Heat shock response protects human peritoneal mesothelial cells from dialysate-induced oxidative stress and mitochondrial injury

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Abstract

Background. Chronic peritoneal dialysis (PD) is one of the major therapies for uremic patients. However, the peritoneal mesothelial cells (PMCs) are subject to the injury by bioincompatible dialysates. The aim of this study is to investigate the protective roles and mechanisms of heat shock response in PMCs.

Methods. Primary cultured human PMCs (HPMCs) were subjected to commercial peritoneal dialysates. The cell viability was assayed by MTT test and Annexin V assay. The expression of HSPs was detected by Western blots analysis. Intracellular hydrogen peroxide and superoxide anion were detected using H2DCFDA and dHE probe, respectively, with flow cytometry. The mitochondrial membrane potential (ΔΨm) of HPMCs was evaluated using JC1 probe with flow-cytometry.

Results. Exposure of HPMCs to 1.5%, 2.5%, and 4.25% dextrose, and 7.5% icodextrin dialysates, respectively, for 60 min resulted in significantly accumulation of intracellular reactive oxygen species (ROS), ΔΨm loss, and cell death in HPMCs. Amino acid dialysates exhibited no significant cytotoxicity. Adjusting the acidity in 1.5% glucose, 2.5% glucose, and 7.5% icodextrin dialysates, respectively, for 60 min resulted in significantly accumulation of intracellular ROS, ΔΨm loss, and cell death in HPMCs.

Conclusions. In conclusion, the acidic bioincompatible dialysates induce oxidative stress, ΔΨm loss, and subsequent cell death in HPMCs. Amino acid dialysates is more biocompatible than glucose and icodextrin dialysates to...
Introduction

Peritoneal dialysis (PD) has become a major mode of therapy for patients with end-stage renal failure. However, chronic exposure to conventional peritoneal dialysates with low biocompatibility is associated with functional and structural alterations of the peritoneal membrane [1, 2]. These changes may lead to progressive worsening of peritoneal membrane function as well as systemic inflammation [3]. Increasing evidence suggests that human peritoneal mesothelial cells (HPMCs) are instrumental in controlling intraperitoneal homeostasis and thereby play a key role in the initiation and control of disease processes affecting the abdominal cavity [4]. Moreover, loss of HPMCs may damage peritoneal membrane integrity and result in ultrafiltration failure in patients receiving continuous ambulatory PD (CAPD) therapy. Toxicity to conventional dialysates may be due to hypertonicity, high glucose concentration, lactate buffering systems, the presence of glucose degradation products (GDPs) and acidic pH. Hence, novel dialysates with improved physicochemical properties have been designed to increase their biocompatibility.

The mechanisms by which dialysates induce cellular injury in HPMCs are still unclear. Changes in intracellular ion concentrations occur as an early response to injurious stimuli in many cells and tissues. An oxidative stress-induced inflammatory event, leading to cellular or tissue injury, is considered to be an unifying mechanism leading to injury in renal, cardiovascular, neoplastic and neurodegenerative disease processes, as well as aging. Prolonged exposure to glucose-containing dialysates may induce diabetes-like changes in structure and function of the peritoneal membrane [5], and glucose has been found to promote generation of reactive oxygen species (ROS) in a number of cell types [6, 7]. Previous studies indicated that the accelerated senescence response in HPMCs exposed to high glucose is strongly related to oxidative stress [8].

Mitochondria are the major source of ROS and are most vulnerable to ROS-induced damage [9]. Here, ROS forms a positive feedback loop, and the subsequent damage induced by oxidative stress increases progressively. Disturbances in mitochondrial function may contribute to initiating death signal transduction through loss of the mitochondrial membrane potential and increases in mitochondrial membrane permeability, followed by a subsequent release of proapoptotic factors involved in various aspects of apoptosis [9]. Redox homeostasis and the maintenance of mitochondrial function are both important factors affecting cell survival during exposure to injurious stimuli. Nevertheless, few studies have examined mitochondrial function and redox homeostasis in HPMCs during dialysate exposure.

