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RESEARCH ARTICLE

DIFFERENT MECHANISMS OF NEPHROTOXICITY CAUSED BY AMPHOTERICIN B AND CYCLOSPORIN AN INVOLVING PKA AND P38 MAPK

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ABSTRACT

Amphotericin B (AmB - antifungal) and Cyclosporine A (CsA - immunosuppressant) are commonly used drugs in medical clinics; however, these often present Nephrotoxicity. Thus, the aim of this study was to evaluate if AmB and CsA nephrotoxicity could be associated to oxidative stress and if the PKA and p38 MAPK are involved in cell death, caused by these drugs, in two different segments of the nephron. For this, we used two different cell lines (LLC-PK1 and MDCK) from different segments of the nephron and treated these cells with AmB and CsA. We evaluated the effect of these treatments on cell death on both cells and the involvement of PKA and p38 MAPK pathways in Amphotericin B and Cyclosporin-induced cell death. Further, we investigated the influence of the treatment with the two drugs in oxidative balance, evaluating ROS production, antioxidant enzymes activity (superoxide dismutase and catalase), as well as gene expression of these enzymes. We also analysed the gene expression of $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$. Data were analyzed by one-way analysis of variance (ANOVA), Bonferroni's Multiple Comparison post-Test or Dunn's Multiple Comparison Test to determine the differences among the groups. AmB and CsA caused cell death in both cells lines, however LLC-PK1 presented a higher percentage of cell death caused by AmB and MDCK presented a higher percentage of cell death caused by CsA. ROS production in both cells treated with AmB or CsA have the same profile observed in DNA fragmentation (cell death). The results showed that the treatment with AmB was able to increase the expression of $p47^{phox}$ and $gp91^{phox}$ in both cell lines. The treatment with AmB was able to reduce SOD activity in both cells lines and the treatment with CsA demonstrated that this drug could reduce the activity of SOD and Catalase in LLC-PK1 and MDCK cells. When p38 MAPK and PKA were inhibited in MDCK cells, the percentage of DNA fragmentation decreased, showing that when these cells were treated with AmB or CsA, probably p38 MAPK and PKA were involved in cell death caused by these compounds. This study demonstrated that cells from different regions of the nephron (proximal –LLC-PK1 or distal tubules - MDCK) present different responses to the oxidative stress caused by Amphotericin B (AmB) or Cyclosporine A (CsA), and also showed a differentiated participation of signalling in this process, involving PKA and p38 MAPK pathways.

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INTRODUCTION

The high blood perfusion rate (20% of cardiac output) and the capability to metabolise, concentrate, extract and secrete toxic compounds, make the kidneys extremely vulnerable to a wide variety of toxins. Xenobiotic induced nephrotoxicity is a major cause of both acute and chronic renal failure and thus represents a significant challenge to the health care and pharmaceutical industries (Fanos and Cataldi, 2001). This phrase was missing: Nephrotoxicity due to drug exposure contributes up to 25% of all acute kidney injuries in critically ill patients. (Alvarez-Lerma *et al*., 2012).ROS production in mitochondria and oxidative stress have been implicated in the pathogenesis of a variety of renal diseases (Damiano *et al*., 2015). Due to the functional and biochemical heterogeneity of the nephron, the susceptibility to a particular nephrotoxic insult will vary among nephron segments (Fanos and Cataldi, 2001). Renal cell lines have been described as important tools for the study of therapeutic products that induce nephrotoxicity (Jung

et al., 2009) and cell cultures allows the study of specific pathways at the cellular level without the influence of other systems (Wilmes *et al*., 2011).That´s why the importance to evaluate drug´s nephrotoxicity in different segments. The identification and dissection of cellular stress mechanisms is fundamental to understanding the susceptibility of the kidney to chemicals and pharmaceuticals and for the development of renal biomarkers indicative of sub lethal injury.

Cyclosporine A (CsA) is a very important immunosuppressive drug and greatly improves the survival rates of patients and grafts after solid-organ transplantation (Zimmermann *et al*., 2017). It is also being used to treat autoimmune diseases such as psoriasis and rheumatoid arthritis (Di Lernia *et al*., 2016). The Cs A treatment shows several limitations related to its nephrotoxic effects, like the decrease of glomerular filtration rate (GFR) and hypertension demonstrated in rat models (Damiano *et al*., 2015) and in clinical practice (Lee *et al*., 2011). It´s well demonstrated that the chronic use of CsA associates with high incidences of nephrotoxicity and the eventual development of chronic renal failure (Caires *et al*., 2018). Some recent studies suggest that oxidative stress is an important mechanism implicated in nephotoxicity by CsA. (El-Bassossy and Eid, 2018). As well as increasing oxidative stress, CsA treatment also greatly decreases the antioxidant capacity of the kidneys. This could lead the kidneys to different stresses, making them more susceptible to nephrotoxic agents (Ghaznavi *et al*., 2007). At present, the cellular and molecular biological mechanisms underlying CsA nephropathy are not completely understood.

