Effect of standard and neutral-pH peritoneal dialysis solutions upon fibroblasts proliferation

Efeito da solução de diálise peritoneal com pH neutro e padrão sobre a proliferação de fibroblastos

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Abstract

Introduction: Continuous exposition of the peritoneal membrane to conventional dialysis solutions is an important risk factor for inducing structural and functional alterations. **Objective:** To compare *in vitro* mouse fibroblast NIH-3T3 cell viability after exposition to a neutral pH dialysis solution in comparison to cells exposed to a standard solution. **Methods:** Experimental study to compare the effects of a conventional standard or a neutral-pH, low-glucose degradation products peritoneal dialysis solution on the viability of exposed fibroblasts in cell culture. Both solutions were tested in all the commercially available glucose concentrations. Cell viability was evaluated with tetrazolium salt colorimetric assay. **Results:** Fibroblast viability was significantly superior in the neutral pH solution in comparison to control, in all three glucose concentrations (Optical density in nm-means ± SD: 1.5% 0.295 ± 0.047 *vs.* 0.372 ± 0.042, $p < 0.001$; 2.3% 0.270 ± 0.036 *vs.* 0.337 ± 0.051, $p < 0.001$; 4.25% 0.284 ± 0.037 *vs.* 0.332 ± 0.032, $p < 0.001$; control *vs.* neutral pH respectively, *Student t* Test). There was no significant difference in cell viability between the three concentrations of glucose when standard solution was used (ANOVA $p = 0.218$), although cell viability was higher after exposition to neutral pH peritoneal dialysis fluid at 1.5% in comparison to 2.3 and 4.25% glucose concentrations (ANOVA $p = 0.008$: Bonferroni 1.5% *vs.* 2.3% $p = 0.033$, 1.5% *vs.* 4.25% $p = 0.014$, 2.3% *vs.* 4.25% $p = 1.00$). **Conclusion:** Cell viability was better in neutral pH dialysis solution, especially in the lower glucose concentration. A more physiological pH and lower glucose degradation products may be responsible for such results.

Keywords: cell culture techniques; fibroblasts; materials testing; peritoneal dialysis.

Resumo

Introdução: A exposição contínua da membrana peritoneal a soluções convencionais de diálise é um importante fator de risco para induzir alterações estruturais e funcionais. **Objetivo:** Comparar a viabilidade *in vitro* dos fibroblastos NIH-3T3 de camundongo após exposição à solução de diálise com pH neutro com células expostas à solução padrão. **Métodos:** Estudo experimental; ambas as soluções foram testadas em todas as concentrações de glicose comercialmente disponíveis. A viabilidade celular foi avaliada por ensaio colorimétrico de sal tetrazólio. **Resultados:** A viabilidade de fibroblastos foi melhor na solução de pH neutro em relação ao controle nas três concentrações de glicose (densidade óptica em nm-médias ± DP: 1,5% 0,295 ± 0,047 *vs.* 0,372 ± 0,042, $p < 0,001$; 2,3% 0,270 ± 0,036 *vs.* 0,337 ± 0,051, $p < 0,001$; 4,25% 0,284 ± 0,037 *vs.* 0,332 ± 0,032, $p < 0,001$; controle *vs.* pH neutro respectivamente, teste *t* de *Student*). Não houve diferença significativa na viabilidade celular entre as três concentrações de glicose quando solução padrão foi utilizada (ANOVA $p = 0,218$), embora a viabilidade celular tenha sido superior após exposição aos fluidos de diálise peritoneal neutros, pH 1,5% em comparação com 2,3 e 4,25% de concentrações de glicose (ANOVA $p = 0,008$: Bonferroni 1,5% *vs.* 2,3% $p = 0,033$, 1,5% *vs.* 4,25% $p = 0,014$, 2,3% *vs.* 4,25% $p = 1,0$). **Conclusão:** A viabilidade celular foi melhor em solução neutra de pH de diálise, especialmente nas menores concentrações de glicose. O pH fisiológico e com menos produtos de degradação de glicose podem ser responsáveis por estes resultados.

Palavras-chave: diálise peritoneal; fibroblastos; teste de materiais; técnicas de cultura de células.

Introduction

Long-term peritoneal dialysis (PD) as therapy for end-stage renal disease (ESRD) patients is only feasible if adequate peritoneal membrane structure and function are preserved. Standard PD solutions are acid (pH = 5.5), lactate-buffered, with high glucose concentrations (75-220 mmol/L), hyperosmolar (334-486 mOsm/L) and contain high concentration of glucose degradation products (GDP) - known to affect peritoneal function.[1] Lasting PD therapy with an intact peritoneal membrane has been, as yet, an unreached goal and the main drive to develop biocompatible PD solutions. Peritoneal membrane continuous exposition to acid solutions seems to be an important risk factor for structural and functional changes. Loss of mesothelial cells, small vessels changes, thickened and fibrotic sub-mesothelial compact zone have been observed at peritoneal biopsies.[2] Functional changes have included ultrafiltration loss, small solutes clearance decline and, eventually, technique failure.[3] Using biocompatible dialysis solutions is paramount in accomplishing long-term membrane survival.[4] A neutral-pH, poor GDP dialysis solution, presented in a double-chambered bag has been developed in anticipation of improved biocompatibility.[3] Previous clinical and experimental studies have suggested its beneficial effects.[3,5,6] The aim of the current study was to compare mouse fibroblast NIH-3T3 - a well characterized cell lineage - viability after exposition to either the standard acid or the neutral-pH dialysis solution.