Heat shock proteins (HSPs) have been recognized as major endogenous cytoprotective molecules that act against deleterious stresses [10]. At the cellular level, protective mechanisms of HSPs involve their chaperone functions that prevent proteins from misfolding and aggregation [11, 12]. In previous studies, we found that heat shock treatment lead to synthesis of HSPs, and this helped to prevent sepsis-induced structural and functional destruction of mitochondria [13, 14]. HSPs may also exert cytoprotective actions by interfering with stress-induced apoptotic programmes [15, 16]. Recent studies demonstrated that peritoneal HSP70 can be induced by chronic PD therapy [17], and that dialysate composition determines HSP expression patterns in HPMCs [18, 19]. However, the role that HSP induction plays and the mechanisms by which heat shock responses protect cells from dialysate-induced cellular damage in HPMCs remains to be identified.

In the present study, we compared the toxic effects of conventional glucose-based dialysates to a novel dialysate (icodextrin- and amino acid-based) on cultured HPMCs, and evaluated the role of heat shock response during low-biocompatibility dialysate-induced injury.

Materials and methods

HPMC culture

HPMCs were isolated as previously described with minor modifications [20]. The samples were concentrated by centrifugation of effluent dialysates from patients receiving PD therapy, and then cultured in Earle’s M199 medium containing 10% fetal calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin. After centrifugation, cells were washed twice with phosphate-buffered saline (PBS) and seeded into 100-mm collagen-coated (Vitrogen 100, Collagen Corp., Palo Alto, CA, USA) culture dishes. These culture dishes were incubated in a humidified 5%-CO2 atmosphere. Non-adherent cells were removed after 1 day by two brief washes with the medium. After confluence, cells were detached by trypsinization and were split (in a ratio of 1:2) two to three times. Cells were identified by morphology and by immuno-histochemical characteristics. HPMCs in the culture medium exhibited a cobblestone appearance at confluence and were confirmed by the expression of cytokeratin and vimentin, and all of them were negative for von Willebrand factor VIII. Cells between passages 2 and 4 were used for experiments.

Peritoneal dialysate treatment

Five kinds of commercial peritoneal dialysates were used. They were conventional glucose lactate-based dialysates (Dianeal®, Baxter) containing 1.5, 2.5 or 4.25% dextrose, respectively, and novel peritoneal dialysates containing 1.1% amino acids (Nutrineal®, Baxter) and 7.5% icodextrin (Extraneal®, Baxter). At confluence, HPMCs were treated with various peritoneal dialysates for various time intervals as indicated, with or without heat pretreatment. All experiments were performed at least in duplicate five times to six times.

Heat shock treatment

Heat shock response treatment of HPMC was carried out by incubation in the complete medium. The cultures were transferred to an oven at various temperatures (39, 40, 41, 42 and 43 °C) for 30 min. For recovery after treatments, the cells were placed in a 37 °C incubator until further analysis. The time course for HSP induction, including HSP90, HSP72, HSP60 and Hsp27, was also evaluated.

Assessment of cell viability

Methylthiazoletetrazolium test. HPMCs were exposed to various peritoneal dialysates for 0 10, 30, 60, 90 and 120 min, respectively. At the end of each incubation, methylthiazoletetrazolium (MTT) assay was used to measure the activity of living cells via mitochondrial dehydrogenases. The

Keywords: heat shock protein; mitochondria; oxidative stress; peritoneal mesothelial cells
Heat shock protects HPMCs

A key characteristic of early apoptosis is the translocation of phosphatidylserine (PS) from the inner to outer membrane leaflet of the plasma membrane. Annexin V (BD Biosciences, San Jose, CA, USA) is a PS-binding protein and was used to detect apoptotic cells. The nuclear stain, 7-aminoactinomycin D (7-AAD), was used to identify late apoptotic or necrotic cells. After treatment with various peritoneal dialysates for 1 h, HPMCs were re-suspended at a concentration of 1 × 10^6 cells/ml in a binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl_2, 1 mM MgCl_2). Aliquots of cells (100 µl) were stained with annexin V-FITC and 7-AAD in the binding buffer for 15 min at room temperature in the dark. A total of 1 × 10^4 cells were analysed, within 60 min of staining, using a FACs Caliber flow cytometer (BD Biosciences) to determine the proportion of apoptotic cells at different stages and of viable cells. The 7-AAD can be used as the viability marker. Quadrant markers were set on dot plots of unstained cells (viable cell population) and then subsequently applied to other samples.