Amphotericin B (AmB) is the antifungal agent that has been most widely used in clinical practice since its discovery in 1955 (Oura and Sternberg, 1955). It exhibits a broad-spectrum antifungal activity and is a crucial therapeutic option for the treatment of severe systemic mycosis (Le and Wolbers, 2017). However, the application of AmB is limited due to its severe toxicities to normal tissues, which induces various side effects, including nephrotoxicity (Chen *et al*, 2018). While there is substantial literature on systemic toxicity, blood toxicity, and adverse reactions to amphotericin B (Leon *et al*., 2007), there is relatively little literature concerned with its effects on local cells involved in the oxidative damage. Tissues adjacent to locally delivered amphotericin B may also be vulnerable to AmB toxicity. Genotoxic-risks have been identified during the treatment of systemic fungal infections with amphotericin B that reflects affinity to damage DNA (Mandal *et al*., 2015). The ROS production by AmB is a universal and important action mechanism that is correlated with the fungicidal effect and might explain the low rate of resistance to the molecule (Mesa-Arango *et al*., 2014).

Indeed, ROS generation by AmB has been described as a consequence of AmB's spontaneous insertion into ergosterol-containing membranes (Cohen, 2016). Elevation of lipid peroxidation product MDA, NO, and SOD activity with reduction of antioxidant enzyme catalase in renal tissue following AmB administration shows that oxidative stress with respect to free-radical damage is one of the possible mechanisms in the pathophysiology of AmB nephrotoxicity (Altuntas *et al*., 2014). It is well known that AmB and CsA are nephrotoxic and that this toxicity could be caused by oxidative stress increase, however the mechanisms involved in this toxicity are still unclear. Some studies demonstrated different possibilities that lead the nephron to death, such as signalling pathways involved in oxidative stress, inflammation and apoptosis. PKA is a ubiquitous protein kinase expressed throughout the renal tissue, which has a wide range of functions, including cell protection, regulation of cell cycles, apoptosis, proliferation, and differentiation (Bichet, 2006). França *et al*., 2014 (1) showed that PKA signa ling pathway can aid in reducing the degree of nephrotoxicity caused by Amphotericin B and by Cyclosporine. MAP kinases are members of signa ling cascades and serve as focal points in response to a variety of extracellular stimuli. One subgroup of this family is the p38 MAPK and this kinase is involved in several process, the activation of the p38 MAPK pathway plays essential roles in the production of proinflammatory cytokines, induction of enzymes such as COX-2 which controls connective tissue remode ling in pathological conditions expression of intracellular enzymes such asiNOS, a regulator of oxidation (Badger *et al*., 1998). So, the involvement of these pathways in nephrotoxicity caused by AmB and CsA should be investigated. The purpose of this study was to evaluate if AmB and CsA nephrotoxicity could be associated to oxidative stress and if the PKA and p38 MAPK are involved in cell death, caused by these drugs, in two different segments of the nephron.

MATERIALS AND METHODS

Reagents: RPMI-culture medium (#R8758), penicillin and streptomycin (#P4333), H89 (PKA inhibitor - #B1427) and PD169316 (p38 inhibitor - #P9248) and Luminol (#A8511) are from Sigma – Aldrich (St. Louis, MO, USA). Trizol reagent (#15596018), propidium iodide (#P1304MP) and bovine fetal serum (#26140079) are from Invitrogen Life Technologies Co Ltd, Carlsbad, CA, USA, Amphotericin B, at a purity of 90%, and Cyclosporin A were donated by Cristália (Produtos Químicos Farmacêuticos Ltd, Itapira, Sau Paulo, Brazil).

Cell culture: The LLC-PK1 (porcine kidney proximal tubular cells) and MDCK cells (canine distal tubular cells) were obtained from the Cell Bank at Universidade Federal do Rio de Janeiro (UFRJ). Cells were cultivated in RPMI-culture medium (Sigma) and supplemented with 10% (v/v) bovine fetal serum (Invitrogen Co Ltd, Carlsbad, CA, USA), 100 IU penicillin ml^{-1} and 100 μg ml^{-1}streptomycin (Sigma). Cells were cultivated in 75-cm^2 bottles and incubated at 37 °C in a humidifier with 5% CO_2.

