Polymyxin B: dose and time dependent nephrotoxicity effect in vitro

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Abstract

Objective: To characterize the toxicity of polymyxin B (PmxB) in renal cell in different dosage and times.

Methods: LLC-PK1 cells grown in 12 well multiwell plates were divided into the following groups: Control (CTL) - cells maintained in DMEM supplemented with 5%; G1 - cells exposed to concentration of 75µM PmxB G2 - cells exposed to concentration of 375µM PmxB. Each group was assessed at 24, 48 and 72 hours as for cell viability (Acridine orange/ethidium bromide) and apoptosis (Hoechst 33342).

Results: The data demonstrate the cell viability and apoptosis exposure of three doses of PmxB in three time intervals, with a significant increase in toxicity to high doses and longer duration of stay in the antibiotic to apoptosis.

Conclusion: Cytotoxicity by PmxB in cell culture model, showed to be time and dose dependent, increasing with increased exposure and higher dose of antibiotic.

Keywords
Polymyxin B; Acute kidney injury; LLC-PK1 cells; Apoptosis

Resumo

Objetivo: Caracterizar a toxicidade da polimixina B (PmxB) em células renais em dosagem e tempos diferentes.

Métodos: Células LLC-PK1, cultivadas em placas multiwell de 12 poços, foram divididas nos seguintes grupos: Controle (CTL) - células mantidas em meio DMEM suplementado a 5%; G1 - células expostas à concentração de 75mM de PmxB; G2 - células expostas às concentrações de 375mM de PmxB. Cada grupo foi avaliado nos tempos de 24, 48 e 72 horas quanto à viabilidade celular (Acridine Orange/ Brometo de Etíldio) e apoptose (Hoechst 33342).

Resultados: Os dados demonstraram a viabilidade celular e a apoptose à exposição de três doses de PmxB em três intervalos de tempo, com um aumento significativo da toxicidade à elevação das doses e ao maior tempo de permanência no antibiótico para apoptose.

Conclusão: A citotoxicidade pela PmxB, no modelo de cultivo celular, mostrou tempo e dose dependente, aumentando com a maior exposição e maior dose de antibiótico.

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**Introduction**

Recent epidemiological studies relate nephrotoxic drugs with 19% to 25% of cases of acute kidney injury (AKI).\(^1\) The applicability of potentially nephrotoxic agents in the hospital environment is inevitable, due to the increasingly frequent occurrence of multidrug-resistant bacteria. Another problem relates to the fact that the nephrotoxic acute kidney injury is underestimated as a factor and possibly modifiable causes of AKI.\(^1\)

Among the nephrotoxic agents, the ones from the group of polymyxins stand out. From the five (polymyxins A, B, C, D and E), only polymyxin B (PmxB) and polymyxin E have been used in intra-hospital environment. It is worth noting that polymyxin E presents a lower nephrotoxic potential than PmxB, in contrast, the bactericidal action of PmxB is more expressive.\(^2\)

The composition of polymyxin B sulfate, also known as polymyxin B or Aerosporin, consisting of a mixture of sulfates of polymyxin B\(_1\) and polymyxin B\(_2\).\(^2-4\) As it acts on the cytoplasmic membrane, the PmxB compromises the osmotic properties and transport mechanisms of the cell membrane, as the composition of this structure in bacteria and human cell is similar.\(^2-5\)

This antimicrobial agent may be accumulated in various organs and tissues such as kidney, liver, brain, lungs, heart and muscles for a period of five days.\(^2-4\) Since the excretion of this pharmacological agent occurs primarily by the kidneys, where 60% of the drug can be found in urine. In the event of renal injury, its half-life may be increased to two or three days. Nephrotoxicity, neurotoxicity and hypersensitivity reactions constitute as main characteristic toxic effects.\(^2-4\)

Experimental studies have demonstrated that the flat portion of the proximal tubule (segment S3) is the most susceptible to ischemic lesion or nephrotoxic. The cells in this part of the nephron lose tubular brush border normal and undergo extensive cellular necrosis. The proximal tubular cells of the cortex (segments S1 and S2) are less susceptible to injury, less necrosis was observed in the cells of the S3 segment. Despite the frequent use of the term necrosis, apoptosis, another mechanism of cell death, is also present in cellular injury of ischemic and nephrotoxic AKIs. Moreover, histological data showing the concrete necrosis in kidney tubules are not available yet. This assumes that its manifestation and sparse variable are responsible, in part, for precipitated functional damage in AKI.\(^1,6\)

The renal tubular cells have potential reactions to renal injury. A sub-lethal cell injury may lead to cellular dysfunction, altered gene expression, the cell dedifferentiation and, finally, the recovery of cell function.\(^6\)