Detection of apoptosis. A key characteristic of early apoptosis is the translocation of phosphatidylserine (PS) from the inner to outer membrane leaflet of the plasma membrane. Annexin V (BD Biosciences, San Jose, CA, USA) is a PS-binding protein and was used to detect apoptotic cells. The nuclear stain, 7-aminoactinomycin D (7-AAD), was used to identify late apoptotic or necrotic cells. After treatment with various peritoneal dialysates for 1 h, HPMCs were re-suspended at a concentration of 1 × 10^6 cells/ml in a binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl_2, 1 mM MgCl_2). Aliquots of cells (100 µl) were stained with annexin V-FITC and 7-AAD in the binding buffer for 15 min at room temperature in the dark. A total of 1 × 10^4 cells were analysed, within 60 min of staining, using a FACs Caliber flow cytometer (BD Biosciences) to determine the proportion of apoptotic cells at different stages and of viable cells. The 7-AAD can be used as the viability marker. Quadrant markers were set on dot plots of unstained cells (viable cell population) and then subsequently applied to other samples.

Detection of $\Delta\psi_m$. The $\Delta\psi_m$ was measured by flow cytometry using the lipophilic cation JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) (Molecular Probes, Invitrogen, Germany). JC-1 stains mitochondria in cells with high mitochondrial potentials by forming orange-red fluorescent J-aggregates that emit at 590 nm upon excitation at 490 nm. In cells with depolarized or damaged mitochondria, JC-1 forms a monomeric form that emits at 525 nm with the same excitation wavelength. For staining, HPMCs were incubated with JC-1 (5 µg/ml) for 30 min at 37°C in the dark. Cells were then washed in PBS and analysed immediately by a flow cytometer (FACScan; BD Biosciences). A total of 10 000 cells were analysed for green fluorescence with a 525-nm filter and for orange fluorescence with a 590-nm filter. All data were analysed with BD Cell Quest Pro Software (BD Biosciences).

Detection of intracellular ROS. Intracellular peroxide and superoxide levels in HPMCs were assessed using 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR, USA) and dihydroethidium (DHE) (Molecular Probe Inc.) probes, respectively, by flow cytometry. H_2DCFDA is mainly oxidized by hydrogen peroxides and hydroxyl radicals, while DHE is mainly oxidized by superoxide anions. However, H_2DCFDA and DHE are the practice probes used to detect oxidative activities in viable cells. H_2DCFDA is deacylated by intracellular esterases forming H_2DCFH, which, in the presence of intracellular hydrogen peroxides and hydroxyl radicals, is oxidized in the cytoplasm to a highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF) ($\lambda_{ex}$ = 485 nm; $\lambda_{em}$ = 530 nm). DHE is oxidized upon reaction with superoxide to ethidium bromide (EB), which is trapped by intercalation with DNA in the nucleus and fluoresces red ($\lambda_{ex}$ = 475 nm; $\lambda_{em}$ = 610 nm). After treatment with peritoneal dialysates, with or without heat shock pretreatment, HPMCs were loaded with the H_2DCFDA (20 µM) and DHE (10 µM) for 20 min at room temperature in the dark. A total of 1 × 10^4 cells were analysed, within 60 min of staining, by a FACs caliber flow cytometer (FACScan; BD Biosciences). DCF-derived fluorescence was analysed for green fluorescence with a 525-nm filter. Ethidium-derived fluorescence was analysed for orange fluorescence with a 590-nm filter. A total of 10 000 cells were analysed.

Western bolt analysis. Equal amounts (10 µg) of protein extract were loaded and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA, USA). Antibodies to HSP60, HSP72, HSP90, HSP27 (StressGen Biotechnologies, Victoria, BC, Canada) and actin (Chemicon, Temecula, CA, USA) were used as the primary antibody, while horseradish peroxidase-conjugated anti-mouse or ant-rabbit immunoglobulin G were used as the secondary antibody. Target proteins were detected by enhanced chemiluminescence. Actin was detected simultaneously and acted as an internal control. The results were quantified by a densitometer and analysis software (Bio-1D V97 software, Vilber Lourmat, France).

Adjustment of dialysate acidity. The pH of the 1.5% dextrose and icodextrin dialysate was adjusted to 6.8, the same pH as of the amino acid dialysate, with NaOH titration. Then, the effects of pH adjustment on cell viability (the MTT test) and intracellular ROS (peroxide and superoxide) in HPMCs after dialysates exposure were evaluated by flow cytometry as previously prescribed.

Statistical analysis. All results are expressed as means ± standard errors (SEMs). The mean values were compared by analysis of variance (ANOVA). In cases of smaller numbers of data points, the Kruskal–Wallis tests and Mann–Whitney U-tests were also used. A $P$-value < 0.05 was used as the criterion for a statistically significant difference.

