POLYMYXIN B TOXICITY IN LLC-PK$_1$ CELLS IS MEDIATED BY THE HEME OXYGENASE-1 ENZYME

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Introduction and Objectives

Polymyxin B (PMB) is one of the few remaining therapeutic options avaliable to treat infections cause by multidrug-resistent gram-negative bacteria. The mechanism of antibacterial activity of PMB is crucial for the development of its toxicity, especially nephrotoxicity. Oxidative injury has been played a key role in the PMB nephrotoxicity. Reactive oxidative species (ROS) generated via mitochondria activity initiates renal cell injury by apoptosis and necrosis, ultimately leading to acute kidney injury (AKI).

Injury induces enhancement of the mechanisms of defense act as "protector genes" and heat shock protein (HSP 32), also known as heme oxygenase-1 (HO-1).

The aim of this study is to distinguish the role of HO-1 enzyme in PMB toxicity in LLC-PK$_1$ cells.

Methods

- Cells culture: Immortalized LLC-PK$_1$ cells, pig proximal tubular epithelial cell line, obtained from the American Type Culture Collection was maintained in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) and 5% fetal bovine serum (FBS). LLC-PK$_1$ cells cultivated on multiwell plates (12 wells) were divided into the following groups:
  - **Control** - (n=8);
  - **PMB** - cells exposed to 375 µM PMB (n = 8);
  - **PMB+Hemin** – cells exposed to 25 µM of Hemin (HO-1 inducer), one hour before 375 µM PMB (n = 8);
  - **PMB+ZnPP** - cells exposed to 10 µM of zinc protoporphyrin - ZnPP (HO-1 inhibitor), one hour before 375 µM PMB (n = 8).
- Cell viability was determined by acridine orange and ethidium bromide method.
- Apoptotic cells were determined using HOE 33342 staining method.
- Membrane damage mediators of LLC-PK1 cells in presence of PMB evaluated were:
  - Intracellular enzyme lactate dehydrogenase (LDH);
  - Lipid peroxidation was determined by the malondialdehyde (MDA) quantification;
  - Nitric oxide (NO) in the cell culture media was determined by Griess.
- Quantitative RT-PCR and imunofluorescence protein synthesis of HO-1.
- Statistical Analysis: differences between groups were analyzed by one way analyses of variance ANOVA and post hoc Bonferroni test. Results are presented as mean±SEM and $p<0.05$ was considered statistically significant.

Results

PMB exposed cells demonstrated decrease in the cellular viability and increase in the population of apoptotic cells. HO-1 inducer treatment improved these parameters (**Table 1**).

Table1: Physiological Parameters

Groups	Viability (%)	Apoptosis (%)
Control	81±5	8±2
PMB	40±2^{A}	36±3^{A}
PMB+Hemin	55±1^{AB}	22±1^{AB}
PMB+ZnPP	53±3^{AB}	24±2^{AB}

Data reported mean±SEM. $^{A}p<0.05$ vs Control, $^{B}p<0.05$ vs PMB.

Cell membrane damage can be evaluated by the release of the intracellular enzyme LDH. LLC-PK1 cells exposed to PMB increased LDH (**Figure 1**). PMB exposed cells exhibited no significant effect on MDA levels (**Figure 2**), NO generation increased in LLC-PK1 cells by HO-1 inducer and inhibitor treatment (**Figure 3**).