The lethal cell injury, in the case of AKI, when not restored homeostasis can lead to necrosis and apoptosis. The morphological characteristics as well as mechanisms that involve processes of necrosis and apoptosis are considerably different.\(^1,6\)

In the AKI, apoptosis and necrosis may be caused by the same cytotoxic events. It seems clear that apoptosis is a response to injury of damaged cells which undergo a short ischemia or toxins at low concentrations compared to those that induce rapid metabolic collapse and necrosis. Some studies demonstrate that the mechanism of renal tubular cell death seems to depend on the severity of the injury.\(^1\)

In an attempt to simulate nephrotoxic effects of this drug in vitro, LLC-PK\(_1\) cells, proximal tubular epithelial cells were strain of choice. Obtained from the Hampshire pig, they have many renal epithelial morphology as apical microvilli, tightjunctions, wraps the basolateral membrane. They also feature a predominantly proximal phenotype, constituting thus a safe model to assess nephrotoxicity.\(^1,6\)

Organisms increasingly multiresistant to conventional antibiotics has led to the rescue of PmxB which has taken a Pharmacotherapeutic important role in recent years, and not surprisingly, already manifest adverse effects similar to well-established drugs in the clinic. This combination of facts justify studies like these that developed nephrotoxicity experiments with PmxB. Given these considerations, the aim of this study was to characterize the toxicity of PmxB in renal cells.
Methods

Experimental quantitative in vitro study conducted by the Laboratory of Experimental Animal Model of School of Nursing, University of São Paulo (LEMA - EEUSP) and in the laboratory of Cell and Molecular Biology in the Department of Nephrology, Federal University of São Paulo (UNIFESP).

LLC-PK₁ cells were used, a line of proximal tubular epithelial of pig, passage 40, obtained from the American Tissue Collection (ATCC), which were taken from a nitrogen container, thawed and maintained in culture bottles containing Dulbecco’s Modified Eagle’s Medium (DMEM) with 5% fetal bovine serum (FBS).

LLC-PK₁ cells grown in 12 multiwell plates were divided into the following groups: Control (CTL) - cells maintained in DMEM supplemented with 5%; G1 - cells exposed to concentration of 75µM polymyxin B, G2 - cells exposed to concentration of 375µM polymyxin B. Each group was assessed at 24, 48 and 72 hours for cell viability and apoptosis.

Proximal tubular cells were grown in culture bottles 25cm², 75cm² or 12 multiwell plates according to the experimental protocol, in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with fetal bovine serum (FBS 5% v/v), NaHCO₃ 2.0 g/L, HEPES 2.6g/L, penicillin 10,000 IU/L, streptomycin 50mg/l neomycin and 100mg/L and kept in incubator at 37° C with 95% air and 5% CO₂. Once they had reached 80% of confluence, cells were detached from the flask by trypsinization and neutralized trypsin activity with DMEM containing FBS. Cells were used for the maintenance of the cultures or for conducting experimental protocols, each protocol was repeated at least eight times. Cells stocks were frozen at passage 40 for maintaining a constant uniformity in the experiments.

Cell viability was determined by exclusion of fluorescent dyes acridine orange and ethidium bromide (100 µg/ml), in a ratio v: v (1:1). The method of viable cell count is based on the selective uptake of the fluorescent dyes mentioned, which is dependent on membrane integrity. Ethidium bromide passes through the intact membrane, by binding to cellular DNA, and emitting green fluorescence (excitation 460nm and emission 650nm) indicating cells are viable. The acridine orange stains RNA but it does not fully pass through the membrane and is picked up only by cells whose membrane have been damaged in color and fluoresces red-orange (excitation 510 nm and emission 595 nm) showing which cells are not viable (necrosis). At the end, at least 200 cells were counted for each representative group through a fluorescence microscope (magnification 40x and 200). Results were expressed as percentage of viable cells.

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Cell viability was determined by exclusion of fluorescent dyes acridine orange and ethidium bromide (Sigma). In order to read viability only 10µL trypsinized cell suspension were mixed with 0.3µL of the solution were used of acridine orange dyes and ethidium bromide (100 µg/ml), in a ratio v: v (1:1). The method of viable cell count is based on the selective uptake of the fluorescent dyes mentioned, which is dependent on membrane integrity. Ethidium bromide passes through the intact membrane, by binding to cellular DNA, and emitting green fluorescence (excitation 460nm and emission 650nm) indicating cells are viable. The acridine orange stains RNA but it does not fully pass through the membrane and is picked up only by cells whose membrane have been damaged in color and fluoresces red-orange (excitation 510 nm and emission 595 nm) showing which cells are not viable (necrosis). At the end, at least 200 cells were counted for each representative group through a fluorescence microscope (magnification 40x and 200). Results were expressed as percentage of viable cells.