DNA Content Determination: A flow cytometric DNA fragmentation assay based on Nicoletti *et al*., 1991 work was used as a quantitative measure of cell death. Cells (LLC-PK1 and MDCK) were treated with Amphotericin B (4.0 μg ml^{-1}) and Cyclosporin A (250μM) for twenty-four hours and then collected by centrifugation, lysed with 300 μl of a hypotonic solution containing 0.5% Triton X-100 and 50 μg ml^{-1} propidium iodide (PI; Invitrogen). Cells were incubated at 4 °C for 1 h and analyzed in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) for shifts in PI fluorescence. The membrane-impermeable dye PI binds directly to the DNA, which is only possible upon membrane damage, occurring at late apoptotic or early necroptotic events. To study the involvement in the inhibition of the PKA and p38 MAPK pathways in Amphotericin B and Cyclosporin A-induced cell death, LLC-PK1 and MDCK cells were pre-treated for 30 min with 1.0 μM H89 (PKA inhibitor) and PD169316 (p38 inhibitor) followed by Amphotericin B (4.0 μg ml^{-1}) and Cyclosporin A (250μM) treatment. Subdiploid DNA content,

indicated by PI fluorescence, and cell viability were measured after 24 h to assess the cellular responses in the presence of the signaling pathway inhibitor.

ROS production: To evaluate ROS generation, we performed a chemiluminescence assay based on the luminol amplification, as described by Chaves *et al*., 2000. Luminol reacts with ROS generated by cells to produce an excited amino phthalate anion that emits light when returning to the ground state (Fä *et al*., 1999). Briefly, to analyze the effect of AmB and CsA in ROS production, cells ($1x10^6mL^{-1}$) were incubated with AmB (4.0 μg ml^{-1}) or CsA (250μM) for 24 hours, then cells were washed with PBS for 2 times and ressuspended in PBS and with 500 μL of 10^{-4} M luminol for 30 min. The photons emitted during this period were recorded every minute using an internal luminometer printer (Lumat LB9501, Berthold, Germany).Values were expressed as relative light units/30 min (RLU/30 min).

Superoxide dismutase activity: To measure the Total SOD activity it was used the kit from Bio Assay Systems (CA, USA). Briefly, cells LLC-PK1 or MDCK ($1x10^6$ mL^{-1}) were incubated with AmB (4.0 μg ml^{-1}) or CsA(250 μM) for 24 hours. Cells were homogenized in cold lysis buffer. Supernatant was used in the test. In the assay, superoxide (O_2^-) is provided by xanthine oxidase (XO) catalyzed reaction. O_2^- reacts with a WST-1 dye to form a colored product. SOD scavenges the O_2^- thus less O_2^- is available for the chromogenic reaction. The color intensity (OD440nm) is used to determine the SOD activity in a sample. BioAssay Systems SOD assay provides a convenient colorimetric means for the quantitative determination of SOD enzyme activity in biological samples.

Catalase activity: CAT is a ubiquitous antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Bio Assay Systems (CA, USA) improved assay directly measures CAT degradation of H_2O_2 using a redox dye. Briefly, cells LLC-PK1 or MDCK ($1 \times 10^6 mL^{-1}$) were incubated with AmB (4.0 μg ml^{-1}) or CsA(250 μM) for 24 hours. Cells were homogenized in the assay buffer (phosphate buffer) and the reaction started by addition of 50μM $H_2O_2$for 30 min. In the end it was used the detection reagent and the absorbance was measured at 570 nm using a plate reader (Biotek ELx808). One unit is the amount of CAT that decomposes 1μmole of H_2O_2 per min.

Real time quantitative RT- PCR assay: After the treatment with AmB or CsA for 24 hours, total RNA was extracted from using Trizol reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's protocol, and resuspended in 30 μL RNase-free water. The concentration and purity of RNA was estimated by the A260/A280 ratio spectrophotometrically (NanoVue, GE Healthcare, UK). A total of 1 μg RNA was converted to cDNA using an oligo (dT) and a HighCapacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer ' s recommendations. Quantitative realtime polymerase chain reaction (qPCR) was performed using Power SYBR ® Green PCR Master Mix reagent (Applied Bio systems, Foster City, CA, USA) in a final reaction volume of 12 μL. The reaction included 0.1 μg of cDNA and 0.5 μL of each primer (forward and reverse, 10 μM). The forward and reverse primer sequences for β2 microglobulin and for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$were obtained from published nucleotide sequences (Vinolo *et al*., 2009).

The primer sequences for NADPH oxidase subunit $gp91^{phox}$ (forward primer 5 ` -tgcccagtaccaaagtttgccg-3 ` and reverse primer 5 ` - aacgccaaaaccggaccaacc-3`) were derived from the National Center for Biotechnology Information Gen Bank (NM 00397 and were constructed using the Primer-BLAST Program (http://www.ncbi.nlm.nih. gov/tools/primer-blast/). The forward and reverse primer sequences for Zn-SOD, Mn-SOD and CAT were obtained from published nucleotide sequences (Xiong *et al*., 2010). The reactions were carried out using the ABI Prism 7300 Sequence Detector (Applied Biosystems) under the following conditions: 50 ° C for 2 min, 95 ° C for 10 min and then 40 cycles of 95 ° C for 15s and 60 ° C for 1 min. The specificity of the products obtained was confirmed by analysis of dissociation curves of the amplified product. As an internal control, expression of the housekeeping gene β2 microglobulin was used. The data obtained were analyzed using the comparative CT method. All analyses were performed in triplicate.

