ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 10, Issue, 11, pp.74946-74954, November, 2018

DOI: https://doi.org/10.24941/ijcr.32824.11.2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

DIFFERENTIAL PROFILE OF P38 MAPK PATHWAY IN CHANGEOXIDATIVE STRESS, NITRIC OXIDE PRODUCTION AND CELLDEATH IN LLC-PK1 AND MDCK CELLS: CASE AMPHOTERICIN B AND CYCLOSPORINE

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ARTICLE INFO

Article History:
Received 04th August, 2018
Received in revised form
26th September, 2018
Accepted 06th October, 2018
Published online 29th November, 2018

Key words:
Amphotericin B,
Cyclosporine, Nephrotoxicity,
Nitric Oxide, Oxidative stress,
p38 MAPK.

ABSTRACT

Amphotericin A and cyclosporine are an important antibiotic and immunosuppressive agents, respectively; however, nephrotoxicity is one the main adverse effects. The purpose of this study was to evaluate the effect of inhibiting the p38 MAPK (p38) signaling pathway in nephrotoxicity caused by amphotericin A and cyclosporine from the assessment of stress oxidative, nitric oxide production and cell death in LLC-PK1 and MDCK cell lines. Amphotericin and cyclosporine proved to be oxidative stress (pro-oxidant status), reduction of NO production and DNA fragmentation, determined by flow cytometry using the Propidium iodide dye. In LLC-PK1 cells, the inhibition of the p38 signaling pathway (PD169316 inhibitor) caused a significant reversion status (pro-oxidant to antioxidant), increase of nitric oxide production and reduction in DNA fragmentation when these cells were stimulated with cyclosporine. In contrast, in MDCK cells the same profile was found when these cells were incubated with amphotericin B. Thus, a significant finding of our study is that pharmacological inhibition of p38 MAPK resulted in a substantial reduction in amphotericin B and cyclosporine nephrotoxicity *in vitro* evaluated by oxidative stress, NO production and cell death parameters. These results suggest that p38 MAPK signaling can contributes to amphotericin B and cyclosporine-induced renal injury *in vivo*. We also demonstrate that cells from different regions of the nephron (proximal or distal tubules) present varying sensitivities to the toxic effects of amphotericin B and cyclosporine and showed a differentiated participation of p38 MAPK pathway signaling in this process.

Citation: Flávia Dayrell França, Cleiber Lucan Alves Araújo, Sandra de Sousa Araújo and Míriam Martins Chaves. 2018. "Differential profile of p38 MAPK pathway in changeoxidative stress, nitric oxide production and celldeath in LLC-PK1 and MDCK cells: case amphotericin B and cyclosporine", *International Journal of Current Research*, 10, (11), 74946-74954.

INTRODUCTION

Kidneys represent the most common target for drugs because of their role in controlling body fluids and electrolyte homeostasis (Wilmes *et al*., 2011). Renal toxicity caused by drugs is a common adverse reaction which brings about severe consequences on a patient's health. In 100 drugs used in intensive care units, 25% present a potential for nephrotoxicity (Peyrou and Cribb, 2007), amphotericin B (antibiotic) being the most common (Wilmes *et al*., 2011). Cyclosporine A is widely used for organ transplantation and autoimmune disorders, but it was proven that long-term use of Cyclosporine could lead to chronic cyclosporine nephrotoxicity (Xiao *et al.*, 2011). The etiology of the nephrotoxicity of amphotericin B and cyclosporine remains to be clarified; however, vasoconstriction and the direct toxic action of amphotericin B on renal tubular epithelial cells have been postulated to be the major causes of amphotericin B-induced nephrotoxicity (Yano *et al.*, 2009).

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Cyclosporine can induce both reversible and irreversible damage to all kidney compartments, including the glomeruli, arterioles, and tubulointerstitial (Naesens *et al.*, 2009). As the toxicity is generally directly related to biological disturbances at the molecular cellular level, the knowledge of these and their impacts on molecular cellular functions are essential (van de Water *et al*., 2006). In recent years, oxidative stress (OS) has been converted as one of the most popular subjects in research of molecular mechanism of renal disease. Factors that induce OS in kidney include systemic diseases such as hypertension, diabetes mellitus, hypercholesterolemia, infection, chemotherapeutics, radiocontrast agents' environmental toxins, radiation, antibiotics, smoking, occupational chemicals, as well as alcohol consumption. In continuing, we will discuss the relationship between these factors and OS in kidney (Tsarouhas *et al.*, 2018). OS is defined as unbalance between the production of free radicals and the ability of the body to counteract their dangerous effects through neutralization by antioxidants (Nasri *et al.*, 2014). In certain pathological conditions, increased production of reactive oxygen species (ROS) and depletion of antioxidants in defense system leads to enhanced ROS activity and OS, resulting tissue damage. OS causes tissue damage by different mechanisms including production of lipid peroxidation, DNA damage, and protein modification. These processes have been related with the pathogenesis of several systemic diseases such as hypertension, diabetes mellitus, and hypercholesterolemia and also kidney disease. In recent years, OS has become one of the most beloved topics in research of molecular mechanism of renal diseases (Hosseini *et al.*, 2018).