This assessment of apoptosis is the morphological method, quantitative Hoechst 33342 [Bisbenzimide HOE 33342 (2’-[4-ethoxyphenyl] -5 - [4-methyl-1-piperazinyl] -2,5 ’-bi-1H-benzimidazole trihydrochloride)], which is a dye specific for adenine - thymine. This dye is easily absorbed by the cell, staining the DNA, chromosomes and nucleus, enabling visualization of apoptotic bodies for fluorescence microscopy in intense blue coloration. For the evaluation of apoptosis solution a 100 µg/mL Hoescht dye 33342 in PBS was prepared. The cells were subjected to the same preparation described for the viability assessment, to obtain the cell pellet. On the blade 5µL of Hoescht 33342 added to 5 µL of cell suspension were pipetted. The blade was stored for 10 minutes at environment temperature and in the absence of light, to chromatin staining. The procedure was then the count of 100 to 200 cells for each group. Results were expressed as percentage of apoptotic cells compared to total cells.

The statistical methodology applied consisted of analysis of variance-ANOVA. In this model, all interactions were tested. When detected differences, there were multiple comparisons through the Bonferroni test and Tukey test. The descriptive statistics with p ≤ 0.05 were considered significant. Data were defined as mean ± standard error and they will be presented in the tables below.
Results

The data in table 1 demonstrate cell viability of exposure to two doses of PmxB in three time intervals, with a significant increase in toxicity with increasing levels within 24 hours, in 48 hours and in 72 hours.

**Table 1.** Viability of LLC-PK₁ cells in response to treatment with Polymyxin B

<table>
<thead>
<tr>
<th>Groups (µM)</th>
<th>n</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>8</td>
<td>80.7±3.4</td>
<td>80.9±1.9</td>
<td>81.4±5.8</td>
</tr>
<tr>
<td>G1</td>
<td>8</td>
<td>57.6±7.7</td>
<td>56.9±8.1</td>
<td>56.9±2.9</td>
</tr>
<tr>
<td>G2</td>
<td>8</td>
<td>41.0±6.7</td>
<td>41.3±9.7</td>
<td>40.9±2.0</td>
</tr>
</tbody>
</table>

Legend: CTL = 0µM; G1 = 75µM; G2 = 375µM; *p < 0.05 vs CTL - 24h/48h/72h; **p < 0.05 vs G1 - 24h/48h/72h.

Table 2 presents the data of cell apoptosis LLC-PK₁ exposed to the groups and times previously mentioned. Raising the dose (group) increased the population of cells undergoing apoptosis within 24 hours, 48 hours and 72 hours. This demonstrates that the cellular response was the same with increasing dose PmxB: more cells died by apoptosis. In general, it was observed significant difference at all times which demonstrates greater toxicity because as the exposure time and the dose of PmxB are increased, larger number of cells undergo apoptosis.

**Table 2.** Apoptosis of LLC-PK₁ cells in response to treatment with Polymyxin B

<table>
<thead>
<tr>
<th>Groups (µM)</th>
<th>n</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>8</td>
<td>4.5±1.2</td>
<td>8.6±5.3</td>
<td>8.9±2.1</td>
</tr>
<tr>
<td>G1</td>
<td>8</td>
<td>20.7±2.4</td>
<td>24.9±2.6</td>
<td>28.0±2.7</td>
</tr>
<tr>
<td>G2</td>
<td>8</td>
<td>29.9±2.6</td>
<td>32.7±3.1</td>
<td>36.1±3.8</td>
</tr>
</tbody>
</table>

Legend: CTL = 0µM; G1 = 75µM; G2 = 375µM; *p < 0.05 vs CTL - 24h/48h/72h; **p < 0.05 vs G1 - 24h/48h/72h; ***p < 0.05 vs G1 - 24h; ****p < 0.05 vs G2 - 24h.

Discussion

The results show that the cytotoxic effect of PmxB was intensified with increasing dose of this drug. This finding was confirmed since the LLC-PK₁ cells showed reduced viability when subjected to an increasingly high dose of PmxB. Conversely, with increasing dose of antibiotic the population of apoptotic cells increased, demonstrating how a cell reacts to the toxic effect of the injury, ie the presence of a biological phenomenon increasing the injury is also greater. Vitro models that mimic the PmxB toxicity in kidney cells are not common. Its mechanism of action in acute kidney injury is unclear. Due to the inconclusive clarifications regarding cellular mechanisms involved in the genesis of this side effect, this research, through techniques of immortalized kidney cell culture, evaluated the effects of PmxB.

These findings confirmed the toxic dose dependent effect of this antibiotic, which in practice may be assumed that a high inhibitory concentration as a therapeutic measure may be a risk factor for renal cell death with eventual functional outcome.

Besides the dose, the time of drug permanence in cells influenced cell death mechanism. The longer time the cells were exposed to toxicity of polymyxin, the bigger number of apoptosis was observed.