Statistical analysis: The data were expressed as the means and standard error medium (SEM). All data were analyzed by the Kolmogorov – Smirnov normality test. Data were analyzed by one-way analysis of variance (ANOVA), and differences were considered statistically significant when $p \leq 0.05$. Bonferroni's Multiple Comparison post-Test was used for the data that with a normal distribution and for the nonparametric data was used Dunn's Multiple Comparison Test to determine the differences among the groups. Tests were performed with GraphPad Prism version 6.00 for Windows (San Diego, CA, USA).

RESULTS

DNA Fragmentation induced by Amphotericin B and Cyclosporine A: After treatments with amphotericin B (AmB - 4.0 μg ml^{-1}) and cyclosporine A (CsA - 250 μM) for 24h, an increase in the percentage of dead cells can be observed in Figure 1. 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. In Panel A the quantitative analysis of DNA fragmentation in two lineages after treatment with AmB shows that LLC-PK1 cells presented a greater percentage of cell death (31.9%), whereas the MDCK cells presented 17.9%. In Panel B the DNA Fragmentation assay showed that in both cell lines (LLC-PK1 and MDCK) the treatment with CsA lead to DNA fragmentation and cell death. It can be observed that in MDCK cells there is a greater percentage of cells death (8.6%) comparing to LLC-PK1 (14.7%).

ROS production in cells treated with Amphotericin B and Cyclosporin A: To evaluate the ROS production in cells after treatment with AmB and CsA, a chemiluminescence assay based on the luminol amplification was done. In figure 2, in Panel A it can be observed that there is an increase in ROS production in cells treated with AmB (AmB - 4.0 μg ml^{-1}). Both cells were incubated with only with H_2O_2 for 30 minutes, for mimetize an oxidative environment, and we can observe that both cells produced a large amount of ROS. AmB induced ROS production in both cells, LLC-PK1 cells presented a greater percentage of ROS production (413%), and MDCK cells presented 280%, compared to control group (only cells). In Panel B, cells were treated with CsA (250 μM), and we can observe that CsA induced ROS production in both cell lines. However, the profile is different from the AmB treated cells. MDCK cells had a higher(395%) ROS production compared with LLC-PK1 cells(300%).

Gene expression of NADPH oxidase subunits: To investigate the molecular mechanism of the AmB and CsA treatment in LLC-PK1 and MDCK cells, expression of the genes for NADPH oxidase ($gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$) was evaluated by qRT-PCR and the results are presented in Figure 3. In Panel A to C is demonstrated the treatment with AmB(4.0 μg ml^{-1}) in both cell lines (LLC-PK1 and MDCK) and the expression of $p47^{phox}$, $gp91^{phox}$ *and* $p22^{phox}$genes respectively. We can observe in Panel A to C that the treatment with AmB increased the expression of all the genes analyzed in LLC-PK1 cells, whereas in MDCK cells there was an increase in expression of $p47^{phox}$ and $gp91^{phox}$ genes but not in $p22^{phox}$. In Panel D to F is demonstrated the treatment with CsA(250 μM), and we can observe that there is an increase in gene expression of $p47^{phox}$ and $gp91^{phox}$in both cell lines (LLC-PK-1 and MDCK) when they were treated with CsA. Inverse that happened with AmB, there was no difference in expression of $p22^{phox}$ in LLC-PK1 cells treated with CsA, while in MDCK there is an increase in this gene expression.

Antioxidant enzyme activities: Total SOD and CAT antioxidant enzyme activities were evaluated in the present study in both cell lines (FIGURE 4). There is no difference in Catalase activity in LLC-PK1cells treated with AmB (4.0 μg ml^{-1}) – Panel A, but we can observe that in MDCK cells, catalase activity decreases significantly when cells were treated with AmB. In Panel B, SOD activity was demonstrated in LLC-PK1 and MDCK cells treated with AmB. We can observe a decrease in SOD activity in both cells after treatment with AmB. The effect of treatment with CsA in LLC-PK1 and MDCK in Catalase and SOD activity can be observed in Panel C and D. In Panel C there is a significantly difference between LLC-PK1 cells without treatment and the cells treated with CsA. However no difference was obtained in MDCK cells treated with CsA compared to control group (cells without treatment). Panel D demonstrated SOD activity in cells (LLC-PK1 and MDCK) treated or not with CsA, and we can observe that in the cells treated with CsA there is a reduction of SOD activity compared to control cells.

Gene expression of antioxidant enzymes: To analyze if the treatment has a relation in gene expression of antioxidants enzymes, real-time reverse-transcriptase-polymerase chain reaction was done (FIGURE 5). There is a decrease in gene expression of Catalase, Zn-SOD and Mn-SOD in LLC-PK1 cells treated with AmB(4.00 μg ml^{-1}) – Panel A, B and C respectively. However, no difference in these genes expression was observed in MDCK cells treated with AmB. Another profile was observed in cells treated with CsA(250 μM) – Panel D, E and F. MDCK cells treated with CsA presented a decreased in expression of Catalase, Zn-SOD and Mn-SOD compared to control cells (without treatment). Nevertheless, LLC-PK1 cells treated with CsA didn´t presented significantly reduction in gene expression of Catalase, Zn-SOD and Mn-SOD.