Results. Heat shock treatment induced HSP production in HPMCs. HSP72 was induced in HPMCs following heat shock treatment for 30 min at various temperatures (39, 40, 41, 42 and 43°C) (Figure 1A), and was induced in a temperature-dependent manner. Heat shock treatment at 42°C was applied for the subsequent studies. Time course studies of HSP induction, including HSP90, HSP72, HSP60 and HSP27, showed that HSP expression in HPMCs occurred as early as 3 h after heat shock treatment, and remained significantly elevated at 96 hours of recovery as compared with controls. Elevations were especially strong in HSP72 and HSP27 (Figure 1B). HSP expression was maintained at higher levels during the 6–48 h following heat treatment. The time point of 24-h recovery after heat shock treatment was chosen for the following studies. Finally, there was no significant change in HSP 60 expression after heat shock treatment (data not shown).

Heat shock treatment attenuated dialysate-induced HMP cell death. HPMCs were exposed to various dialysates containing different concentrations of glucose (1.5, 2.5 and 4.25% dextrose), 1.1% amino acids and 7.5% icodextrin for up to 120 min. Among these, the glucose-based and icodextrin dialysates significantly suppressed HPMC viability in a time-dependent manner as assessed by MTT assay (Figure 2). Heat pretreatment (42°C for 30 min) attenuated the dialysate-induced cytotoxicity and increased HPMC viability. The cytotoxicity of the amino acid-based dialysate was significantly lower than that of the other dialysates, and it did not affect HMPC viability. Dialysate-induced cytotoxicity characteristics were also assessed by annexin V and 7-AAD staining and were analysed by flow cytometry. After incubating HPMCs with various dialysates for 1 h, the apoptotic rate as well as early and late apoptosis increased significantly with the glucose-based dialysates (1.5, 2.5 4.25% dextrose) and
Fig. 1. Induction of HSPs in HPMCs after heat shock treatment. Panel A: HSP72 expression was detected at 24 h after recovery from 30 min of heat shock treatment at various temperatures as indicated. Actin was detected simultaneously and acted as the internal standard. Panel B: time course expressions of HSP27, HSP72 and HSP90 after heat shock treatment at 42°C were detected by western blot analysis. Actin was detected simultaneously and acted as the internal standard. One representative expression out of six is shown. Panel C: statistical analyses of the relative content of HSP27 (OD ratio HSP27/actin), HSP72 (OD ratio HSP72/actin) and HSP90 (OD ratio HSP90/actin). Values are means ± SEMs of six samples in each group. *P < 0.05 versus non-heating control.

Heat pretreatment prevented the loss of Δψm induced by dialysate exposure

In cells with mitochondria having a high Δψm, JC-1 forms orange-red fluorescent J-aggregates (R1 region), while in cells with depolarized or damaged mitochondria, the sensor dye exists as green fluorescent monomers (R2 region) (Figure 4A). During control conditions, with or without heat treatment, most of the cells were localized in the R1 region (high Δψm). After 60-min exposure to the glucose and icodextrin dialysates, most cellular dots shifted from R1 to the R2 region (indicating a loss of Δψm). The relative ratio of cellular dots in the R1 to R2 regions decreased significantly after exposure to the glucose-based and icodextrin dialysates compared with controls. There was no significant shift in the cellular dots following exposure to the amino acid dialysate. Moreover, heat pretreatment attenuated the shift of cellular dots from R1 to R2. Heat pretreatment also prevented the loss of Δψm induced by peritoneal dialysate exposure.

Heat pretreatment protected HPMCs from dialysate-induced oxidative stress

After exposure of HPMCs to 1.5% dextrose dialysate using different time courses, the intensity of both the DCF green fluorescence and EB red fluorescence increased, and the fluorescence shifted to the right as shown in the histogram. Both the accumulation of intracellular hydrogen peroxide and superoxide anions showed some kind of time dependence (Figure 5A and C; left panel). Increasing the glucose concentration, as shown with the 2.5 and 4.25% dextrose dialysates, did not further increase intracellular ROS. Intracellular ROS generation was also induced by exposure to 7.5% icodextrin dialysates. In contrast, the amino acid dialysate induced less ROS formation compared with the other dialysates.

To further characterize the low-fluorescent population detected by DCF after dialysate exposure, we simultaneously stained dialysate-treated HPMCs with DCF and 7-AAD, which are standard flow cytometric viability probes (Figure 5B). We found that 7-AAD fluorescence was high in low-DCF fluorescent cell population, indicating that low-DCF fluorescent HPMCs were mainly dead cells, but not less-stressed cells. We concluded that dialysate treatment leads to ROS accumulation and even to cell death in HPMCs.

