The *In vitro* Toxicity of Peritoneal Dialysis Fluid

Wasin Manuprasert MSc*, Sirigul Kanchanabuch MSc*,
Somchai Eiam-Ong MD**, Talerngsak Kanjanabuch MD*,**

** Kidney Ureter Bladder & Metabolic Disorder Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*
*** Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Bangkok, Thailand*

Objective: *To investigate the toxicity of peritoneal dialysis fluid (PDF) components on peritoneal changes in primary human mesothelial cell.*
Material and Method: *To investigate the mechanism of changes, primary human peritoneal mesothelial cells (HPMCs) were isolated from human omental tissue and were exposed for 15 hours with the various concentrations of conventional PDF and various PDF components. The mesothelial injury was determined by calculating a ratio of supernatant and total intracellular LDH while mesothelial apoptosis was assessed and counted by positive TUNEL staining and flow cytometry, respectively.*
Results: *PDF caused mesothelial detachment, de-differentiation, cell injuries, and apoptosis and this depended on the concentrations of PDF. The acidic condition and high glucose concentration likely played a major role in the HPMC injuries and detachment while individual PDF component could not yield mesothelial apoptosis as severe as the whole PDF effects. Thus, the additive effects of PDF composition, instead of the effect of each component, contributed to dialysis-related HPMC damages.*
Conclusion: *PDF showed concentration dependent fashion-induced HPMC injury, dedifferentiation, and apoptosis. All of the abnormalities occurred by the additive effects of PDF components.*

Keywords: *Peritoneal dialysis fluid, Apoptosis, Glucose, Mesothelial cells*

J Med Assoc Thai 2011; 94 (Suppl. 4): S140-S147
Full text. e-Journal: *http://www.mat.or.th/journal*

Peritoneal dialysis (PD) is an effectively established treatment for patients with end stage renal disease (ESRD). The peritoneal membrane acts as a filter between peritoneal dialysis fluid (PDF) and the blood stream. The waste products and excess water are removed from the body through diffusion and convection processes[1]. Although PD is an effective treatment for ESRD patients, long term exposure to PDF results in functional and anatomical changes of the peritoneal membrane. Patients treated with long term PD show time-dependent pathological features, consisting of mesothelial loss, submesothelium thickening, and neoangiogenesis. These changes result from bioincompatibility of the PDF solution[2]. The unphysiological compositions include high concentration of glucose, hyperosmolarity, low pH, and glucose degradation products (GDPs). However, the effects of each PDF component on mesothelial cells have not yet been fully elucidated in the same experiment. The present study was conducted to investigate the toxicity of individual PDF component on mesothelial cell changes.

Correspondence to:
Kanjanabuch T, Division of Nephrology, Department of Medicine and Kidney & Metabolic Disorders Research Center, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
Phone: 0-2256-4321 ext. 211
E-mail: golfnephro@hotmail.com

Material and Method

Cell culture and characterization

The human peritoneal mesothelial cells (HPMCs) were isolated from omental tissue and the preparation of primary culture was performed as previously described[3]. Briefly, the omental tissue was digested with 0.125% trypsin and 0.53 mM EDTA in shaker water bath at 37°C for 20 minutes and then was centrifuged at 1,200 rpm 4°C for 5 minutes to pellet the cells. The cells were re-suspended by fresh complete culture medium (M199 supplemented with 10% FCS, penicillin 50 U/ml, streptomycin 50 µg/ml, insulin 0.1 µg/ml, transferrin 5 µg/ml, hydrocortisone 0.4 µg/ml,

and L-glutamine 5 mM; all chemicals were provided from Sigma) and the cells were seeded in collagen coated culture dish. The culture dish was incubated in a humidified 5% CO_2 atmosphere at 37°C and the medium was changed after the first day and every third day. HPMCs were characterized by their morphological appearance and immunohistochemistry staining of mesothelial marker, cytokeratin 5/8 (Fitzgerald Industries Intl, MA, US).

PDFs and PDF components solutions

All concentrations of conventional PDF (dextrose in lactate buffer pH 5.5) were provided by Baxter Healthcare, Thailand. To further explore the respective effects of glucose, osmolarity, acidity, and GDPs, we prepared culture solutions containing glucose with different concentrations (15, 25, and 42.5 g/L), mannitol with equal osmolarity to dextrose (14, 23, and 41.2 g/L), and solutions in the presence or absence of MGO (all of these solutions contained 4% BSA, and the pH was adjusted to 7.4 with sodium bicarbonate) as well as the acidic culture medium solution (containing 4% BSA, pH adjusted to 5.5 with HCl).