Effects of Protein Kinase A Inhibitor on DNA Fragmentation: To evaluate if PKA pathway has participation on cell death in cells treated with AmB or CsA, LLC-PK1 and MDCK cells were pre-treated for 30 minutes with H89 – PKA inhibitor (1.0 μM) followed by AmB (4.00 μg ml^{-1}) or CsA(250 μM).The results showed that the treatment with AmB increased significantly sub-diploid DNA content compared to control cells in both lineages (LLC-PK1 and MDCK cells) and the inhibition of the PKA pathway (H89)decreased DNA fragmentation in MDCK cells compared to cells treated with AmB (FIGURE 6 – Panel A), and this decrease was not observed in LLC-PK1 cells. In Panel B the results demonstrated that there is an increase in cell death when cells were treated with CsA compared to control cells (LLC-PK1 and MDCK) and the inhibition of PKA pathway(H89) decreased significantly cell death in both cell lines compared with cells treated with CsA.

Effects of p38 MAPK Inhibitor on ROS production: To analyze the influence of p38 MAPK pathway in DNA fragmentation in renal cell lines, cells were pre-incubated with the p38 MAPK inhibitor (PD169316) for 30 minutes followed by AmB (4.0 μg ml^{-1}) or CsA(250 μM) treatment. In Figure 7 – Panel A, cells (LLC-PK1 and MDCK) treated with AmB had an increased in sub-diploid DNA content compared to cells without treatment, and when cells were incubated with the p38 MAPK inhibitor (PD169316), they presented different response. In MDCK cells, DNA fragmentation was decreased when they were incubated with AmB and PD169316, whereas in LLC-PK1 no difference was observed. In Panel B the results show that there was an increase in cell death in both cell lines (LLC-PK1 and MDCK) treated with CsA, compared to control cells. However, the inhibition of p38 MAPK pathway (PD169316) decreased significantly the cell death in both cell lines.

DISCUSSION

Some studies had showed that drugs have different actions in different segment of the nephron, due to the functional and biochemical heterogeneity of the nephron (França *et al*., 2014 (1, 2)). In vitro cell culture technology allows the study of specific pathways at a cellular level without the influence of higher order systems. This study demonstrated that cells from different regions of the nephron (proximal –LLC-PK1 or distal tubules - MDCK) present different responses to the oxidative stress caused by Amphotericin B (AmB) or Cyclosporine A (CsA), and also showed a differentiated participation of signaling in this process, involving PKA and p38 MAPK pathways.

Many studies have demonstrated the toxic effect of drugs and that the kidney is specially affected, exactly because it is responsible for the blood filtration, and it is really important to eliminate toxic substances from the body. Accordingly to many studies, drugs can be nephrotoxic and cause cell death, leading people to acute and chronic diseases. The first result that we showed is that the percentage of DNA fragmentation in AmB or CsA treated cells is different in both cells lines. We observed that in LLC-PK1 cells, AmB has a toxic effect more accentuated than in MDCK cells. The opposite effect was observed when cells were treated with CsA, showing that MDCK cells were more susceptible to DNA fragmentation than LLC-PK1. The increased fragmentation of DNA observed in flow cytometry can be interpreted as cell death (Nicoletti *et al*., 1991). Therefore, it can be concluded that AmB and CsA caused cell death in both cells lines, however LLC-PK1 presented a higher percentage of cell death caused by AmB and MDCK presented a higher percentage of cell death caused by CsA. PI is widely used in the study of cell death, 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).

