% \pm 0.31\%$ vs $0.36\% \pm 0.35\%$, $p = 0.1832$), but was elevated in stains of effluent from the PDS group ($1.62\% \pm 0.68\%$ vs $0.41\% \pm 0.31\%$, $p = 0.0031$). The presence of MCs in peritoneal effluent from the PDS group was further confirmed by May–Grünwald–Giemsa stain [Figure 5(C)], evidenced by the presence of MCs in cell smears of peritoneal effluents from the PDS group; such cells were absent in cell smears of effluents from the HPG groups. Those data demonstrate that less mesothelial injury and cellular detachment is induced by HPG solutions than by PDS after *in vivo* exposure.

HPG SOLUTION IS BETTER TOLERATED IN HPMC CULTURES

As shown in Figure 6(A), more viable HPMCs, negatively stained by trypan blue, were present after 3 hours of exposure to HPG than to PDS ($p < 0.0001$, 7.5% HPG vs PDS; $p = 0.0044$, 15% HPG vs PDS). Similar results were seen in SV40-immortalized HPMCs [$p < 0.0001$, 7.5% HPG vs PDS; $p = 0.0067$, 15% HPG vs PDS; Figure 6(B)]. Furthermore, as shown in Figure 7(A), cytoplasmic vacuolation was present in microscopic views of cultured HPMCs incubated with PDS, but absent in cells treated with HPG solutions or with culture medium. Those results were further confirmed with flow cytometric analysis, in

Figure 4 — Hyperbranched polyglycerol (HPG) solution induces less neutrophilic infiltration. The presence of neutrophils in recovered fluids was used as a biomarker for peritoneal inflammation and was quantitatively determined using flow cytometry. (A) Typical percentage of neutrophils in a side scatter (SSC)/forward scatter (FSC) plot of each group. (B) Mean $\pm$ standard deviation of neutrophils in glucose-based peritoneal dialysis solution (PDS) compared with pooled HPG solutions.

Figure 5 — Hyperbranched polyglycerol (HPG) solution causes less mesothelial cell injury. Peritoneal membrane injury was determined by the presence of detached human peritoneal mesothelial cells (HPMCs) in the recovered effluent. The HPMCs were identified using flow cytometric analysis or May–Grünwald–Giemsa (MGG) stain. (A) Cells were stained with either rabbit anti-mouse immunoglobulin A polyclonal (control) immunoglobulin–fluorescein isothiocyanate (FITC) or rabbit anti-human bone marrow endothelial cell (HBME) 1 polyclonal immunoglobulin–FITC. Typical percentage of FITC-stained cells in fluorescence-activated cell sorter histograms (BD Biosciences, Mississauga, ON, Canada) for each group. (B) Mean $\pm$ standard deviation of FITC-stained cells in glucose-based peritoneal dialysis solution (PDS) and pooled HPG solutions. (C) Typical microscopic view of MGG-stained smears of cells in PDS and HPG. Leukocytes are dark blue; HPMCs are blue-violet.

which measurement of side scatter indicated the level of cytoplasmic granularity. As shown in Figure 7(B), PDS induced significant cytoplasmic granularity of HPMCs, which was indicated by an increase in the mean intensity of side scatter from 27.4 ± 0.8 ($\times 1000$) in cells in culture medium to 77.0 ± 1.2 ($\times 1000$) in cells treated with PDS

($p < 0.0001$). In contrast, incubation with HPG solutions did not increase the granularity of HPMCs.

In vitro HPMC survival, apoptosis, and necrosis were further examined using flow cytometric and Western blot analysis during recovery after 1 hour of hyperosmotic stress induced by HPG or PDS solutions. As

Figure 6 — More cultured human peritoneal mesothelial cells (HPMCs) survived after exposure to hyperbranched polyglycerol (HPG) solutions. Primary or immortalized HPMCs were grown in K1 culture medium overnight and were then incubated with HPG solutions (7.5% and 15%) or glucose-based peritoneal dialysis solution (PDS). Viable cells were counted by trypan blue exclusion assay at various time points. Data are presented as mean $\pm$ standard deviation of three separate experiments. (A) Primary HPMCs: $p < 0.0001$, PDS vs 7.5% HPG; $p = 0.0044$, PDS vs 15% HPG. (B) Immortalized HPMCs: $p < 0.0001$, PDS vs 7.5% HPG; $p = 0.0067$, PDS vs 15% HPG.