In contrast, antioxidants are free radical scavengers that react with the free radicals and delay the cellular damage. The lack of balance in production of free radicals and the ability of the body to negate their dangerous effects through neutralization by antioxidants produce oxidative stress (OS). There are many experimental evidences suggesting a key role for OS and inflammation on renal failure (Himmelfarb and Hakim, 2003; Galle,2001). There are many reports which suggest that the use of antioxidants help in the disease prevention (Halliwell, 1995; Willcox *et al.*, 2004; Hajhashemi *et al.*, 2010). One of the major scientific advances in the past decade in understanding of the renal function and disease is the prolific growth of literature incriminating nitric oxide (NO) in renal physiology and pathophysiology. Although most actions of NO are mediated by cyclic guanosine monophosphate (cGMP) signaling, S-nitrosylation of cysteine residues in target proteins constitutes another well-defined non-cGMP dependent mechanism of NO effects. While NO is considered beneficial in general in regulation of vasomotor tone, immune defense modulation and neurotransmission, excessive NO generation is cytotoxic due to the effects on generation of reactive oxygen and nitrogen species and nitrosylation of proteins. While the vast amount of NO literature has enhanced our understanding of its relevance in kidney disease and health, it has also contributed to significant confusion in view of the conflicting data role of NO in nephrology (Oshiro *et al.*, 2018;Sumayao*et al.*, 2018). The p38 MAPK plays an important role in the regulation of cell survival in a wide range of cell types, including renal tubular cells (Yano *et al*., 2009). Mitogen-activated protein kinase (MAPK) activities (p38) is markedly enhanced after ischemia *in vivo* and chemical anoxia *in vitro* (Park *et al.*, 2001). The relative extent of p38 activation has been proposed to determine cell fate after injury. p38 MAPK which lies in a pathway that is activated strongly by cellular stress, pro-inflammatory cytokinesand bacterial lipopolysaccharide (Davies *et al.*, 2000). Next context, renal cell lines have been employed as alternative methods for the study of therapeutic products that cause nephrotoxicity (Jung *et al.*, 2009; Lincopan *et al.*, 2005; Pfaller and Gstraunthaler, 1998; Price *et al.*, 2004) and the use of *in vitro* techniques has enhanced the comprehension of molecular mechanisms of nephrotoxicity (Wilmes *et al.*, 2011). The LLC-PK1 (porcine proximal tubular cells) and MDCK cells (canine distal cells) are considered acceptable models to study drug nephrotoxicity (El Mouedden *et al.*, 2000; Ramseyer and Garvin, 2013; Servais *et al.*, 2006; Shin *et al.*, 2010; Yano *et al.*, 2009; Yuan *et al.*, 2011). Thus, in the present work, the question has been raised as to whether or not nephrotoxicity generated by amphotericin B and cyclosporine depends on p38 MAPK pathway signaling by evaluating stress metabolism, NO production and cell deathusing the LLC-PK1 and MDCK renal cell lines.

MATERIALS AND METHODS

Drugs

Amphotericin Bwasdonated by Cristália (Produtos Químicos Farmacêuticos Ltda- Itapira, SP, Brazil(purity of 90%). Astock solutionof300µg/mLo famphotericin Binsterile buffer solution (PBS) was prepared and different volumes were added to theRPMI-1640(Sigma St. Louis, MO, USA) to generate eight different concentrations:2, 4, 6, 8, 10, 15, 20and30µg/mL. The choice of concentrations of amphotericin Bwasbased on the work of Wasan *et al.*, 1994. Cyclosporine was kindly donated by Cristália (Produtos Químicos Farmacêuticos Ltda- Itapira, SP, Brazil). A stock solution of 500 µM of cyclosporine was prepared in a phosphate buffer saline (PBS) solution and different volumes were added to the RPMI-1640 medium (Sigma St. Louis, MO, USA) to generate 8 different concentrations: 5, 10, 20, 25, 30, 40, 45, and 50 µM. The choice of cyclosporine concentrations was based on findings from Nascimento *et al.*, 2005. The Inhibitor of p38 MAPK pathway, PD169316 (Calbiochem Merck KGaA, Darmstadt, Germany) was dissolved in anhydrous dimethylsulfoxide (DMSO) to form a concentrated solution that was 1000 times the required final concentration. The inhibitor was aliquoted and stored at -20°C. The concentrated solution was diluted immediately prior to use and the cells were pretreated with 10 µM of PD169316 for 30 minutes (Yano *et al.*, 2009). H_2O_2 10.5% v/v as the stimulus dose chosen (curve concentration response) for LLC-PK1 and MDCK cells since it increases ROS production and the cells remained viable (80.12%).

Cell culture: The LLC-PK1 cell lines (kidney proximal tubular cells from pigs – passages 5 to 15), and MDCK (distal tubular cells from dogs – passages 5 to 15) were obtained from the Cell Bank at Federal University of Rio de Janeiro (UFRJ). These were cultivated in an DMEM culture medium (Sigma St. Louis, MO, USA) and supplemented with 10% (v/v) bovine fetal serum (Invitrogen Co Ltd., Carlsbad, CA, USA), 100 IU penicillin/mL, and 100 µg streptomycin/mL (Sigma St. Louis, MO, USA). Cells were cultivated in 75 cm^2 bottles and incubated at 37 °C in a humidified with 5% CO_2.