Cell death and cytotoxicity

HPMCs were prepared by depleting serum overnight prior to the experiments. The cells were incubated for 15 hours with various concentrations of PDF, different glucose concentrations, different mannitol concentrations with equal osmolarity to glucose, acidic culture medium, and culture medium containing MGO. Subsequently, the cell viability, toxicity, and apoptosis were assessed and compared with control.

The cytotoxicity of PDF and PDF components on the HPMCs were measured by lactate dehydrogenase (LDH) assay kit (Cytotox; Promega, WI, US) according to the manufacturer's protocol. The results were determined as percentage of supernatant LDH compared with total intracellular LDH.

The numbers of HPMCs apoptosis were counted using flow cytometry after staining the cells with TUNEL (APO-BrdU™ TUNEL assay kit; Invitrogen corporation, Oregon, US) and counterstained with propidium iodide (PI). The results were expressed as a percentage of apoptotic (TUNEL-positive) over the total (PI-positive) cells.

Statistical analysis

All data were expressed as mean ± SEM. Morphological differences among groups were evaluated statistically using the analysis of variance (F-test). The chosen level of significance was $p < 0.05$.

Results

HPMCs detachment and loss of differentiation marker induced by PDF

After 15-hour incubation, cell detachment of HPMCs in the PDF groups occurred in a significantly concentration-dependent fashion (control 100.0 ± 0.0%, 1.5% dextrose PDF 70.0 ± 5.0%, 2.5% dextrose PDF 30.0 ± 5.0%, 4.25% dextrose PDF 20.0 ± 5.0% of attached cells). The main PDF compositions which enhanced cell detachment were high glucose and acidic condition. All of the osmotic agents (glucose and mannitol) induced cell detachment in a concentration-dependent fashion, especially in the glucose aspect (control = 100%, 1.5% dextrose in culture medium = 91.0 ± 13.89%, 2.5% dextrose in culture medium = 60.0 ± 17.32%, 4.25% dextrose in culture medium = 26.67 ± 11.55%, 1.4% mannitol in culture medium = 98.0%, 2.3% mannitol in culture medium = 93.67 ± 5.13%, 4.12% mannitol in culture medium = 88.34 ± 2.89%, acidic culture medium = 46.67 ± 15.28% and 20 μM MGO in culture medium = 94.34 ± 4.04% of attached cells) (Fig. 1-3). Expressions of cytokeratin, the mature differentiation marker of mesothelial cells, were in conformity with the cell detachment result (Fig. 4).

PDF induced apoptosis and cell injuries

To demonstrate apoptosis, apoptotic cells were assessed by TUNEL assay and the nucleus was counterstained by PI. The positive anti-BrdU labeled in the PDF groups showed a concentration-dependent fashion (control = 3.1 ± 0.48%, 1.5% dextrose PDF = 16.09 ± 0.93%, 2.5% dextrose PDF = 16.40 ± 1.62%, and 4.25% dextrose PDF = 17.64 ± 2.87%) (Fig. 5 and 6). The PDF compositions which mainly increased cell detachment were high osmolality and the presence of MGO. All of the osmotic agents (glucose and mannitol) induced apoptosis in a concentration-dependent pattern (control = 3.1 ± 0.48%, 1.5% dextrose in culture medium = 4.75 ± 0.63%, 2.5% dextrose in culture medium = 6.37 ± 1.34%, 4.25% dextrose in culture medium = 7.95 ± 2.87%, 1.4% mannitol in culture medium = 5.74 ± 2.53%, 2.3% mannitol in culture medium = 7.94 ± 1.69%, 4.12% mannitol in culture medium = 9.15 ± 2.44%, acidic culture medium = 4.86 ± 0.48%, and 20 μM MGO in culture medium = 7.07 ± 0.60%) (Fig. 7).

Mesothelial cell injuries were measured by the percentage of supernatant LDH compared with the total intracellular LDH. PDF groups showed the