Figure 7 — Induction by hyperbranched polyglycerol (HPG) solutions of various cellular changes in cultured human peritoneal mesothelial cells (HPMCs). Immortalized HPMCs were grown in K1 culture medium overnight and were then incubated with K1 culture medium (CM), HPG solutions (7.5% and 15%), or glucose-based peritoneal dialysis solution (PDS). (A) A typical microscopic view of immortalized HPMCs after 3 hours of incubation. Arrows point to vacuoles in the cytoplasm. (B) After 30 minutes of incubation, cellular granularity was determined by measuring the intensity of side scatter (SSC) using flow cytometric analysis. Data are presented as mean $\pm$ standard deviation of four separate experiments. $p < 0.0001$, PDS vs CM; $p < 0.0001$ CM vs HPG.

shown in Figure 8(A) and Table 3, absolute cell survival was higher in HPG-treated than in PDS-treated cells: there were more viable double-negative stained cells in HPG-treated groups ($75.77\% \pm 1.35\%$ in 7.5% HPG, $75.27\% \pm 0.61\%$ in 15% HPG) than in the PDS-treated group ($61.7 \pm 2.73\%$, $p < 0.0001$). The HPMCs treated with PDS died mostly by necrosis or late apoptosis and

were stained positively with 7-AAD alone or in combination with annexin V–phycoerythrin. Alternatively, after incubation with 7.5% or 15% HPG solution, HPMCs were mostly committed to apoptosis and stained positively by annexin V–phycoerythrin alone or in combination with 7-AAD. The apoptosis induced by HPG was further confirmed in Western blot analysis by the presence of

Figure 8 — Induction by hyperbranched polyglycerol (HPG) solutions of cell apoptosis in cultured human peritoneal mesothelial cells (HPMCs). Immortalized HPMCs were grown in K1 culture medium overnight and then incubated with culture medium (CM), HPG solutions (7.5% and 15%), or glucose-based peritoneal dialysis solution (PDS) for 1 hour. The HPMCs were then allowed to recover in K1 culture medium for 6 hours. Cell death was examined by flow cytometry and Western blot analysis. (A) Representative fluorescence-activated cell sorter histograms (BD Biosciences, Mississauga, ON, Canada) of cell death, indicating 7-amino-actinomycin D (7-AAD)–stained nuclear DNA and annexin V–stained phospholipids on the cell surface. (B) Representative Western blot from four separate experiments, showing the active forms of caspase 3 (19 kDa and 17 kDa).

TABLE 3
Induction of Cell Apoptosis by Hyperbranched Polyglycerol (HPG) Solutions^a

Stain	CM	7.5% HPG	15% HPG	PDS	p Value (n=4)
7-AAD–positive	2.98±1.61	5.72±0.61	4.64±0.74	12.9±0.51	<0.0001 (PDS vs CM) 0.0178 (HPG vs CM)
7-AAD– or annexin V–positive	3.9±2.55	7.13±0.42	8.31±0.52	17.8±1.81	0.0001 (PDS vs CM) 0.0071 (HPG vs CM)
Annexin V–positive	7.03±0.08	9.52±0.82	11.8±1.23	7.84±0.85	0.1062 (PDS vs CM) <0.0001 (HPG vs CM)
Negative stain	86.07±4.02	75.77±1.35	75.27±0.61	61.7±2.73	<0.0001 (PDS vs 7.5% or 15% HPG)

CM = culture medium; PDS = glucose-based peritoneal dialysis solution; 7-AAD = 7-amino-actinomycin D.

^a Mean ± standard deviation of four separate experiments; analyzed by two-tailed Student t-test (PDS vs CM) or one-way analysis of variance (HPG vs CM).

active forms of caspase 3 in cellular protein extracts of HPG-treated HPMCs and by their absence in PDS-treated cells [Figure 8(B)]. Taken together, these data suggest that hyperosmotic HPG solutions induce less HPMC death (which occurs mostly by apoptosis) and that PDS induces more HPMC death associated with necrosis and cytoplasmic vacuolation.

DISCUSSION

Because of excellent biocompatibility, HPG has received much attention in the field of biomedical applications (11,12). The present study demonstrates, for the first time, the potential of HPG as an osmotic agent in the development of new PD solutions. In a rodent model of PD, 3 kDa HPG solution shows concentration- and osmolality-dependent UF and is more effective than glucose-based PDS in urea clearance at the higher concentration levels tested. We demonstrated significantly reduced peritoneal membrane injury and inflammation *in vivo* with HPG solution compared with PDS, which is consistent with the finding that more viable HPMCs were present *in vitro* after exposure to HPG solution.

Hyperbranched polyglycerol is a compact, dendritic, narrow-dispersed macromolecule with low intrinsic viscosity that can be used as a primary osmotic agent in PD solution, indicated by the fact that increasing the number of HPG molecules in an electrolyte solution results in an increase in its osmolality (Table 1). During PD, fluid flow across the peritoneal membrane is driven by osmotic pressure between the PD fluid and the plasma. Osmotic pressure is generated by an osmotic gradient of small solutes (crystalloid osmosis) and large impermeable solutes (colloid osmosis) (26). Indeed, significant fluid removal is seen in rats after dialysis with 7.5% or 15% HPG solution (Figure 2), for which osmolality is 324 mOsm/kg or 424 mOsm/kg respectively (Table 1), which is higher than the normal range of plasma osmolality in Sprague-Dawley rats (295 ± 3 mOsm/kg) (30). A 5% HPG solution (294 mOsm/kg) is considered iso-osmotic to rodent plasma and appears to balance plasma colloid osmotic pressure, resulting in an even fluid balance (Table 1, Figure 2).