Heat pretreatment did not influence the basal levels of DCF- and DHE-derived fluorescence in control cells (without dialysate exposure). However, heat pretreatment did attenuate dialysate-induced ROS accumulation in HPMCs...
Heat shock protects HPMCs

Fig. 2. Cytotoxicity detection after dialysate exposure. HPMCs were incubated in various dialysates for 0, 10, 30, 60, 90 and 120 min, and the cell death was assessed by MTT assay. Values are means ± SEMs of eight samples in each group. *P < 0.05 versus relative non-heating group. (Unclear)

1.5% D: glucose lactate-based dialysate containing 1.5% dextrose; 2.5% D: glucose lactate-based dialysate containing 2.5% dextrose; 4.25% D: glucose lactate-based dialysate containing 4.25% dextrose; A.A.: dialysate with 1.1% amino acids; Icod: dialysate with 7.5% icodextrin.

(Figure 5A and C; right panel). The shifting of the DCF- and DHE-derived fluorescent histogram was dramatically decreased. The dialysate-induced EB fluorescent accumulation was significantly decreased after heat shock treatment (Figure 5D).

**Peritoneal dialysate exposure induced HSP72 expression**

HPMCs expressed HSP72 in response to 60 min of exposure to the dialysates (Figure 6). The induction of HSP72 was detected at 24 h after exposure to conventional glucose-based (1.5, 2.5 and 4.25% dextrose) and icodextrin dialysates. The expression of HSP72 was not visibly increased after exposure to the amino acid dialysate as compared with control cells.

**Adjustment of dialysate acidity attenuated dialysate-induced cell death and oxidative stress**

The pH of the 1.5% dextrose and icodextrin dialysates was increased to 6.8, the same pH as the amino acid dialysate, using NaOH titration. HPMCs were exposed to dialysates containing 1.5% dextrose and 7.5% icodextrin, with and without adjustment of acidity, for up to 180 min. MTT assay showed that the commercial 1.5 glucose-based and icodextrin dialysates with a low pH significantly suppressed HPMC viability in a time-dependent manner. Decreasing the dialysate acidity significantly improved HPMC viability (Figure 7). Adjustment of acidity also attenuated the dialysate-induced intracellular ROS accumulation. After exposure of HPMCs to the 1.5% dextrose and icodextrin dialysate, the intensity of both DCF green fluorescence (Figure 8A) and EB red fluorescence (Figure 8B) increased, and the fluorescence shifted to the right as shown in the histogram. In contrast, the dialysate with more neutral pH induced less ROS accumulation.

**Discussion**

In the present study, conventional glucose-based peritoneal dialysates (1.5, 2.5 and 4.25% dextrose) and novel dialysates (7.5% icodextrin and 1.1% amino acid) were utilized to investigate dialysate-induced cellular injury in HPMCs. Cell susceptibility was higher with conventional glucose dialysates and the 7.5% icodextrin dialysate than with the 1.1% amino acid dialysate. Our results showed that the conventional glucose dialysates and 7.5% icodextrin dialysate exerted significant cytotoxicity on HPMCs, while the amino acid dialysate was more biocompatible. After exposure to the low-biocompatibility dialysates, intracellular superoxide promptly accumulated in HPMCs and the mitochondrial membrane potential collapsed, producing significant increases in cell death. The accumulation of intracellular hydrogen peroxide and superoxide anions is considered a critical characteristic of oxidative stress that is induced by low-biocompatibility dialysates. Following induction of oxidative stress, our low-biocompatibility dialysates led to mitochondrial functional perturbations that included collapse of the mitochondrial membrane potential (ΔΨm). These data indicate that mitochondria are a susceptible target for low-biocompatibility dialysates and
Fig. 3. Apoptosis detection after PDF exposure. Panel A: apoptosis detected by flow cytometry with an annexin V/7-AAD staining assay. One representative expression out of six is shown. The horizontal (green fluorescence) and vertical (red fluorescence) axes represent labelling with annexin V and 7-AAD, respectively. The lower left quadrant of the dot-plot graph represents viable non-apoptotic cells. Early apoptotic cells bind to annexin V in the lower right (LR); late apoptotic cells bind to annexin V and take up 7-ADD in the upper right (UR). The upper left contains cells that take up 7-ADD but do not bind to annexin V. These cells are most likely necrotic. Left panel: exposures to different dialysates, but without heat pretreatment. Right panel: exposures to different dialysates with heat pretreatment. Panel B: statistical analysis of cellular population in apoptosis. The percentage of apoptosis (RU + RL) is represented and the indicated data show means ± SEMs of six samples in each group. *P < 0.05 versus each other as indicated. #P < 0.05 versus non-dialysate treated group.