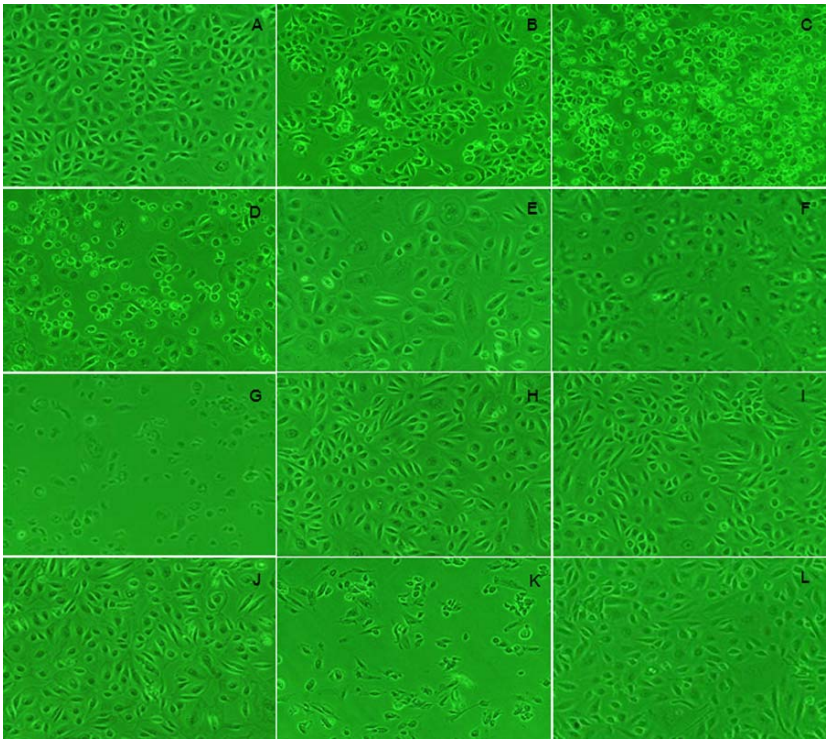

Fig. 1 The effect of PDF on mesothelial morphological changes. After 15 hours treatment, cultured mesothelial cells were observed for the morphologic changes and cell detachment from culture dish. A) culture medium pH 7.4 (control), B) 1.5% dextrose PDF, C) 2.5% dextrose PDF, D) 4.25% dextrose PDF, E) 1.5% dextrose in culture medium, F) 2.5% dextrose in culture medium, G) 4.25% dextrose in culture medium, H) 1.4% mannitol in culture medium, I) 2.3% mannitol in culture medium, J) 4.12% mannitol in culture medium, K) acidic culture medium (pH 5.5), and L) 20 μM MGO in culture medium

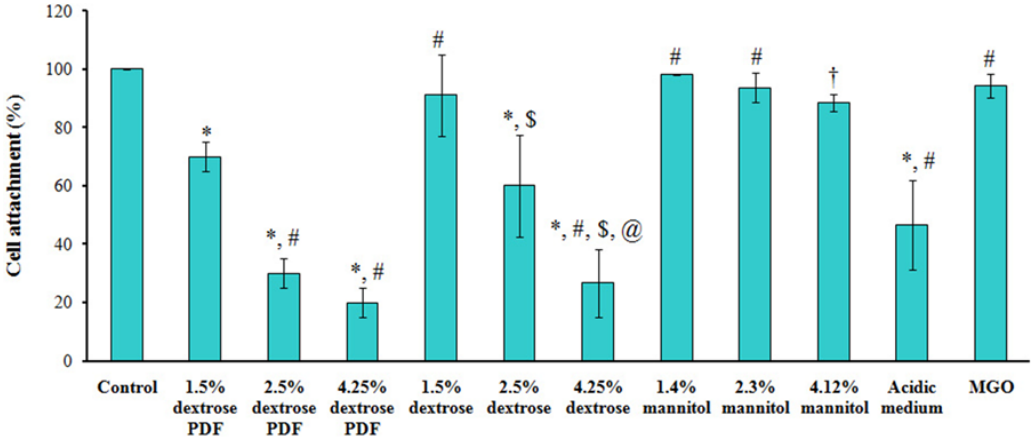

Fig. 2 Percentage of cell attachment when mesothelial cells were exposed to various dosages of PDF at 15 hours. * $p \leq 0.01$ vs. control, $^{\#}$ $p \leq 0.01$ vs. 1.5% dextrose PDF, $^{\$}$ $p \leq 0.01$ vs. 1.5% dextrose, $^{@}$ $p \leq 0.01$ vs. 2.5% dextrose, † $p \leq 0.05$ vs. 1.5% dextrose PDF

concentration-dependent induction of cell injuries (control = 9.30 ± 4.39 %, 1.5% dextrose PDF = 24.51 ± 3.80 %, 2.5% dextrose PDF = 30.41 ± 6.99 %, and 4.25% dextrose PDF = 90.90 ± 5.46%). The PDF compositions which mainly facilitated cell injuries were high glucose and the acidic condition (control = 9.30 ± 4.39 %, 1.5%