Reactive oxygen species (ROS) quantification: The quantitative basal ROS determination was performed in a luminol-dependent chemiluminescence assay. A luminol (Sigma Co.) stock solution was made by dissolving 1.77 mg of

luminol in 1.0 mL DMSO to give a concentration of 10^{-2} M. Before using it, was diluted to 10^{-4} M in PBS (pH 7.3). The tubes were incubated with 1 x 10^6 cells/100µL DMEM medium), the amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment and luminol for 30 minutes. The chemiluminescence measurements were performed in a luminometer 1250-101 (Lumat, LB 9501, EG and G Berthold - Germany). The experiments were performed in duplicate and carried out at 37°C. The results were expressed in Relative Light Units/min (RLU/min). The control experiments were done simultaneously. To study the involvement in the inhibition of p38 MAPK pathway in amphotericin B and cyclosporine induced ROS production, LLC-PK1 and MDCK cells were pretreated for 30 minutes with 10 µM (PD169316 – p38 MAPK inhibitor) followed by amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment. The ROS production was measured after 30 min in the presence of the signaling pathway inhibitor.

MTT Assay: The intracellular antioxidant response was performed by the quantitative MTT [3-4(4-5-dimethythiazol-2yl) 2,5-diphenyltetrazolium bromide], dye reduction was performed as described by Chaves *et al*. (Chaves *et al.*, 1998, 2007). Briefly, LLC-PK1 and MDCK cells were incubated for 24 hours into 24-well plates (5,0 x 10^5cells/well and after this time were treated with amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) and 20 µL of MTT (5.0mg/mL in PBS) during 120 min at 37 °C. The final volume was adjusted to 1000µL with PBS. The reaction was stopped by adding 100µL of DMSO, and the absorbance was read at 570nm in microplate Elisa (Beckman). The experiments were performed in duplicate. The control experiments were done simultaneously. To study the involvement in the inhibition of p38 MAPK pathway in amphotericin B and Cyclosporine induced reduction response, LLC-PK1 and MDCK cells were pretreated for 30 minutes with 10 µM (PD169316 – p38 MAPK inhibitor) followed by amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment. The reduction response was measured after 120 min after incubation of MTT (5.0mg/mL in PBS) in the presence of the signaling pathway inhibitor.

Evaluation of the Oxidizing and Reducing Capacities: Simultaneous evaluation of oxidizing – O (luminol-dependent chemiluminescence) and reducing – R (MTT dye reduction) responses have been used. The cellular metabolic balance involving oxidizing/reducing (O/R) responses provide an evaluation of the oxidant status. An antioxidant status occurs when the O/R of the experiment is lower than the O/R of the control and the pro-oxidant status occurs when the O/R of the experiment is higher than the O/R of the control. The simultaneous evaluation of both cellular oxidizing and reducing responses are important parameters to study the metabolic cellular equilibrium (Oliveira *et al.*, 2012). To study the involvement in the inhibition of p38 MAPK pathway in amphotericin B and Cyclosporine induced O/R balance, LLC-PK1 and MDCK cells were pretreated for 30 minutes with 10 µM (PD169316 – p38 MAPK inhibitor) followed by amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment. The O/R balance was measured through mathematical calculation in the presence of the signaling pathway inhibitor.

Nitric Oxide (NO) quantification: LLC-PK1 and MDCK cells were incubated for 24 hours into 24-well plates (5,0 x 10^5cells/well and after this time were treated with amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM). After 24 h supernatants were obtained by centrifugation 1500 rpm, 10 min. and nitric oxide production was measured by means the Griess reaction. This involved comparing 100µL aliquots of culture supernatant with serial dilutions $NANO_2$ (from 7.81 mM to 1000mM). To this an equal volume of Griess reagent (N-1-naphthylethylenediamine 0.1% in H2O+ sulfanilamide 1% in 2,5 % H3PO4) was added and then incubated at room temperature for 10 minutes and read at 540 nm (Green *et al.*, 1982). To study the involvement in the inhibition of p38 MAPK pathway in amphotericin B and Cyclosporine induced NO production, LLC-PK1 and MDCK cells were pretreated for 30 minutes with 10 µM (PD169316 – p38 MAPK inhibitor) followed by amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment. The NO quantification was measured after 10 min after incubation of Griess reaction in the presence of the signaling pathway inhibitor.

Sub-diploid DNA content determination: A flow cytometric DNA fragmentation assay was employed as a quantitative measure of cell death (Nicoletti *et al.*, 1991). Twenty-four hours after treatment with amphotericin B and cyclosporine, the cells were collected by centrifugation, lysed with 300 µL of a hypotonic solution containing 0.5% Triton X-100 and 50 µg/mL propidium iodide (PI, Invitrogen, USA). Cells were incubated at 4°C for 1h and analyzed in a FACScan flow cytometer (Becton Dickinson, Germany) for shifts in PI fluorescence that were indicative of nuclei with hypodiploid DNA content. To study the involvement in the inhibition of p38 MAPK pathway in AmphotericinB and Cyclosporine induced cell death, LLC-PK1 and MDCK cells were pretreated for 30 minutes with 10 µM (PD169316 – p38 MAPK inhibitor) followed by amphotericin B (4.0 µg/mL) and cyclosporine (5.0 µM) treatment. Subdiploid DNA content and cell viability were measured after 24 h to assess the cellular responses in the presence of the signaling pathway inhibitor.