that they may act as a high-sensitivity biosensor during dialysate-induced oxidative stress. Analysis of ROS accumulation and ΔΨm changes in HPMCs may provide a new, convenient and rapid (up to 60 min) means to assess biocompatibility of dialysates ex vivo.

The loss of HPMCs may represent a disadvantage for chronic PD therapy [5]. Loss of the mesothelial layer and replacement of the normal peritoneal structure with fibrotic tissue is sometimes associated with the loss of peritoneal membrane function, even leading to the discontinuation of PD therapy. Mitochondria, in addition to acting as the powerhouse, have been shown to play a central role in regulating cellular life [21,22]. Reduction in the mitochondrial membrane potential is thought to be an early event and possible cause of programmed cell death [23,24]. The opening of mitochondrial permeability transition (MPT) pores, located on the inner mitochondrial membrane, is thought to underlie the loss of the mitochondrial membrane potential, which then leads to leakage of cell death signalling factors from mitochondria resulting in cell death [21,23]. Our results showed that low-biocompatibility dialysates might lead to mitochondrial injury, as demonstrated by loss of
the mitochondrial membrane potential and subsequent cell death in HPMCs.

Our findings also demonstrated that low-bio-compatibility dialysates led to oxidative stress. Previous studies showed that ROS, generated by conventional dialysates, mediated functional and structural alterations in peritoneal membranes in vivo [25]. We also found that the amino acid dialysate caused negligible ROS generation in HPMCs compared with conventional glucose dialysates and icodextrin dialysate. Glucose-mediated oxidative stress is most pronounced in senescent cells and may be related to mitochondrial dysfunction [26], and glucose-based dialysates can induce mitochondrial DNA damage in peritoneal mesothelial cells [27]. Generation of ROS, such as superoxide anions and hydrogen peroxide, is unavoidable in mitochondria during functioning of the mitochondrial electron transport chain. In addition, mitochondria are the first to be affected by ROS [9]. They facilitate MPT pore induction via oxidation of SH (sulfhydryl) groups in ANT (adenine nucleotide translocator) and facilitate cytochrome c detachment from cardiolipin [25]. Accumulation of superoxide, an important mediator of mitochondrial depolarization, may lead to loss of the mitochondrial membrane potential. Furthermore, mitochondrial functional disturbances also contribute to the acceleration of ROS accumulation [9]. Our results showed that basal DCF and DHE levels were detectable in the control group. Though basal DCF and DHE levels were
Fig. 5. Detection of hydrogen peroxide and superoxide anion. Panel A: detection of intracellular hydrogen peroxide. Hydrogen peroxide generation was monitored by H₂DCFDA dye and analysed by flow cytometry. One representative expression out of six is shown. The horizontal axis shows the geometric green fluorescence intensity and the vertical axis shows cell number. Left panel: time course of hydrogen peroxide accumulation after 0, 10, 30, 60, 90 and 120 min of exposure to 1.5% dextrose dialysate. Right panel: DCF green fluorescence is overlaid on the histogram of the non-heating and heating groups at 1 h after exposure to different dialysates. Panel B: double staining with H₂DCFDA and 7-AAD dye. Upper histogram panel corresponds to the results from lower dot blot gram. Left panel is the control group and right panel shows 1.5% dextrose dialysate-treated cells. Dead cells negative for DCF and positive for 7-AAD are margined in the tetragon. Panel C: detection of intracellular superoxide anion. Superoxide anion generation was monitored by DHE dye and analysed by flow cytometry. One representative expression out of six is shown. The horizontal axis shows the geometric red fluorescence intensity and the vertical axis shows cell number. Left panel: time course of superoxide anion accumulation after 0, 10, 30, 60, 90 and 120 min of exposure to 1.5% dextrose dialysate. Right panel: EB red fluorescence is overlaid on the histogram of non-heating and heating groups at 1 h after exposure to the different dialysates. Panel D: statistical analysis of the ratio of EB fluorescent intensity. The data are means ± SEMs of six samples in each group. *P < 0.05 versus each other as indicated.
detectable in HPMCs in the control group, bioincompatible dialysates stimulated greater ROS generation in HPMCs in the experimental group. DCF and DHE levels of HPMCs were significantly higher in the experimental groups than in the control group. Significant increases in ROS levels were observed after dialysate treatment. We suggest that mitochondrial functional disturbances and collapse of the mitochondrial membrane potential in HPMCs exposed to bioincompatible dialysates may be due to prompt accumulation of ROS, superoxide anions, and especially hydrogen peroxide. The mechanisms by which dialysates cause ROS accumulation have not been well studied. In our study, increasing the glucose concentration, as with the 2.5 and 4.25% dextrose dialysates, did not further increase intracellular ROS. There was no difference in the lactate concentration among the different dialysates, and the osmolarity of the biocompatible amino acid dialysate was even higher than that of the 1.5% dextrose and icodextrin dialysates (Table 1). Dialysate acidity was the major difference between the biocompatible and bioincompatible dialysates. Our results further confirmed that decreasing the acidity in the 1.5% dextrose and icodextrin dialysates significantly attenuated the dialysate-induced cell death and intracellular ROS accumulation in HPMCs. The acidity of the dialysate is the major stimulator of ROS in dialysate-induced HPMC injury in vitro. We propose that decreasing ROS accumulation and prevention of subsequent mitochondrial injury by correcting the acidity of dialysates may contribute to the decrease in dialysate-induced HPMC injury. Although a limitation in the present study was that the pH of the instilled dialysates equilibrated with the body fluids in vivo, we believe that repeat acute acidic injuries to HPMCs during CAPD may