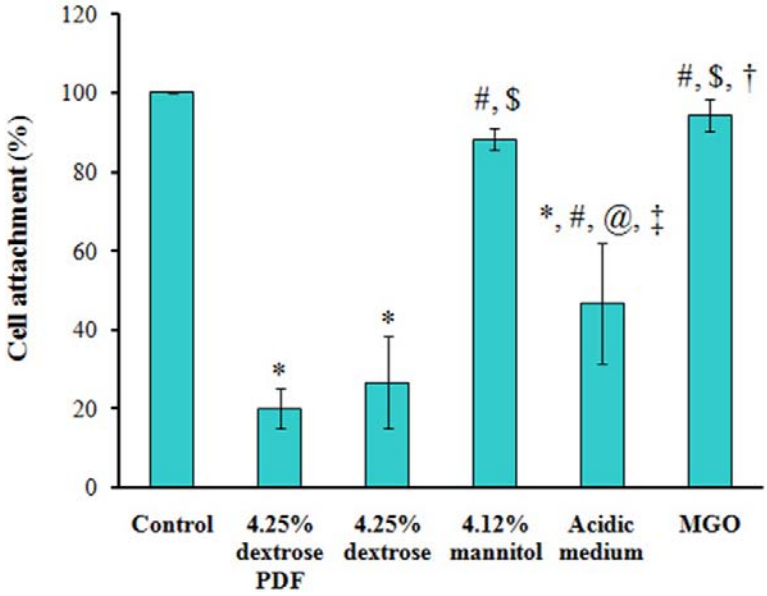

Fig. 3 Percentage of cell attachment when mesothelial cells were exposed to various PDF components at 15 hours. * $p \leq 0.01$ vs. control, $^{\#}p \leq 0.01$ vs. 4.25% dextrose PDF, $^{\$}p \leq 0.01$ vs. 4.25% dextrose, $^{@}p \leq 0.01$ vs. 4.12% mannitol, $^{\dagger}p \leq 0.01$ vs. acid medium, $^{\ddagger}p < 0.05$ vs. 4.25% dextrose

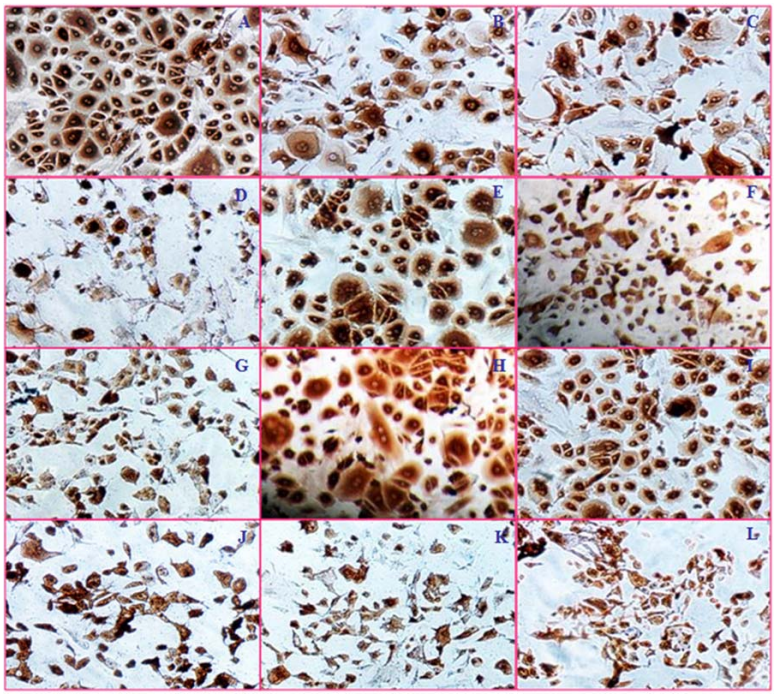

Fig. 4 The effect of PDF-related mesothelial differentiation marker loss. Mesothelial differentiation marker, cytokeratin, is expressed in the mesothelial cytoplasm where brown staining by immunohistochemistry represents the cytokeratin expression. A) culture medium pH 7.4 (control), B) 1.5% dextrose PDF, C) 2.5% dextrose PDF, D) 4.25% dextrose PDF, E) 1.5% dextrose in culture medium, F) 2.5% dextrose in culture medium, G) 4.25% dextrose in culture medium, H) 1.4% mannitol in culture medium, I) 2.3% mannitol in culture medium, J) 4.12% mannitol in culture medium, K) acidic culture medium (pH 5.5), and L) 20 µM MGO in culture medium