It has been suggested that the mechanism of the nephrotoxicity PmxB is a consequence of increased cell membrane permeability, resulting in increased influx of cations, anions and water, leading to edema and cell lysis. PmxB acts on the plasma membrane of the bacteria, impairing transport mechanisms. Its actions are not selective, it may also act at the cell membrane of other organisms. Its therapeutic effect depends on the minimal inhibitory concentration for the infecting bacteria.

Nephrotoxicity of PmxB is an infrequent clinical problem in patients with adequate renal function and is related to the dose of this drug. Studies on topical or parenteral use of PmxB in daily doses of 2.5mg.kg⁻¹ caused proteinuria, hematuria and formation of cylinders in some cases.
Evidences show that the bactericidal action of PmxB seems to be more effective in vitro than in vivo, so it is advised that during parenteral therapy serum levels are assessed and maintained below 10 mg/l.\(^1\)\(^{10}\)

A recent study performed in an intra-hospital environment with 60 patients investigated with more emphasizes its nephrotoxic effect. The following criteria were considered: demographic characteristics, underlying diseases, infection environments, microorganisms, length of stay, PmxB dosage, frequency and duration of dose, other administered medications, serum creatinine, development of neurological and skin alterations, and microbiological and clinical results. The overall mortality rate was 20%. Among patients who developed renal injury, the mortality rate was 57%, and the mortality rate of patients who did not develop renal injury was 15%. From the group who developed kidney injury, three survived; from these two restored renal function and one required renal replacement therapy. Contradicting earlier studies where ratios nephrotoxicity ranged from 17-100%, the PmxB was well tolerated by most patients, of which 14% developed nephrotoxicity. Its application has provided clinical and microbiological efficacy, with less impairment of renal function.\(^{12}\)

Another study on PmxB reemphasizes its nephrotoxicity, but provides a new approach to this antimicrobial drug. Endotoxin is a lipopolysaccharides (LPS) found in the outer membrane of gram-negative bacteria. The PmxB binds to endotoxin neutralizing its activity, which includes cell death. The PmxB is a nonapeptide derived from PmxB which controls anti-lipopolysaccharides activity, but with less toxic characteristics. In this experimental study, dogs which were given high doses of PmxB showed hyperthermia, abdominal and facial flushing, and increased urea nitrogen and serum creatinine. Such changes were not observed in control animals or those receiving equal dose of PmxB nonapeptide. Sprague-Dawley rats which received doses of PmxB developed dyspnea, cyanosis, decreased physical activity and ataxia. In the control group or the group that was administered to PmxB nonapeptide these changes were not recorded. Importantly, the PmxB nonapeptide has no antibiotic activity, but is able to disrupt the membrane of gram-negative bacteria, showing that the chemical structure of the antimicrobial effect of dissociated PmxB is showing to be related to its toxicity.\(^{13}\)

It is important to emphasize that the detection of apoptosis in the model developed here standardizes toxicity induced PmxB. Apoptosis is a coordinated event that acts in physiological processes such as tissue homeostasis, embryonic development and immune response. The mitotic cycle and apoptosis have similar characteristics. Therefore, the study and understanding of this phenomenon in ischemic and nephrotoxic AKI can provide directions for efficient prevention or recovery.

It should be noted that success in the prevention of AKI requires knowledge of the mechanisms pathogens involved, the identification of individual risk factors and measures of pre-emptive associated with surveillance and early intervention. In this context models to encourage research on the cellular changes involved in functional disorder precipitated by nephrotoxins such as PmxB.

This research will enable the Nursing to correlate basic research to clinical research allowing this professional a more enhanced biological, physiological and pathological understanding of mechanisms that affect the nephrotoxic AKI by PmxB, besides the establishment of nephroprotection preventive measures.

The present study will enable the establishment of protocols for in vitro models; recognition model of nephroprotection from techniques of molecular and cell biology; interpretation of molecular, cellular and pathophysiological events of nephrotoxic AKI from studies with cell cultures; early identification of clinical situations that influence the occurrence of renal dysfunction; understanding of used therapies in the treatment of patients with renal dysfunction, identification of risk factors related to the occurrence of AKI by nephrotoxic PmxB; elaboration of preventive measures for AKI by PmxB and establishing Clinical strategies to minimize the occurrence of AKI Nephrotoxic by PmxB.
Conclusion

The PmxB confirmed its cytotoxic effect in LLC-PK₁ cells, triggering a reduction in viability and increase in apoptosis as it increased the time and dose of the antibiotic. Cytotoxicity by PmxB in cell culture model, showed time and dose dependency, increasing time exposure and higher dose of antibiotic.

Contribution

Neiva LB; Fonseca CD; Watanabe M and Vattimo MFF participated in the conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and final approval of the version to be published.

References