Statistical analysis: All resultswere analyzed by One-Way ANOVAandTukeypost-test using GraphPad Prism version 5.00 for windows (San Diego, CA). $p<0.01$ and $p<0.05$ was considered to indicate statistical significance.

RESULTS

Pro-oxidant effect of the amphotericin B and cyclosporine in LLC-PK1 and MDCK cells: The ROS production expressed as RLU/min in LLC-PK1 and MDCK cells in the experiment with amphotericin B and cyclosporine (E) and control (C) are show in Tables 1 and 2 respectively. Both amphotericin B and cyclosporine generated a significant activation of ROS production in relation of control. The similar profile was observed when the cells were incubated with hydrogen peroxide (H_2O_2), powerful activator of ROS production showing a profile pro-oxidant (Tables 1 and 2). The pre-incubation with PD169316 showed a reversion of the metabolic status. The status pro-oxidant changed to status antioxidant. LLPC-K1 cells incubated with cyclosporine (72%) showed best reversion of the status pro/antioxidant than presented with incubation with amphotericin B (30%) (Table 1). MDCK cells showed the inverse profile of the status pro/antioxidant, cyclosporine (7%) and amphotericin B (65%) (Table 2).

Oxidative Stress production by the amphotericin B and cyclosporine in LLC-PK1 and MDCK cells: The balance (O/R) of oxidizing (O) and reducing responses (R) was used to

identify oxidative stress caused by the amphotericin B and cyclosporine (Tables 1 and 2). Both amphotericin B and cyclosporine were able of altering the balance (experiment's O/R is different to control's (O/R) when compared to control with H_2O_2. Amphotericin B and cyclosporine increased ROS production (O) and lowered the reducing response (R), generating a pro-oxidant status (experiment's O/R is higher than control's O/R) when compared to control (Tables 1 and 2). The pre-incubation with PD169316 showed a reversion of the O/R balance. The O/R balance pro-oxidant changed to O/R balance antioxidant. LLPC-K1 cells incubated with cyclosporine (83%) showed best reversion of the O/R balance pro/antioxidant than presented with incubation with amphotericin B (47%) (Table 1). MDCK cells showed the inverse profile of the O/R balance pro/antioxidant, cyclosporine (46%) and amphotericin B (88%) (Table 2).

Effects of amphotericin B and cyclosporine on the production of nitric oxide (NO) in LLC-PK1 and MDCK cells: In both cells lines, amphotericin B and cyclosporine provoked a significant decrease in nitric oxide (NO) production: in LLC-PK1 cells, the nitric oxide decreased by 23% (cyclosporine) and 56% (amphotericin), whereas in MDCK cells, it fell by 80% (cyclosporine) and 48% (amphotericin) (Figure 1). The pre-incubation with PD169316 showed a reversion of the NO production. Only LLPC-K1 cells incubated with cyclosporine showed significant reversion of NO production (Figure 2.A). On the other hand, MDCK cells showed significant reversion of NO production only with amphotericin B incubation (Figure2.B).

DNA Fragmentation Induced by Amphotericin B and Cyclosporine: An increase in the percentage of dead cells occurred 24 h after treatment with amphotericin B and cyclosporine and the quantitative analysis of DNA fragmentation in two lineages is demonstrated in Figure 3. This cell population consists of a sub-diploid DNA content that is indicative of DNA fragmentation and cell death. This alteration could be observed in LLC-PK1 and MDCK cell lines. LLC-PK1 cells presenteda smaller percentage of cell death (14%), whereas the MDCK cells presented 25%, after treatment with Amphotericin B. With Cyclosporine, LLC-PK1 cells presented 32% of cell death, while the MDCK cells presented 12%.

Effects of p38 MAPK Inhibitor on DNA fragmentation in Renal Cell Lines: Pretreatment with PD169316 failed to reduce the fragmentation of DNA caused by amphotericin B in LLC-PK1 cells (Figure4.A). In MDCK cells, the inhibition of the p38 MAPK pathway caused a significant reduction in DNA fragmentation (Figure4.B). However, the inhibition of the p38 MAPK pathway caused a significant reduction in DNA fragmentation caused by Cyclosporine in LLC-PK1 cells (Figure 4.A). In MDCK cells, pretreatment with PD169316 failed to reduce the fragmentation of DNA caused by cyclosporine (Figure 4.B), which were assayed by determining sub-diploid DNA content after 24 hours of treatment with amphotericin B and/or cyclosporine.

DISCUSSION

Nephrotoxicity is an important side effect of amphotericin B and cyclosporine. The combined administration of these drugs is frequent in patients with hematological diseases undergoing allogeneic stem cell transplantation. Recent studies have shown that one of the antimicrobial and immunological modes of action of certain drugs involves cellular oxidative stress response. Thus, these types of drugs could be defined as oxidative stress drugs (Kim *et al.*, 2012). Examples include amphotericin B (Sokol-Anderson *et al.*, 1986; Jukic*et al.*, 2017).