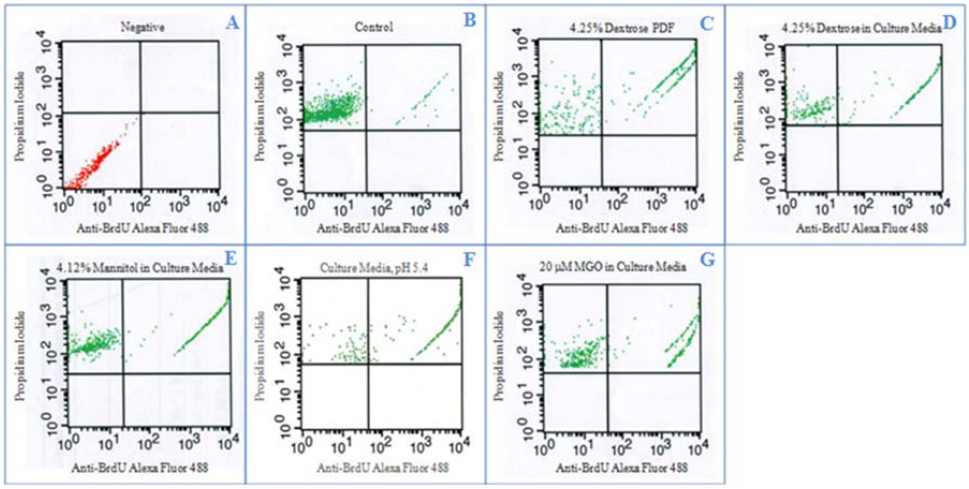

Fig. 5 Apoptotic mesothelial cells counted by flow cytometry. Apoptotic cells were assessed by TUNEL/PI method. BrdU was labeled for DNA fragmentation that results from apoptosis while PI stained cells represent the total cells.

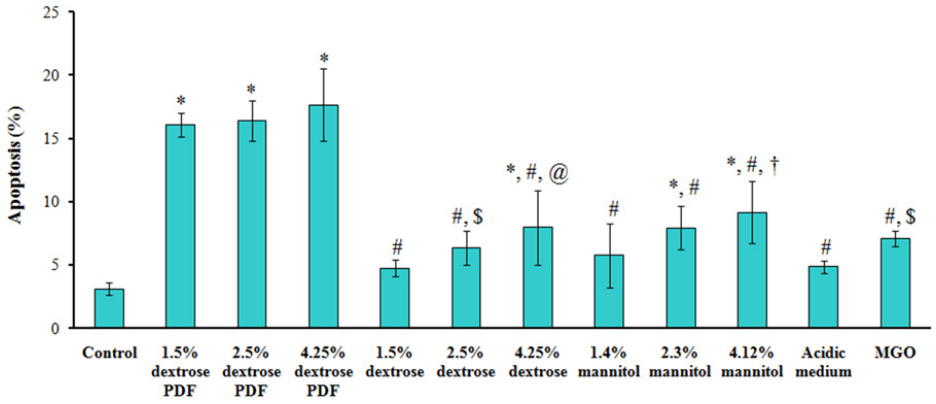

Fig. 6 Percentage of apoptotic cells when mesothelial cells were exposed to various dosages of PDF at 15 hours. * $p \leq 0.01$ vs. control, # $p \leq 0.01$ vs. 1.5% dextrose PDF, $ $p \leq 0.05$ vs. control, @ $p \leq 0.05$ vs. 1.5% dextrose, † $p \leq 0.05$ vs. 1.4% mannitol

dextrose in culture medium = 24.86 ± 2.58 %, 2.5% dextrose in culture medium = 26.25 ± 2.57 %, 4.25% dextrose in culture medium = 93.19 ± 6.10%, 1.4% mannitol in culture medium = 26.13 ± 1.37%, 2.3% mannitol in culture medium = 27.70 ± 3.29%, 4.12% mannitol in culture medium = 31.03 ± 5.26%, acidic culture medium = 86.83 ± 0.94 %, and 20 μM MGO in culture medium = 12.48 ± 0.75 %) (Fig. 8 and 9).

Discussion

The present studies have demonstrated that PDF could contribute in major part to mesothelial morphologic changes, cell injuries, and apoptosis and these changes were dependent on the concentrations of PDF. Yanez-Mo et al[4], reported that conventional PDF induced mesothelial morphological changes (epithelial-to-mesenchymal transition; EMT) and loss of differentiation marker (cytokeratin). Mesothelial injuries determined by LDH release ratio (Fig. 8 and 9) also showed PDF concentration-dependent fashion that was also demonstrated by Hunjoo Ha et al[5]. In the present study, the PDF compositions showed additive effects to mesothelial morphologic changes and cell injuries but the major components to induce all above abnormalities were high glucose and acidic condition (Fig. 7-9). Lee and Ha[6] reported the effect of high glucose concentration in inducing EMT formation of $HPMC_s$. Ishibashi et al[7] reported that high glucose