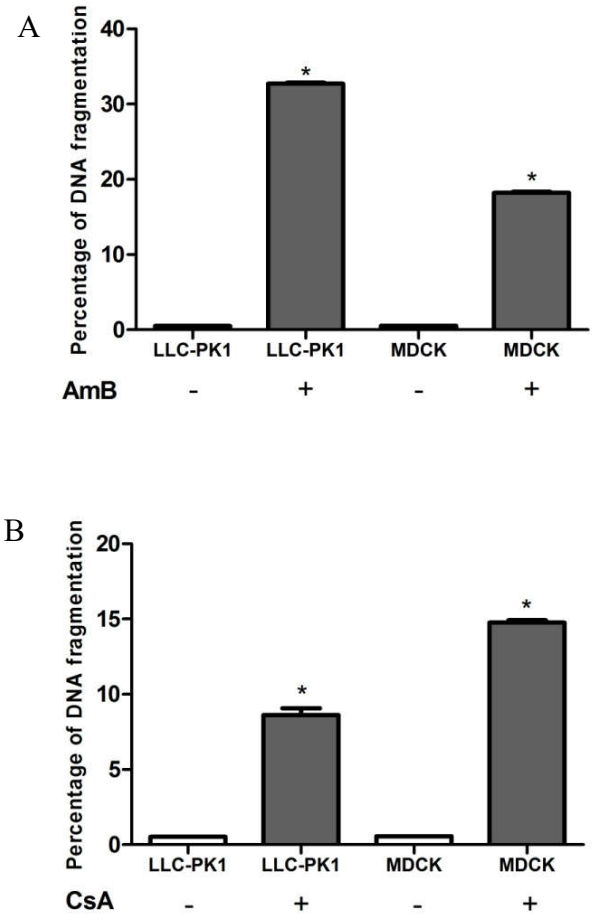

Figure 1: DNA fragmentation induced by Amphotericin B (AmB) and Cyclosporin A (CsA). Cells from both cell lines (LLC-PK1 and MDCK) were seeded in 24-well plates (1.0 x10^4 cells/well) and were treated with AmB (4.0 µg ml^{-1}) and CsA (250µM). DNA fragmentation was analyzed after staining with propidium 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 5000 events. Results represent the mean + standard deviation (SD) of triplicates (n = 3) from three independent experiments. (*) significantly different from control relative to each cell (p<0.05).

Inflammation, apoptosis, and fibrosis associated with oxidative stress play a pivotal role in producing structural and functional kidney impairment in CsA-induced renal injury (Camilleri *et al*., 2016). AmB-associated nephrotoxicity is characterized by acute renal failure due to acute tubular necrosis. Acute renal failure occurs in about a quarter of the patients receiving amphotericin B, and higher dosage and longer duration of therapy are associated with a higher risk of nephrotoxicity (Bates *et al*., 2001). Previous studies from our group concluded that AmB caused cell death in the two studied cell lines (LLC-PK1 and MDCK), and these can be found in the late stages of apoptosis/necrosis (França *et al*., 2014) (1). Apoptosis is involved in the occurrence and development of a variety of renal pathological injuries. As an inducer of apoptosis, oxidative stress can cause kidney damage (Ma *et al*., 2018). Thus, the second objective of this study was evaluated if the cell death observed in the first result could be associated with ROS production. As observed in Figure 2, ROS production in both cells treated with AmB or CsA have the same profile observed in DNA fragmentation. LLC-PK1 treated with AmB presented higher ROS production than MDCK cells. And MDCK cells produced more ROS than LLC-PK1 when treated with CsA. These results corroborate the first result, showing that the percentage of cell death could be explained by ROS production. Among the ROS, superoxide concentration (O_2^-) is the most powerful which is mainly produced in the kidney by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Wilcox, 2005). NADPH-oxidase subunits are located in the kidney in interstitial cells, blood vessels, glomeruli, and tubules (Radeke *et al*., 1991) and play a crucial role in various signaling pathways leading to regulation of gene and protein functions under normal conditions of oxidative balance. The NADPH oxidase consists of six hetero-subunits, which associate in a stimulus-dependent manner to form the active enzyme complex and produce O_2^-. Two NADPH oxidase subunits, $gp91^{phox}$ and $p22^{phox}$, are integral membrane proteins. They form a heterodimeric flavocytochrome b558 ('cyt b558') that constitutes the catalytic core of the enzyme, but exists in a dormant state in the absence of the other subunits. These play mostly regulatory roles, and are located in the cytosol during the resting state.

They include the multidomain proteins $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$, as well as the small GTPase Rac, which is a member of the Rho family of small GTPases. Reversible protein–protein interactions mediated by modular protein interaction domains are key to NADPH oxidase assembly, and much effort has been put into identifying the regions that are responsible for mediating complex formation during the different stages of the activation process (Groemping and Rittinger, 2005). Based on this information and that is so important for ROS production, we analyzed the gene expression of $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$. The results obtained were according to the ROS production, showing that the treatment with AmB was able to increase the expression of $p47^{phox}$ and $gp91^{phox}$ in both cell lines (LLC-PK1 and MDCK). $p47^{phox}$ expression was more expressive in LLC-PK1 cells treated with AmB compared to MDCK cells. $p22^{phox}$ expression was increased in LLC-PK-1 cells treated with AmB, but not in MDCK. The opposite effect was observed in cells treated with CsA, that we observed an increase in expression of subunits in MDCK cells, and it is more expressive in these cells than in LLC-PK1 cells. So, this can explain the results obtained by ROS production, this overproduction can be correlated to the increased expression of $p22^{phox}$ and $p47^{phox}$ subunits of NADPH oxidase. Enhanced superoxide generation in drug toxicity is induced by $p47^{phox}$, a key component of the NADPH oxidase. There are a plenty of antioxidant systems against excessive amounts of ROS produced oxidative stress (Urso and Clarkson, 2003). Initially, the SOD detoxifies the superoxide anion into hydrogen peroxide (Kim *et al*., 2014). Then, the enzymes, CAT and glutathione peroxidase (GSH-Px), try to produce water by converting hydrogen peroxide to prevent ROS injury in the cell. SODs have been classified into two types, Cu/Zn-SOD and MnSOD, which reside in the cytoplasm or mitochondria, respectively (Uchimura *et al*., 1999). Many reports have found that antioxidant enzyme activities can be elevated, decreased or unchanged depending of the tissue or cell type evaluated (Maritim *et al*., 2003). Our results demonstrated that the treatment with AmB reduced SOD activity in both cells lines. Catalase activity was decreased in MDCK cells, however in LLC-PK1 cells the activity is decreased, but is not significantly. The treatment with CsA demonstrated that this drug can reduce the activity of SOD and Catalase in LLC-PK1 and MDCK cells.