Methods

Mouse fibroblast NIH-3T3 cell cultures were placed in media containing conventional (Control) PD solution or a neutral-pH PD fluid. All the commercially available glucose (1.5, 2.3 and 4.25%) concentrations were tested.

Dialysis solutions

Both solutions used glucose as osmotic agent. The control solution was Peritosteril® (Fresenius Medical Care, Jaguariúna, Brazil) and the neutral-pH solution was Balance® (Fresenius Medical Care, Bad Homburg, Germany) provided in a dual-chambered bag. The composition of both dialysis solutions is presented in Table 1. The 1.5% glucose Balance® bag contains an alkaline chamber with sodium lactate (sodium: 75 mmol/L; lactate: 70 mmol/L) and a second acid fluid chamber with electrolytes and glucose (sodium: 193 mmol/L; calcium: 3.5 mmol/L; magnesium: 1 mmol/L; chloride: 203 mmol/L; glucose: 166.5 mmol/L). The Balance® solution is ready for use by opening the seal between the two chambers and mixing their contents, resulting in a fluid with pH in the range of 6.8 to 7.4. Differently, Peritosteril® has a pH of 5.5. Both Peritosteril® and Balance® bags were provided by Fresenius Medical Care.

Table 1 Composition of the peritoneal dialysis solutions before mixing with culture medium

Components in mmol/L	Control	Neutral pH
Sodium	134	134
Calcium	1.75	1.75
Magnesium	0.5	0.5
Chloride	103.5	101.5
Lactate	35	35
pH	5.5	7.0
Osmolarity (mOsm/L)	358	358

Cell cultures

Mouse fibroblast cell line NIH-3T3 (American Type Culture Collection CRL-1658, Manassas, VA, USA) grown in 25 ml bottles, containing Dulbecco's modified eagle's medium (DMEM) cell culture media, gentamycin at 0.025 g/L, streptomycin/penicillin at 0.1 g/L and supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Incubations were performed in a humid atmosphere at 37 °C and 5% CO_2. All the procedures were performed on a laminar flow hood, observing strict aseptic techniques to minimize the risk of microbial contamination. Fibroblast growth in culture occurred as an adherent monolayer. Cells were washed with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA) and detached by trypsin-EDTA 0.05% (Gibco, Grand Island, NY, USA). FBS was used to inactivate trypsin action. Cells were counted on a Neubauer chamber and seeded in DMEM on 96-well tissue culture plates at a density of 0.5 x 10^4 cells/well and allowed to grow for 24 hours, when 50 µL of the test solutions (control/DMEM or pH-neutral/DMEM) were added to each well, followed by a 48-hour incubation time. Tests were performed in triplicate by mixing the two PD solution to DMEM at a 1:1 ratio, at different glucose concentrations. The 1:1 ratio of PD solutions and culture medium and the 48-hour incubation time was

reached after a series of pilot tests that evaluated cell proliferation with different proportions of test solution and culture media (25, 50, 75 and 100% of peritoneal dialysis fluid), or different incubation times (24, 48 and 72-hour). Fibroblast growth was poor at any incubation time in the presence of 75 or 100% PD solutions. Control wells (DMEM 100%) best cell viability was obtained at 48-hour incubation time.

MTT cell viability assessment

Cell viability was evaluated with 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide (MTT)(Sigma, Saint Louis, MO, USA). After a 48-hour incubation, added PD solution/DMEM was removed from each well and cells washed with PBS (100 µL). DMEM with 10% MTT 5 mg/ml solution (50 µL/well) was added for a further 4-hour incubation time. Next, MTT was taken out, dimethylsulphoxide [(DMSO); 100 µL; Sigma Co.) was added. Conversion of the MTT product - an estimate of cell viability - was measured by optical density (OD) at 570 nm, using a spectrophotometer fitted with a Microplate Reader (Bio-Rad, Hercules, CA, USA). The OD for the negative control (simply DMEM) represented 100% cell viability. All the experiments were thrice repeated and sample analyses were performed in triplicate.

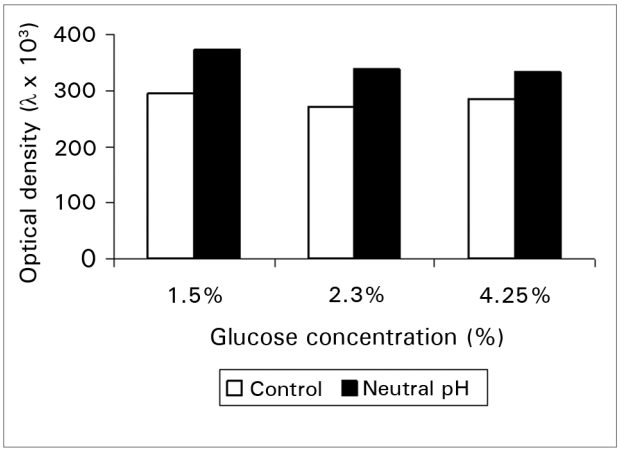
Statistical analysis

Variables are presented as mean and standard deviation (SD). *Student's t* test was used to compare two continuous variables; normality was tested by Kolmogorov-Smirnov test. ANOVA, with ad hoc Bonferroni test, was employed for multiple comparisons. Differences were considered significant at a p value of 0.05 or less. A Statistical Package for Social Sciences s100 µL software (SPSS, version 17 for Windows, SPSS Inc, Chicago, IL, USA) was used in all statistical analyses.