Table 1. Oxidative stress generation by amphotericin B and cyclosporine in LLC-PK1

	O (RLU/min) Mean ± SD	R (O.D. 570 nm) Mean ± SD	O/R balance	Status
Controls	128 ± 4.3	0.392 ± 0.02	326.5	–
LLC-PK1 + DMEM				
LLC-PK1 + DMEM + PD169316	132 ± 8.9	0.401 ± 0.04	329.1	–
Experiments				
LLC-PK1 + H_2O_2	705 ± 11.4	0.173 ± 0.01	4,075 ↑	pro-oxidant
LLC-PK1 + H_2O_2 + PD169316	205 ± 13.6*	0. 385 ± 0.02*	532.4 ↓ (87%)	antioxidant
LLC-PK1 + Amphotericin B	531 ± 4.4	0.201 ± 0.04	2,641 ↑	pro-oxidant
LLC-PK1 + Amphotericin B + PD169316	369 ± 2.5*	0.265 ± 0.06*	1,392 ↓ (47%)	antioxidant
LLC-PK1 + Cyclosporine	386 ± 2.9	0.242 ± 0.03	1,595 ↑	pro-oxidant
LLC-PK1 + Cyclosporine + PD169316	105± 1.8*	0.405 ± 0.08*	259.2 ↓ (83%)	antioxidant

The values represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. *p<0,05 when compared with the respective control (absence of PD169316). Antioxidant status: when O/R of the experiment is lower than the O/R of the control; Pro-oxidant status: when O/R of the experiment is higher than the O/R of the control.

Table 2. Oxidative stress generation by amphotericin B and cyclosporine in MDCK

	O (RLU/min) Mean ± SD	R (O.D. 570 nm) Mean ± SD	O/R balance	Status
Controls MDCK + DMEM	124 ± 11.4	0.356 ± 0.04	348.3	–
MDCK+ DMEM + PD169316	128 ± 9.6	0. 394 ± 0.08	324.8	–
Experiments				
MDCK+ H_2O_2	641 ± 3.2	0.184 ± 0.05	3,483 ↑	pro-oxidant
MDCK+ H_2O_2 + PD169316	168 ± 1.8*	0.327 ± 0.08*	513,76 ↓ (85%)	antioxidant
MDCK+ Amphotericin B	349 ± 2.5	0.169 ± 0.02	2,065 ↑	pro-oxidant
MDCK+ Amphotericin B + PD169316	122 ± 1,9*	0.501 ± 0,04*	243.5 ↓ (88%)	antioxidant
MDCK+ Cyclosporine	492 ± 3.8	0.172 ± 0.03	2,860 ↑	pro-oxidant
MDCK+ Cyclosporine + PD169316	458 ± 3.4*	0.301 ± 0.02*	1,521 ↓ (46%)	antioxidant

The values represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. *p<0,05 when compared with the respective control (absence of PD169316). **Antioxidant status**: when O/R of the experiment is lower than the O/R of the control; **Pro-oxidant status**: when O/R of the experiment is higher than the O/R of the control.

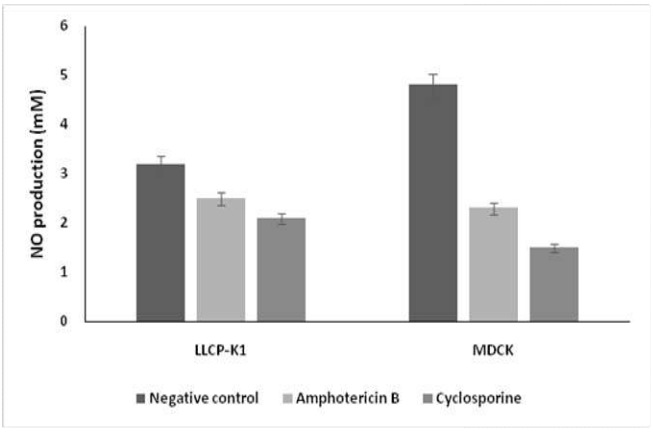

Figure 1. Effects os amphotericin B and cyclosporine on nitric oxide (NO) production in renal cells lines. The production of NO in the LLC-PK1 and MDCK supernatants cultures was determined by Griess reaction after 24-h incubation with amphotericin B (4.0 µg ml $^{-1}$)and cyclosporine (5.0 µM). The results represent the mean ± standard deviation (SD) of the results of six independent experimentsperformed in sextuplicate. *p <0,05 when compared with the negative controlgroup (untreated cells).#p < 0,01 when compared with the negative control group (untreated cells).