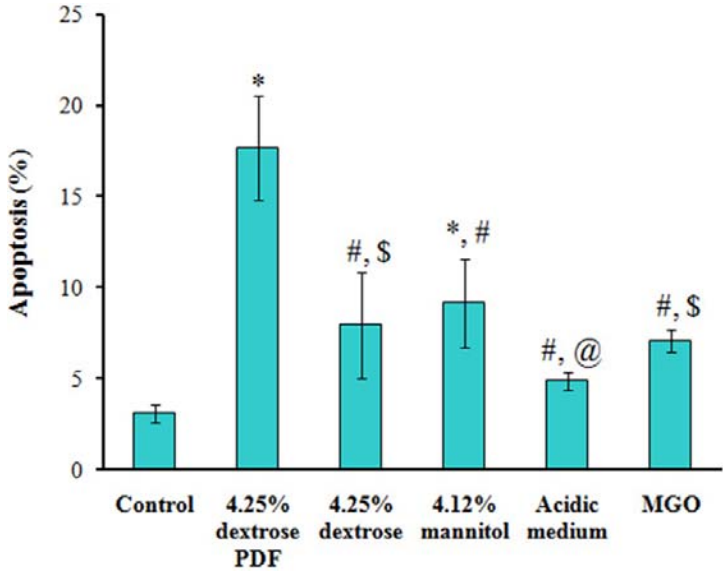

Fig. 7 Percentage of apoptotic cells when mesothelial cells were exposed to various PDF components at 15 hours. * $p \leq 0.01$ vs. control, # $p \leq 0.01$ vs. 4.25% dextrose PDF, $ $p \leq 0.05$ vs. control, @ $p \leq 0.05$ vs. 4.12% mannitol

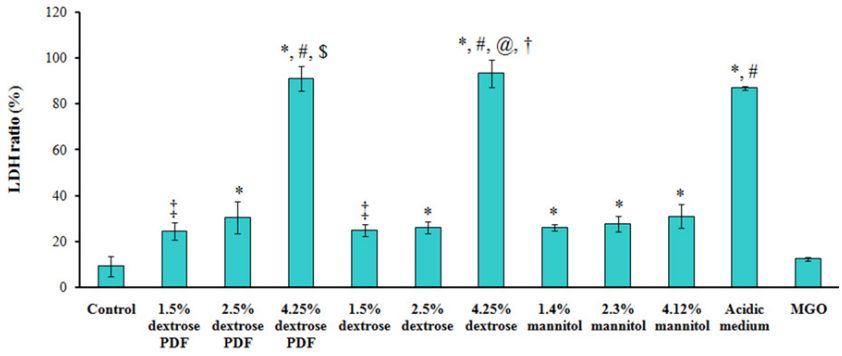

Fig. 8 Percentage of LDH ratio when mesothelial cells were exposed to various dosages of PDF at 15 hours. * $p \leq 0.01$ vs. control, # $p \leq 0.01$ vs. 1.5% dextrose PDF, $ $p \leq 0.01$ vs. 2.5% dextrose PDF, @ $p \leq 0.01$ vs. 1.5% dextrose, † $p \leq 0.01$ vs. 2.5% dextrose, ‡ $p \leq 0.05$ vs. control

concentration caused mesothelial cell injuries similar to human endothelial cell injuries when exposed to high glucose condition while hyperosmotic environment (concentration of mannitol equal to concentration of glucose) had no effects. Yermolaieva et al[8] demonstrated that cultured neuron was injured by acidic condition. Regarding other PDF components, GDP also caused mesothelial cell injuries. MGO increased LDH release to cultured medium and induced co-expression of α-smooth muscle actin (α-SMA) and cytokeratin in mesothelial cells while E-cadherin expression was decreased, indicating that mesothelial cells underwent EMT[9,10].

The effects of concentration dependent-induced mesothelial apoptosis of PDF were previously illustrated by Alscher et al and Yang et al[11,12]. The PDF composition had additive effects to mesothelial apoptosis while the major factors were high glucose and high osmolarity condition (Fig. 5-7). Alscher et al[11] reported that mesothelial apoptosis was induced by high osmolarity (hyperosmolarity) and the hyperosmolarity caused human corneal epithelial cells apoptosis via c-jun N-terminal kinase/extracellular-regulated kinase (JNK/ERK) pathway[13]. Kaifu et al reported that mesothelial apoptosis was enhanced by high glucose concentration-induced caspase 3 activation[14]. Regarding other PDF components, GDP and acidic condition could also augment mesothelial