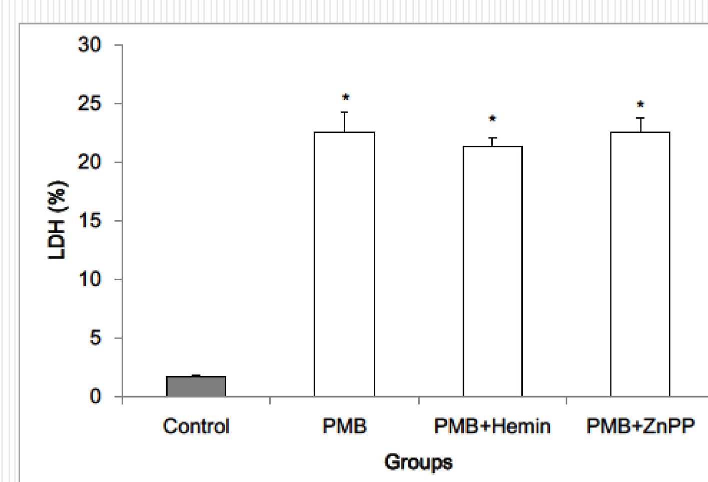

Figure 1: Intracellular enzyme LDH.

Data reported mean±SEM. *p<0.05 vs Control, •p<0.05 vs PMB, •p<0.05 vs PMB+Hemin.

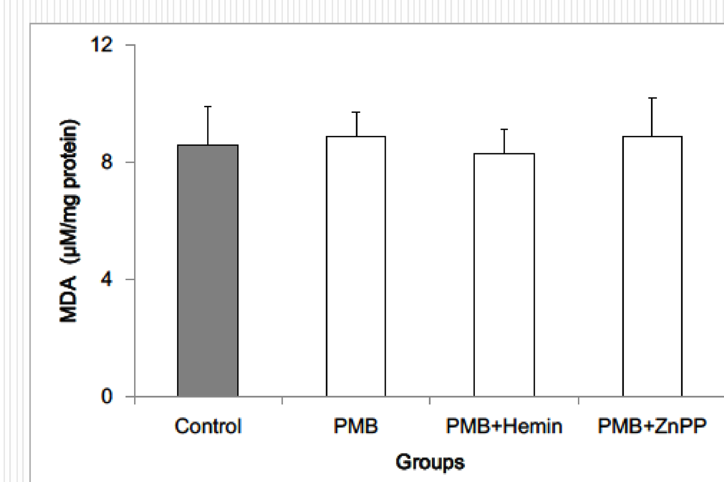

Figure 2: Malondialdehyde (MDA) quantification.

Data reported mean±SEM.

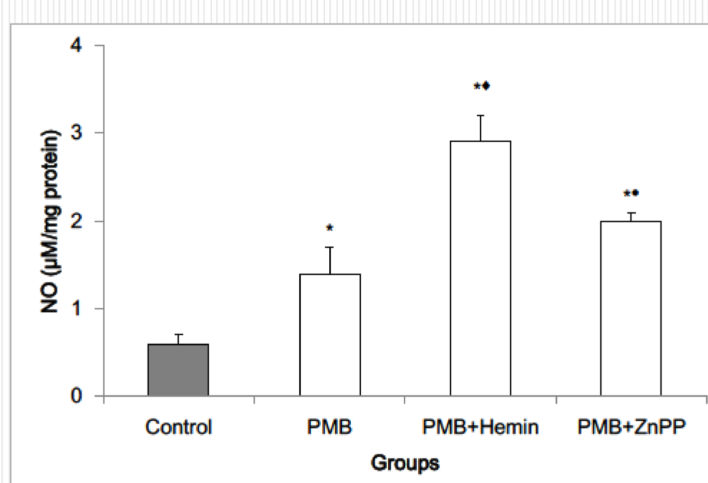

Figure 3: Nitric oxide (NO) in the cell culture media.

Data reported mean±SEM. *p<0.05 vs Control, •p<0.05 vs PMB, •p<0.05 vs PMB+Hemin.