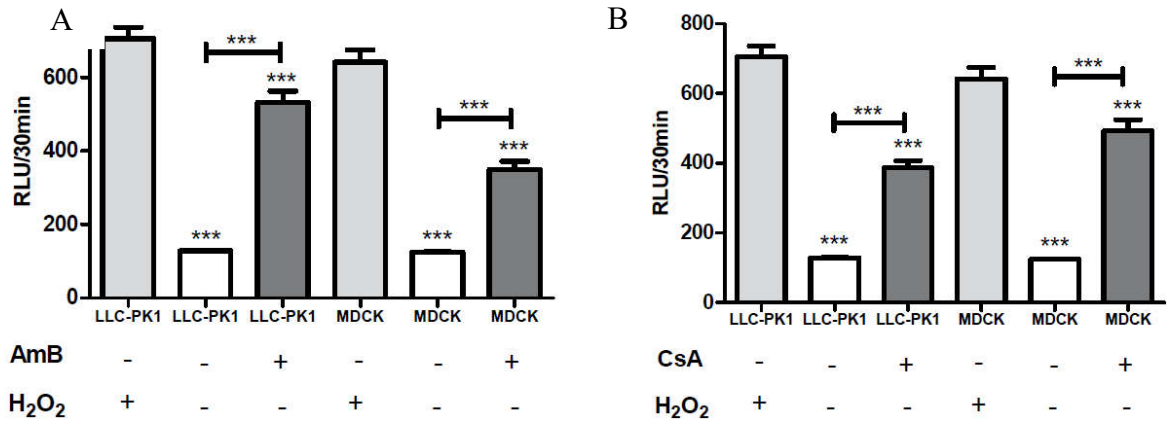

Figure 2. Evaluation of reactive oxygen species (ROS) in cells (LLC-PK1 and MDCK) treated with Amphotericin B (AmB) and Cyclosporin A (CsA). Cells (1x10^6) were exposed to AmB (4.0 µg ml^{-1}) or CsA (250µM for24 hours. As a control to compare the ROS production induced by AmB and CsA , cells were exposed to H2O2 for 30 minutes and then a chemiluminescence assay was performed. The data are presented as the mean ±SD (n= 6) (***) represents significantly difference (p<0.0001).