Table 1. Characteristics of dialysates

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contribute to peritoneal injury. Further studies are needed to confirm the effects of repeat acidity on oxidative stress in vivo. The controlled conditions in the ex vivo study amplified the pathologic changes in HPMCs after dialysate exposure, and provided a convenient means to investigate the protective effects and mechanisms of the heat shock response. Our results showed that the acidity of dialysate is one of the critical causes of acute dialysate-induced damage in HPMCs. The accumulation of ROS and induction of oxidative stress are important mechanisms of dialysate toxicity. Mitochondria are susceptible targets for low-biocompatibility dialysates and they may act as a high-sensitivity biosensor during dialysate-induced oxidative stress. An analysis of ROS accumulation and ΔΨm changes in HPMCs may provide a new, convenient and rapid (within 60 min) means to assess the biocompatibility of dialysates ex vivo.

The present findings have provided new information for further understanding the mechanisms of dialysate-induced HPMC injury.

Several investigators have indicated that sublethal stresses not only result in injury but also induce a particular subset of cellular processes, called the stress response or heat shock response. The stress response induces cellular mechanisms that facilitate cellular repair and survival after acute injury, and induce cytoprotection against subsequent injury. HSPs are the key players of the heat shock response. HSPs, which are a series of endogenous proteins synthesized concomitantly with heat shock response induction, are regarded as multi-functional proteins that protect living organisms against subsequent lethal injury. HSPs can be induced by various exogenous or endogenous pathophysiological stresses. Furthermore, peritoneal HSP70 can be induced by chronic PD therapy [17], and peritoneal dialysate composition determines HSP expression patterns in human mesothelial cells [18,19]. The results of our present study showed that HSP72 induction was detected at 24 h after exposure to dialysates with low biocompatibility, to the conventional glucose-base dialysates and to the 7.5% composition determines HSP expression patterns in human mesothelial cells [18,19]. The results of our present study showed that HSP72 induction was detected at 24 h after exposure to dialysates with low biocompatibility, to the conventional glucose-base dialysates and to the 7.5% icodextrin dialysate, while HSP72 expression was unaltered after amino acid dialysate exposure. In other studies, overexpression of HSP72 was specifically found in mesothelial cells that had detached following in vivo exposure to a PD dialysate but not in mesothelial cells that remained resident in the peritoneal monolayer until the end of the dwell [28].

The induction of HSP72 can be utilized as a tool to assess mesothelial cell injury in the early phase of dialysate exposure [18].