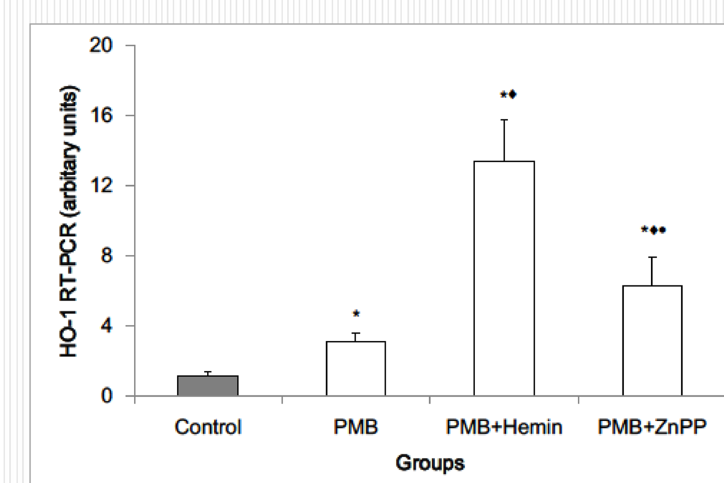

Figure 4: Heme oxygenase-1 (HO-1) quantitative RT-PCR.

Data reported mean±SEM. *p<0.05 vs Control, •p<0.05 vs PMB, •p<0.05 vs PMB+Hemin.

Hemin treatment demonstrated gene expression and protein synthesis of HO-1 in PMB exposed LLC-PK$_1$ (**Figure 4** and **5**).

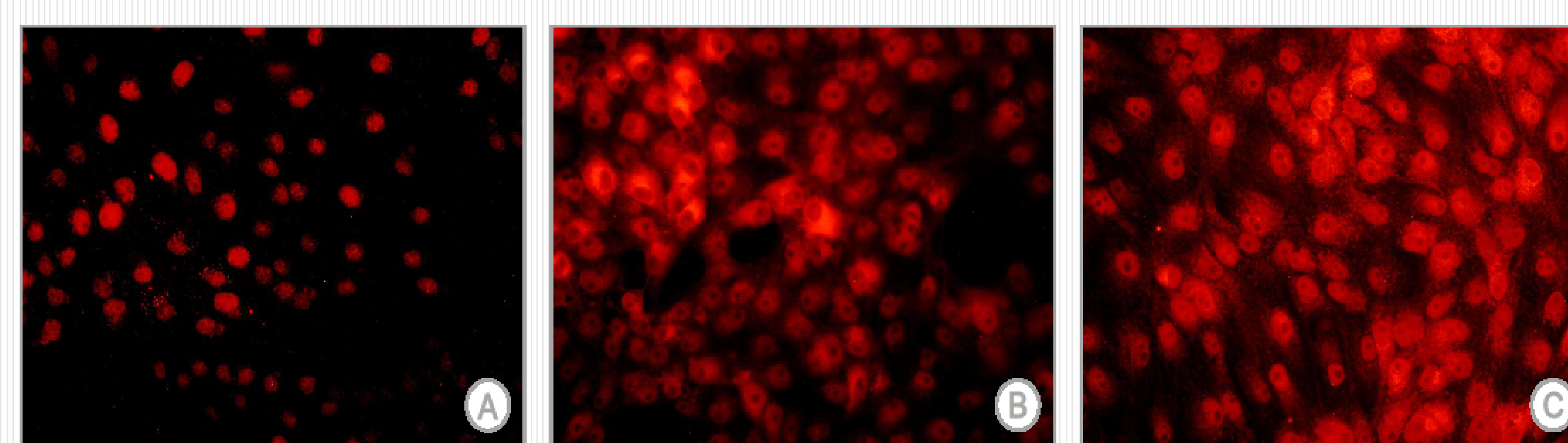

Figure 5: Imunofluorescence protein synthesis of HO-1. (**A**) Control, (**B**) PMB, (**C**) PMB+Hemin.

Conclusions

PMB was confirmed as a cytotoxic drug by increasing apoptosis, membrane cell damage and reducing viability of LLC-PK$_1$ cells. Hemin or ZnPP preconditioning improved cellular viability and reduced apoptosis, confirming HO-1 role in this model.

References

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