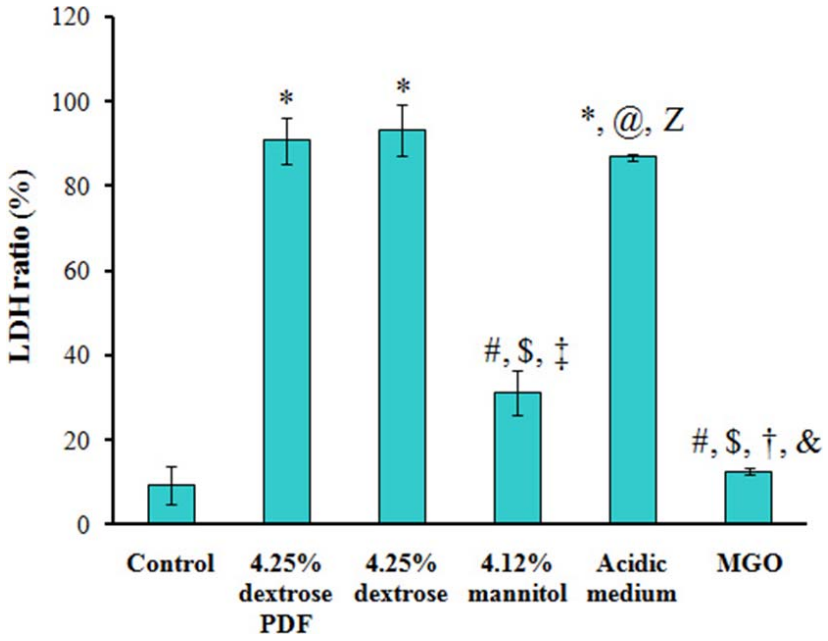

Fig. 9 Percentage of LDH ratio when mesothelial cells were exposed to various PDF components at 15 hours. * $p \leq 0.01$ vs. control, # $p \leq 0.01$ vs. 4.25% dextrose PDF, $ $p \leq 0.01$ vs. 4.25% dextrose, @ $p \leq 0.01$ vs. 4.12% mannitol, † $p \leq 0.01$ vs. acidic medium, ‡ $p \leq 0.05$ vs. control, z $p \leq 0.05$ vs. 4.25% dextrose, & $p \leq 0.05$ vs. 4.12% mannitol

apoptosis. MGO induced mesothelial apoptosis by increasing the expression of caspase-9 and tumor protein-53 (p53) and acidic environment up-regulated pro-apoptotic protein, Bcl-2-associated X protein (Bax) by activating interleukin-1b converting enzyme (ICE)-like caspases[15,16].

In conclusion, PDF-induced peritoneal alteration and PDF component contribute to peritoneal morphologic changes by the additive effects.

Acknowledgement

The present study was supported by Government budget (year 2007-8) and Ratchadapisek-sompotch Fund, Faculty of Medicine, Chulalongkorn University. The authors gratefully acknowledged the staffs of KUB-MED Research Unit, Chulalongkorn University, for technical assistance. The authors would like thank Mr. Preecha Ruangvejvorachai together with Ms. Jutamas Wongphoom for their expertise in histologic and immunohistochemical analyses.

Potential conflicts of interest

None.

References

1. Khanna R, Nolph KD, Oreopoulos DG. Peritoneal dialysis in diabetics. In: Khanna R, Nolph KD, Oreopoulos DG, editors. The essentials of peritoneal dialysis. Boston: Kluwer Academic Publishers; 1993: 113-7.
2. Di Paolo N, Sacchi G, De Mia M, Gaggiotti E, Capotondo L, Rossi P, et al. Morphology of the peritoneal membrane during continuous ambulatory peritoneal dialysis. Nephron 1986; 44: 204-11.
3. Stylianou E, Jenner LA, Davies M, Coles GA, Williams JD. Isolation, culture and characterization of human peritoneal mesothelial cells. Kidney Int 1990; 37: 1563-70.
4. Yanez-Mo M, Lara-Pezzi E, Selgas R, Ramirez-Huesca M, Dominguez-Jimenez C, Jimenez-Heffernan JA, et al. Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 2003; 348: 403-13.
5. Ha H, Yu MR, Choi HN, Cha MK, Kang HS, Kim MH, et al. Effects of conventional and new peritoneal dialysis solutions on human peritoneal mesothelial cell viability and proliferation. Perit Dial Int 2000; 20 (Suppl 5): S10-8.
6. Lee HB, Ha H. Mechanisms of epithelial-mesenchymal transition of peritoneal mesothelial cells during peritoneal dialysis. J Korean Med Sci 2007; 22: 943-5.
7. Ishibashi Y, Sugimoto T, Ichikawa Y, Akatsuka A, Miyata T, Nangaku M, et al. Glucose dialysate in-