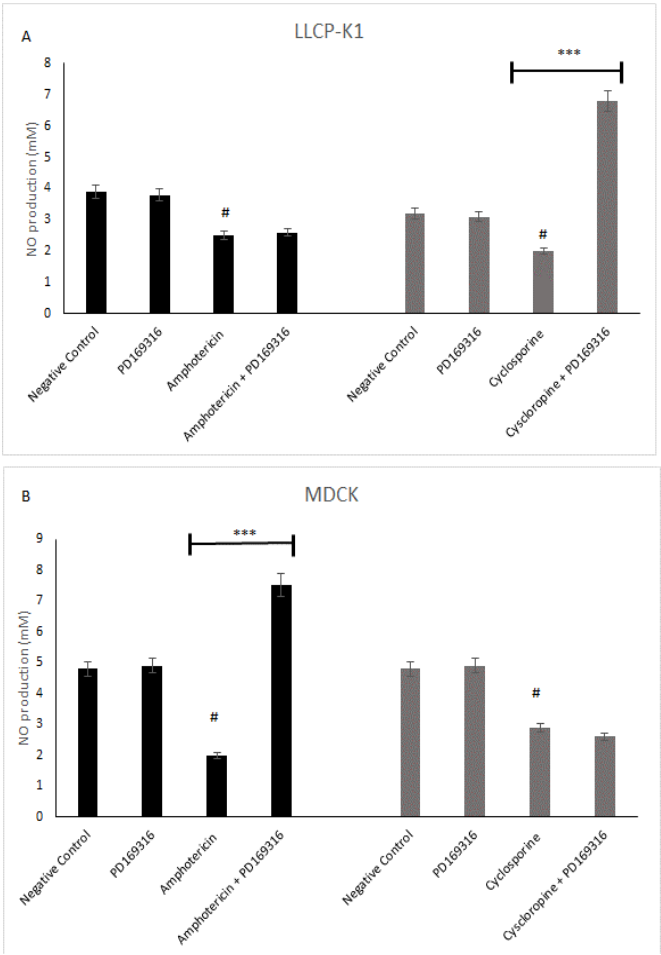

Figure 2. Effects of PD169316 on nitric oxide (NO) production in renal cells lines. The production of NO in the LLC-PK1 and MDCK supernatants cultures was determined by Griess reaction after 24-h incubation with amphotericin B (4.0 µg ml $^{-1}$) and cyclosporine (5.0 µM), pre-treatment for 30 minutes com PD169316 (10 µM) in triplicate. The results represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate.*p <0,001 when compared with cyclosporine or amphotericin.#p < 0,05 when compared withnegative control group (untreated cells).

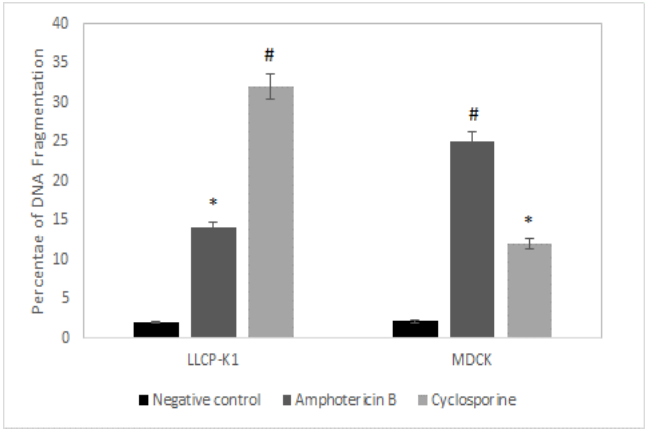

Figure 3. Effect of amphotericin and cyclosporine on DNA fragmentation in renal cells. The cells were placed at the density of 1.0 x 10^4 cells/well in a 24-well plate and were treated with Amphotericin B and Cycloporine in triplicate. DNA fragmentation was analyzed after staining with propidium iodide (PI). A flow cytometric assay was employed as a quantitative measure of cell death. Results are expressed as percentage of events from a total of 5,000 events. Results represent mean ± SD of triplicates from three independent experiments. * p <0,05 and #p < 0,01 significantly diferente from group treated with cyclosporine in relation negative control group respectively.

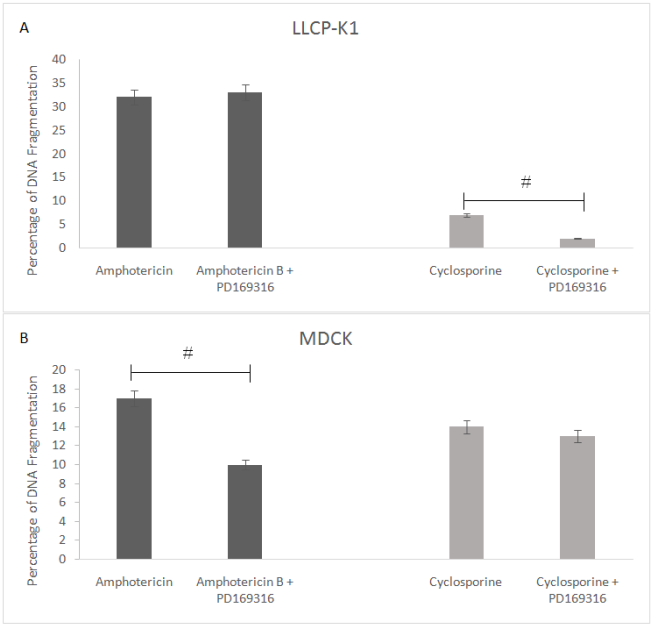

Figure 4. Effect of pre-treatment with PD169316 on DNA Fragmentation induced by Amphotericin B and Cyclosporine. The cells were placed at the density of 1.0 x 10^4 cells/well in a 24-well plate and were treated with amphotericin B (4.0 µg ml $^{-1}$) and cyclosporine (5.0 µM) with or without PD 169316 pre-treatment (10 µM, 30 min) in triplicate. DNA fragmentation was analyzed after staining with propidium iodide (PI). A flow cytometric assay was employed as a quantitative measure of cell death. Results are expressed as percentage of events from a total of 5,000 events. Results represent mean ± SD of triplicates from three independent experiments. #p < 0,05 significantly different from group treated with cyclosporine in relation group treated with PD169316.