HSP induction has been shown to exert its cytoprotective actions against PD dialysate-induced cell damage in HPMCs. For example, Bidmon et al. [29] demonstrated that overexpression of HSP72, either by non-lethal exposure to dialysate or by transient transfection with HSP72, protected HPMCs against lethal exposure to dialysate. In our study, we utilized heat pretreatment as an instrument to induce HSP production in HPMCs, and this treatment helped to prevent HPMCs from dialysate-induced cytotoxicity. However, we did not detect a difference between dialysate—and heat shock treatment-induced cross-tolerance. Nevertheless, the sensitivity of cell responses to various HSP inducers is different and the type of HSP induction in response to various stresses can also vary. Both heat shock pretreatment and sublethal pretreatment with dialysates can induce HSP synthesis in HPMCs, which leads to the effect of ‘cross-tolerance’ that protects cells against subsequent lethal damage. Importantly, HSP induction by heat shock pretreatment results in less cell viability decreases, ROS accumulation and mitochondrial changes than by dialysate pretreatment. The search for a safe and efficient HSP inducer should provide a novel approach to decrease dialysate-induced cell damage.

In our study, overexpression of HSP72 and HSP27 was observed in HPMCs after heat pretreatment. Significant expression of both HSP72 and HSP27 was observed for at least 24–48 h after heat treatment that comprised the entire experimental period. HSP72 is one of the most inducible HSPs in mammals and its induction can be regarded as a marker of successful heat shock response induction. It may act as a molecular chaperone to prevent protein malfolding and aggregation, and may assist protein transportation through cellular compartmentalization [30]. During maintenance of mitochondrial homeostasis, HSPs are necessary for transportation and assembly of mitochondrial proteins [30]. Our previous studies showed that HSP synthesis can participate in preventing disturbances in mitochondrial morphology induced by sepsis, and counter the failure of mitochondrial energy metabolism also during sepsis [13,14]. In addition to acting as molecular chaperones, HSP27 can also participate in controlling the redox state and confer resistance to oxidative stress [31,32]. A role for HSP27 in modulating mitochondrial function has also been described. For example, it antagonizes Bax-mediated mitochondrial injury and apoptosis [33]. Prior heat shock or overexpression of HSP27 delayed cytochrome c release as well as caspase activation, and reduced the level of apoptosis [34]. HSP27 also protects mammalian cells against the deleterious effects of oxidative stress.

Both HSP72 and HSP27 contribute to inhibiting cell death by several mechanisms upstream and downstream of caspase inactivation, and by caspase-independent pathways [32,35]. HSPs have been suggested to exert their cytoprotective actions by interfering with stress-induced apoptotic programmes, which may be important in the preservation of mesothelial cells. In addition, HSP expression can act as an endogenous antioxidant to decrease ROS accumulation, to prevent dialysate-induced ΔΨm loss and mitochondrial disturbances, as well as cell death.

A limitation of the present study was that the experimental design indirectly linked HSPs to the protective effect of heat pretreatment in HPMCs exposed to dialysate toxicity. However, the heat shock responses and their subsequent effects, such as HSP induction and cross-tolerant protective actions may play important roles in protecting HPMCs from dialysate-induced injury.

We propose that the induction of HSP72 and HSP27 in HPMCs may provide a very important means to protect HPMCs against ‘lethal’ exposure to low-biocompatibility dialysates. We further suggest that ROS accumulation and collapse of the mitochondrial membrane potential are critical mechanisms of dialysate-induced cell injury in HPMCs. Moreover, HSP induction in HMPCs may also offer a plural potency against dialysate-induced cell damage.

The heat shock response has been widely used as means to investigate possible cellular self-protective mechanisms.
Exposure of PD fluids to HPMCs causes stress, damage and even cell death. Our results demonstrated that the heat shock response can protect HPMCs against PD fluid-induced cell death through an anti-oxidative action. They further show that preservation of mitochondrial function is an important means to protect against PD fluid-induced damage. We hypothesize that antioxidants, which also act as HSP inducers, may provide a novel tool to manipulate HSP expression in HPMCs and to induce cytoprotection during in vivo exposure of the peritoneum to low-biocompatibility dialysates. Antioxidants and mitochondrial functional preservation may provide ideal protection for HPMCs against PD fluid-induced damage. Further study will be needed to confirm these possibilities.

In conclusion, the amino acid dialysate was more biocompatible than the glucose- and icodextrin-based dialysates to HPMCs. Exposure to low-biocompatibility dialysates induced ROS accumulation, which is characteristic of oxidative stress, and lead to mitochondrial membrane potential collapse and subsequent cell death. Dialysate acidity is the major stimulator of ROS generation. Heat shock pretreatment, which preferentially induced HSP27 and HSP72, helped to protect HPMCs against low-biocompatibility dialysate-induced cellular damage.

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