duces mitochondrial DNA damage in peritoneal mesothelial cells. Perit Dial Int 2002; 22: 11-21.
8. Yermolaieva O, Leonard AS, Schnizler MK, Abboud FM, Welsh MJ. Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. Proc Natl Acad Sci U S A 2004; 101: 6752-7.
9. Witowski J, Korybalska K, Wisniewska J, Breborowicz A, Gahl GM, Frei U, et al. Effect of glucose degradation products on human peritoneal mesothelial cell function. J Am Soc Nephrol 2000; 11: 729-39.
10. Hirahara I, Ishibashi Y, Kaname S, Kusano E, Fujita T. Methylglyoxal induces peritoneal thickening by mesenchymal-like mesothelial cells in rats. Nephrol Dial Transplant 2009; 24: 437-47.
11. Alscher DM, Biegger D, Mettang T, van der KH, Kuhlmann U, Fritz P. Apoptosis of mesothelial cells caused by unphysiological characteristics of peritoneal dialysis fluids. Artif Organs 2003; 27: 1035-40.
12. Yang AH, Chen JY, Lin YP, Huang TP, Wu CW. Peritoneal dialysis solution induces apoptosis of mesothelial cells. Kidney Int 1997; 51: 1280-8.
13. Luo L, Li DQ, Pflugfelder SC. Hyperosmolarity-induced apoptosis in human corneal epithelial cells is mediated by cytochrome c and MAPK pathways. Cornea 2007; 26: 452-60.
14. Kaifu K, Kiyomoto H, Hitomi H, Matsubara K, Hara T, Moriwaki K, et al. Insulin attenuates apoptosis induced by high glucose via the PI3-kinase/Akt pathway in rat peritoneal mesothelial cells. Nephrol Dial Transplant 2009; 24: 809-15.
15. Amore A, Cappelli G, Cirina P, Conti G, Gambaruto C, Silvestro L, et al. Glucose degradation products increase apoptosis of human mesothelial cells. Nephrol Dial Transplant 2003; 18: 677-88.
16. Park HJ, Lyons JC, Ohtsubo T, Song CW. Acidic environment causes apoptosis by increasing caspase activity. Br J Cancer 1999; 80: 1892-7.

ภาวะเป็นพิษของน้ำยาล้างไตทางช่องท้องในเซลล์เพาะเลี้ยง

วศิน มนูประเสริฐ, สิริกุล กาญจนบุษย์, สมชาย เอี่ยมอ่อง, เถลิงศักดิ์ กาญจนบุษย์

วัตถุประสงค์: *เพื่อศึกษาความเป็นพิษของส่วนประกอบภายในน้ำยาล้างไตทางช่องท้องต่อเซลล์บุผนังช่องท้อง*
วัสดุและวิธีการ: *นำเซลล์เยื่อบุผนังช่องท้องมนุษย์มาเลี้ยงในน้ำยาล้างไตทางช่องท้องที่ความเข้มข้นต่างกัน และในสารละลายส่วนประกอบของน้ำยาเป็นเวลานาน 15 ชั่วโมง ประเมินการบาดเจ็บของเซลล์โดยการคำนวณจากสัดส่วนปริมาณการหลั่งของโปรตีนแอลดีเอชในน้ำยาเพาะเลี้ยงเทียบกับภายในเซลล์ และประเมินการตายของเซลล์ด้วยกระบวนการ aptosis ด้วยการย้อมด้วย เทคนิค TUNEL และนับด้วยเครื่อง flow cytometry*
ผลการศึกษา: *จากการศึกษาพบว่าการสัมผัสกับน้ำยาล้างไตทางช่องท้องก่อให้เกิดเซลล์เยื่อบุผนังช่องท้องบาดเจ็บหลุดลอก กลายเป็นเซลล์ตัวอ่อน และตายด้วยกระบวนการการ aptosis โดยการเปลี่ยนแปลงทั้งหมดข้างต้น มีความสัมพันธ์เป็นเส้นตรงกับความเข้มข้นของน้ำยา ซึ่งความเข้มข้นของน้ำตาลกลูโคสที่สูง และความเป็นกรดของน้ำยาเป็นกลไกสำคัญในการทำให้เซลล์บาดเจ็บและหลุดลอก อย่างไรก็ตามไม่ใช่ปัจจัยใดปัจจัยหนึ่งของน้ำยาที่ส่งผลกระทบรุนแรงต่อการตายของเซลล์ หากเกิดขึ้นจากผลรวมของส่วนประกอบทั้งหมดของน้ำยา*
สรุป: *น้ำยาล้างไตทางช่องท้องทำให้เซลล์เยื่อบุผนังช่องท้องบาดเจ็บ กลายเป็นเซลล์ตัวอ่อน และล้มตายด้วยกระบวนการ aptosis ซึ่งการเปลี่ยนแปลงสัมพันธ์กับขนาดความเข้มข้นของน้ำยา และเกิดจากผลลัพธ์รวมของทุกองค์ประกอบของน้ำยาล้างไตทางช่องท้อง ไม่ได้เกิดจากองค์ประกอบใดองค์ประกอบหนึ่งเป็นหลัก*