Although amphotericin B is known as a fungicidal drug by causing ion leakage, studies have shown that forming channels in the cellular membrane was not the sole mechanism of amphotericin B activity (Palacios *et al.*, 2007). Instead, oxidative stress triggered by AMB could be one of the contributing mechanisms for amphotericin Bfungicidally.

Already the proposed mechanism for cyclosporine A is to induce endoplasmic reticulum stress and increases mitochondrial reactive oxygen species production: this modifies the redox balance, which causes lipid peroxidation and thereby induces nephrotoxicity (Wu *et al.*, 2018). Our results showed that both amphotericin B and cyclosporine were able to induced ROS generation. However, this production it turned out different in both lineages. LLC-PK1 was sensitive to cyclosporine A (72% of ROS production) and amphotericin B(30% of ROS production) (Table 1).The principle renal transport systems, which contribute to drug nephrotoxicity, reside in the proximal tubule (Andankar *et al.*, 2018; Fedecostante *et al.*, 2018). The role of the proximal tubule in concentrating and reabsorbing the glomerular filtrate renders it vulnerable to toxic injury (inflammation). This involves the cellular transport systems mentioned previously and is thus dose dependent to a degree. The S3-segment of the proximal tubule has the highest rate of oxygen delivery/ oxygen consumption of all functional entities in the body and hence most susceptible to ischemia (Brezis*et al.*, 1984). MDCK, in contrast, is more sensitive to amphotericin B (65% of ROS production) than thecyclosporine A (7% of ROS production) (Table 2). Amphotericin B is a highly effective antifungal agent that binds with ergosterol, a key component of fungal cell membranes, and forms pores that result in leakage of monovalent ions and cell death (Nett and Andes, 2016). Because human and fungal cell membranes share common structures, amphotericin B can also lead to pore formation in human cell membranes with resulting toxicity (Bolard, 1986). The consequences of the distal tubular membrane injury and changes in cellular permeability include prominent electrolyte disorders, such as hypokalemia, hypomagnesemia, and renal tubular acidosis (Rosner, 2017).

Chronic kidney disease (CKD) is associated with enhanced oxidative stress that is triggered when the antioxidants are unable to counteract the harmful oxidative insults caused by excessive production of reactive oxygen species (Fukai and Ushio-Fukai, 2011). In both cell lines amphotericin B and cyclosporine, were capable of decreasing the cellular antioxidant capacity (Tables 1 and 2). We have reported a strong parallelism between oxidizing species generation and cellular reducing power response. Simultaneous evaluation of the oxidizing (luminol-dependent chemiluminescence) and reducing (MTT dye reduction) responses have been demonstrate. Thus, the presence of a cellular metabolic disequilibrium involved oxidizing /reducing (O/R) cellular responses might be involved in a significant altered inflammatory or immunologic response in an age population (Chaves *et al.*, 1998, 2007; Oliveira *et al.*, 2012). O/R balance in LLC-PK1 and MDCK cells stimulated with amphotericin B and cyclosporine showed a profile pro-oxidant (Tables 1 and 2). LLC-PK1 was more sensitive to cyclosporine (O/R) balance – 83%) than amphotericin B (O/R balance – 47%). In contrast, MDCK was more sensitive to amphotericin B (O/R balance – 88%) than cyclosporine (O/R balance – 46%) (Tables 1 and 2). Nitric oxide (NO) is formed from the amino acid L-arginine by means of nitric oxide synthases (NOSs). Efforts to supplement NO levels with L-arginine have been shown to afford protection to the obstructed kidney, in which vasodilatory actions of nitric oxide are likely to be involved (Hegarty *et al.*, 2002). Previous studies have proposed renal tubular injury as possible consequence of renal vasoconstriction and endothelial injury leading to ischemia (Zhu *et al.*, 2012).

Amphotericin B and cyclosporine decreases significantly nitric oxide (NO) production in both cell lines (Figure 1). Reactive oxygen or nitrogen species (ROS/RNS) generated endogenously or in response to environmental stress have long been implicated in tissue injury in the context of a variety of disease states. ROS/RNS can cause cell death by nonphysiologically (necrotic) or regulated pathways (apoptotic). Cell death mechanisms have been studied across a broad spectrum of models of oxidative stress, including H_2O_2, nitric oxide and derivatives, endotoxin-induced inflammation, photodynamic therapy, ultraviolet-A and ionizing radiations, and cigarette smoke (Ryter *et al.*, 2007).

The increased fragmentation of DNA observed in flow cytometry (Figure 3) can be interpreted as cell death (Nicoletti *et al.*,1991). Therefore, it can be concluded the nephrotoxic drugs caused cell death in the two studied cell lines, and these can be found in the late stages of apoptosis/necrosis. Propidium iodide (PI) is widely used in the study of cell death, as it does not penetrate through the cell membrane, thus differentiating among normal cells of apoptotic and necrotic cells. A characteristic of the cells in the early stages of apoptosis is the maintenance of the integrity of the membrane and the ability to exclude dyes, such as PI (Aubry *et al.*, 1999). Late phases of apoptosis are commonly accompanied by an increased permeability of the cell membrane, which allows for an intake of PI within the cells (Hashimoto *et al.*, 2003). LLC-PK1 cells presented a higher percentage of cell death (DNA fragmentation), showing themselves to be more sensitive to the toxic effects of cyclosporine and MDCK cells showing themselves to be more sensitive to the toxic effects of amphotericin B.