Clearance of nitrogenous waste through the peritoneal membrane occurs by simple diffusion from the bloodstream and is also coupled with UF via the convective phenomenon of solvent drag (31). The small-solute transport seen with HPG solutions, measured as the D/P ratio of urea, was similar to that with PDS. The latter finding supports the biocompatibility of HPG, given that increased solute transport may have suggested the

increased vascular permeability that occurs in acute peritoneal inflammation (32,33). Moreover, urea clearance values for HPG appear to follow the UF profiles observed in our experiments. Nevertheless, in this rodent model of PD, urea clearance with 7.5% HPG solution was significantly higher than that with PDS despite the similar UF and solute transport profiles of both solutions. That finding suggests that the exact relationship between solute transport, UF, and waste clearance with HPG solutions has yet to be fully characterized.

In both the *in vivo* rodent model and cultured HPMCs, HPG solutions induced less peritoneal membrane injury, less leukocyte infiltration (Figures 3 – 5), and higher cell survival (Figures 6 and 8) that were seen with conventional glucose-based PD solution. Increased submesothelial thickness after a single exposure to PD may correlate better with tissue edema than with inflammation and should be interpreted cautiously (34). Hyperosmotic glucose-based PDS induces cellular injury in all types of peritoneal cells, including polymorphonuclear cells, phagocytes (that is, macrophages), and MCs (24,25,35–39). There are three potential explanations for those findings. First, cells may survive better in the neutral physiologic pH of HPG solutions than in the acidic pH of approximately 5.2 in PDS. As shown in Table 1, dissolving HPG in the same electrolyte solution as PDS maintains a physiologic pH, which has been shown to confer survival benefits for HPMCs (35,40). The ability to easily deliver HPG PD therapy at a physiologic pH should be considered an advantage over conventional glucose-based solutions. Second, because hypertonic glucose (41,42) and glucose degradation products (43–45) can both directly damage the peritoneal membrane and contribute independently to cellular death, the lack of either of those substances in HPG solutions may account for improved cell viability. Third, it is possible that 3 kDa HPG and glucose may induce different degrees of cellular damage because of differences in chemical structure, molecular size, and cellular uptake. That hypothesis might be supported by the observed severe vacuolation induced by PDS in the cytoplasm of HPMCs that was not seen with HPG-based solution (Figure 7). Cytoplasmic vacuolation is a nonspecific but distinct pathologic feature of human cells in response to various stress conditions, including hypertonicity, low pH, or toxic chemical exposure (46). Our findings might suggest that human cells interact differently with 3 kDa HPG and with PDS, which contributes to the attenuated cellular injury observed with HPG PD solution. Our findings might also be explained by the fact that these two solutions activate different cell death pathways *in vitro*. It has been reported that acute osmotic stress—induced by a hypertonic environment—results in cell death in many

forms: apoptosis, anoikis, secondary necrosis, and pure necrosis (47). In our experiments, most HPMCs appeared to die by necrosis without caspase 3 activation after exposure to PDS, whereas HPG solution induced apoptosis (Table 3, Figure 8). It has been documented that different dying cells trigger different immune responses—apoptotic cells induce immune tolerance; necrotic cells stimulate cytotoxic immunity (48,49)—implying that the apoptosis induced by hyperosmotic HPG-based PD solution may not cause secondary peritoneal injury because of induction of immune tolerance.

The present study has some limitations that need to be acknowledged. As an initial proof of concept for our novel HPG solution, the current animal model used only a single simulated PD dwell. Ultimately, superior biocompatibility with HPG solution can be established only if the reductions in inflammation and peritoneal injury observed in the present experiments translate into reduced fibrosis, epithelial-to-mesenchymal transition, and angiogenesis over many weeks of exposure (50–55). Moreover, our experiments compared HPG with the best standard-practice PD solution in use worldwide; it will be valuable in the future to compare HPG PD with newer PD solutions having a physiologic pH and using glucose polymer as the osmotic agent. In long-term animal experiments, the presence of uremia also becomes more pertinent—particularly when studying the pharmacokinetic and metabolic properties of HPG. Further studies are also warranted to characterize the crystalloid and colloid properties of HPG and to determine the ideal size of HPG to optimize UF and waste clearance parameters.

CONCLUSIONS

Our series of *in vivo* and *in vitro* experiments demonstrate that, compared with a conventional glucose-based PD solution, a novel HPG-based PD solution can achieve successful UF and waste removal while reducing peritoneal injury and inflammation. This preliminary study suggests that HPG may be a promising alternative to glucose in the development of next-generation PD solutions for patients with end-stage renal disease.

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DISCLOSURES

On behalf of authors (AAM, GR, JNK, CD), the University of British Columbia filed a provisional patent based on the invention described herein.

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