Results

Cells viability after exposition to standard (Control) and neutral-pH solutions with different glucose concentrations is depicted in Figure 1. Cells viability was significantly greater on the neutral pH than on the acid solution, at all tested glucose concentrations, measured by optical density (in nm): 0.295 ± 0.047 *vs.* 0.372 ± 0.042, $p < 0.001$ for 1.5% glucose control and neutral pH, respectively; 0.270 ± 0.036 *vs.* 0.337 ± 0.051, $p < 0.001$ for 2.3% control and neutral pH, respectively; 0.284 ± 0.037 *vs.* 0.332 ± 0.032, $p < 0.001$ for 4.25% control and neutral pH solution, respectively. No significant differences in cell viability among the three control solution glucose concentrations were observed (repeated-measures ANOVA: $p = 0.218$). However, cells viability on neutral-pH solution was greater after exposition to 1.5 than to 2.3 or 4.25% glucose concentrations [ANOVA with post hoc Bonferroni: $p = 0.033$ (1.5% *vs.* 2.3%); $p = 0.014$ (1.5% *vs.* 4.25%); $p = 1.00$ (2.3% *vs.* 4.25%)].

Figure 1. Fibroblast viability after cells were exposed to acid and neutral pH solutions at different glucose concentrations.



Discussion

The current experiments demonstrated that cells viability is greater when fibroblasts are exposed, *in vitro*, to a neutral pH than to an standard PD solution. Besides, greater viability of cells exposed to 1.5%, in comparison to 2.3 and 4.25% glucose on the neutral pH solution was evidenced. The significance of such findings is not yet clear, but it is conceivable that a neutral pH and less glucose degradation products are better for cell viability and that such effect may be amplified at lower glucose concentrations. Improved cell viability with the neutral pH solution does not mean that it is nontoxic or does not induce fibrosis. Fibroblasts were used as an experimental model[7-9] and evaluating the effects of such exposition in other cell models, or in experimental animals, would be desired.[10,11] Use of the neutral pH dialysis solution, *in vivo,* resulted in improved peritoneal ultrafiltration and peritoneal membrane integrity markers.[3,12] It also improved mesothelial cells proliferation and viability.[13]

A 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide-based (MTT) colorimetric assay was used to evaluate cell viability. Its use allows measuring citotoxicity, cell proliferation and cell activation. The assay is based on viable cell mitochondrial dehydrogenases to transform MTT into a water-insoluble blue formazan. The assay method is not time consuming, cheap and allows for using multiwall scanning spectrophotometry.[14] Fibroblasts have also been used in a previous study, which found no difference in cell proliferation with incubation in either icodextrin-containing or standard glucose dialysis solution.[15] The study was carried out on pH-adjusted solutions mixed with culture medium, a major distinction from the present work, where test solutions pH difference was a major characteristic being evaluated. Interestingly, heat sterilized 4.25% glucose solution caused a significant reduction in *in vitro* cell growth, compared with filter-sterilized solution, supposedly associated with increased GDP.[15] Occurrence of GDP and low pH seem to be important factors related with biocompatibility of PD solutions. GDP have been implicated in the formation of advanced glycation end products (AGEs) occurring during the heat sterilization process. The presence of AGEs has been associated with reduce mesothelial cell growth *in vitro*.[16] It has been previously shown that *in vitro* cells exposed to lower glucose concentrations (1.5 or 2.27%) grow better than at higher (3.86%).[17] Such results are in agreement with findings of this study, suggesting that GDP are highly inhibitory of *in vitro* cell growth (17) and could be related with clinical consequences.[18,19] The neutral pH peritoneal dialysis solution was designed as a two-chamber bag, one containing the alkaline component with lactate and the other with electrolytes and glucose. Such approach allows for a higher pH and sterilization with reduced formation of GDP. The two-compartments fluids are mixed immediately before infusion, by rupturing a seal between the chambers.

In conclusion, fibroblasts viability was higher on neutral-pH dialysis solution, especially with lower glucose concentrations. It is possible that a more physiological pH and lower GDP are associated with such results.

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Conflict of interests/disclosure

Both Peritosteril® and Balance® bags were provided by Fresenius Medical Care. André A. Poitevin, Ana E. Figueiredo, Domingos O. d'Avila and Carlos E. Poli-de-Figueiredo have received travel awards by both companies with commercially available peritoneal dialysis products in Brazil (Fresenius Medical Care and Baxter) in the past.

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