The p38 MAPK pathway is activated by a variety of stresses, e.g., oxidants, UV irradiation, hyperosmolality, and inflammatory cytokines, and have been linked to cell death (Ramesh and Reeves, 2005). Thus, we decided to evaluate whether the inhibition of this signaling pathway alters cellular death due to alteration of oxidative stress, antioxidant capacity increased and nitric oxide production caused by the nephrotoxic drugs amphotericin B and cyclosporine. Both cell lines were sensitive to PD169316 (inhibitor of p38 MAPK pathway) in relation of ROS production. The ROS production by amphotericin B and cyclosporin is decrease when LLC-PK1 and MDCK were preincubated which PD169316 (Tables 1 and 2). The inhibition of the metabolic pathway caused a change in metabolic status from pro-oxidant to anti-oxidant (Tables 1 and 2). However, the reversal of metabolic status was more effective in LLC-PK1 cells incubated with cyclosporine and MDCK cellswere more sensitive to amphotericin (tables 1 and 2). The cellular reduction profile and O/R balance presented the same profile found the ROS production (change the pro-oxidant for antioxidant status). Once more though, the metabolic sensitivity was different (LLC-PK1 is more sensitive to cyclosporine and MDCK is more sensitive to amphotericin B) (Tables 1 and 2). Redox changes initiate various cellular signals in the cells, and the redox environment can determine if a cell will proliferate, differentiate, or die. Imbalance of the redox status such as during oxidative stress can trigger a series of events, leading to cellular dysfunction. Reactive oxygen species (ROS) are increasingly considered as being involved in the initiation and progression of chronic renal disease. The proximal tubule is a major site of ROS production, due to its high transport activity supported by an oxygen consuming metabolism (Terryn and Devuyst, 2010). The NO production

was also altered when both cell lines were preincubated with PD169316. However, again we observed a different profile (Figure 2). PD169316 was only able to increase the production of nitric oxide in LLC-PK1 cells line stimulated with cyclosporine (Figure 2). Unlike, MDCK cells only able to increase the production of nitric oxide (NO) when these were incubated with amphotericin B (Figure 2). A strong link has been established between the p38 pathway and inflammation. There are several pre-clinical studies on the involvement of NO in inflammation.

From this large amount of information, it is clear that virtually every cell and many immunological parameters are modulated by NO. Thus, the final outcome is that NO cannot be rigidly classified as an anti-inflammatory or pro-inflammatory molecule (Cirino *et al.*, 2006). The activation of the p38 pathway plays essential roles in the production of proinflammatory cytokines (IL-1β, TNF-α and IL-6) (Xia *et al.*, 1995) and a regulator of oxidation (Da Silva *et al.*, 1997; Craxton *et al.*, 1998). After having evaluated the fragmentation of DNA caused by amphotericin B and cyclosporine, the present study aimed to assess whether or not the inhibition of the p38 MAPK pathway could influence cell death detected in this study, given that this pathway is directly linked to cell cycles and survival (Deacon *et al.*, 2003). This study's results showed that the LLC-PK1 inhibition of the p38 MAPK pathway did not alter the DNA fragmentation caused by Amphotericin B. In the MDCK cell, the inhibition of the p38 MAPK pathway reduced in cell death caused by amphotericin B (Fig. 4). However, the LLC-PK1 inhibition of the p38 MAPK pathway reduced in cell death caused by cyclosporine and in MDCK cell, the inhibition of the p38 MAPK pathway did not alter the DNA fragmentation caused by cyclosporine (Fig.4).

As the p38 MAPK signaling pathway is involved in a variety of cellular responses, including inflammation, cell cycle, cell death and cell differentiation, emphasis on p38 MAPK functions should be given in each cell type (Ono and Han, 2000). In addition, the role of p38 MAPK is dependent on stimulus and cell type (De Borst *et al.*, 2006). Therefore, our results suggest that inhibition of the p38 MAPK pathway may decrease cell death caused by amphotericin B and cyclosporin. According to Mansouri *et al.* (2003), the inhibition of p38 MAPK in ovarian carcinoma cells increases their resistance to cisplatin-induced apoptosis, suggesting that activation of p38 MAPK contributes to cell death in response to cisplatin (another nephrotoxic drug). Furuichi *et al.* (2002) reported that inhibition of p38 MAPK reduced renal ischemia-reperfusion injury. Thus, p38 MAPK activation may be a common element in the mechanism of acute renal injury. Thus, a significant finding of our study is that pharmacological inhibition of p38 MAPK resulted in a substantial reduction in amphotericin B and cyclosporine nephrotoxicity *in vitro* evaluated by oxidative stress, NO production and cell death parameters. These results suggest that p38 MAPK signaling can contributes to amphotericin B and cyclosporine-induced renal injury *in vivo*. We also demonstrate that cells from different regions of the nephron (proximal or distal tubules) present varying sensitivities to the toxic effects of amphotericin B and cyclosporine and showed a differentiated participation of p38 MAPK pathway signaling in this process. These results are important for evaluating future therapeutic interventions in the nephrotoxicity process.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements: FAPEMIG (Fundação de Amparo e Pesquisa de Minas Gerais log number: APQ-02574-14), CAPES, CNPQ, UFMG- PRPqand IVC Health – Testes em Saúde Humana LTDA supportedthispaper.

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