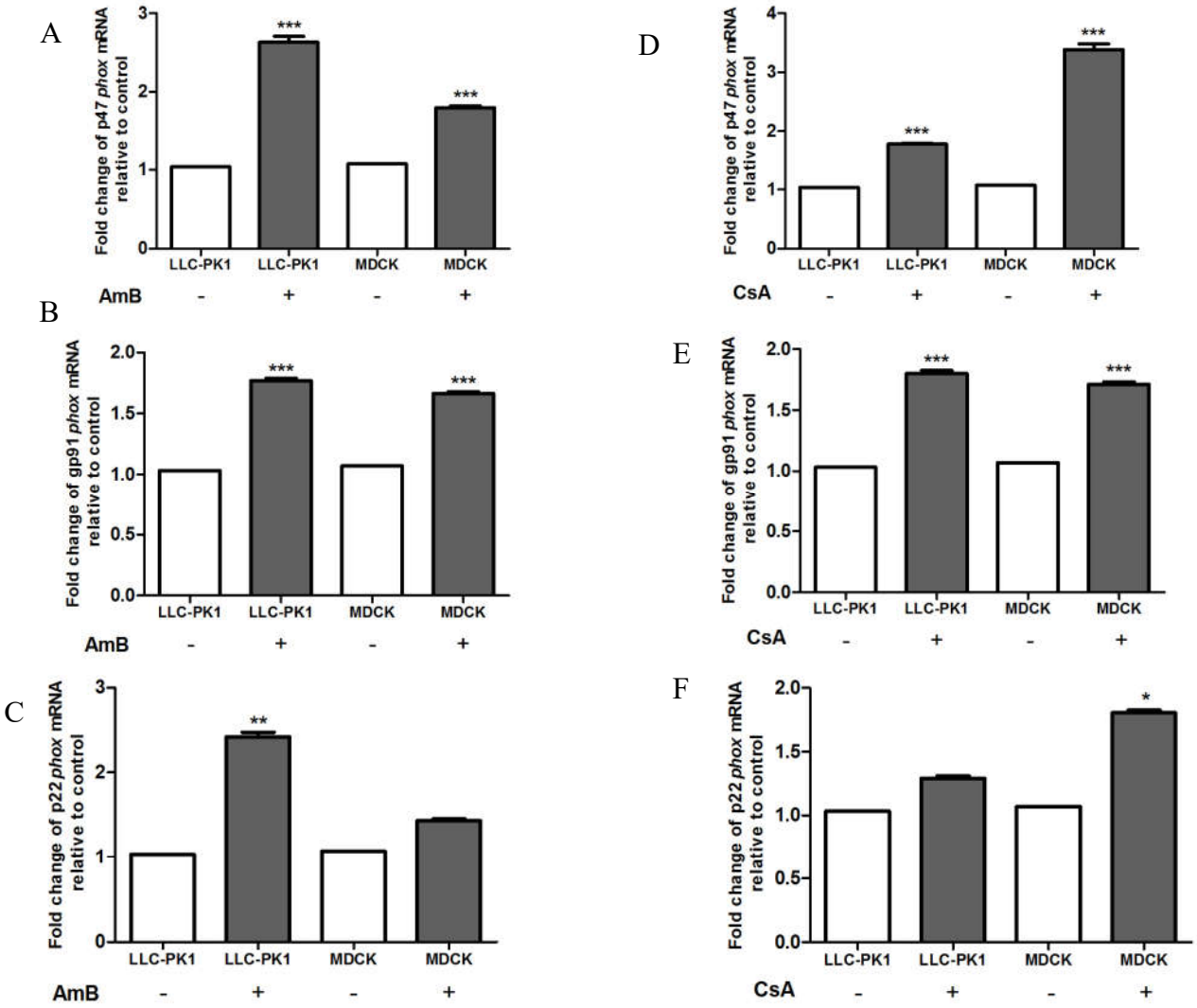

Figure 3. Effects of Amphotericin B (AmB) and Cyclosporine A (CsA) on mRNA expression of the genes for the NADPH oxidase. (A)p47^{phox}, (B) gp91^{phox} *and* (C) p22^{phox} relative to cells treated with AmB (4.0 µg ml^{-1}). (D) p47^{phox}; (E) gp91^{phox} *and* (F) p22^{phox} relative to cells treated with CsA (250µM). The data are presented as the mean ±SD (n= 6). (**), (***) represent significantly difference (p<0,001) (p<0.0001) respectively, relative to each control (cells without treatment).

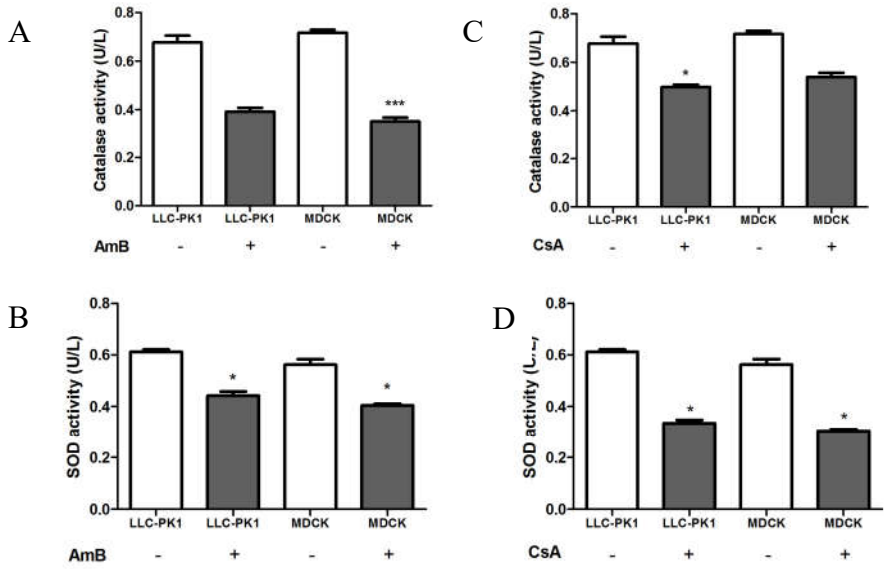

Figure 4. Effects of Amphotericin (AmB) and Cyclosporine A (CsA) in antioxidant enzyme activities in LLC-PK1 and MDCK cells. (A) and (C) Catalase activity in LLC-PK1 and MDCK cells ($1x10^6$ treated with AmB (4.0 µg ml^{-1}) and CsA (250µM) respectively. (C) and (D) Total Superoxide dismutase (SOD) activity in LLC-PK1 and MDCK cells treated with AmB and CsA respectively. The data are presented as the mean ±SD (n= 6). (*) represents significantly difference (p<0,05) relative to each control (cells without treatment).

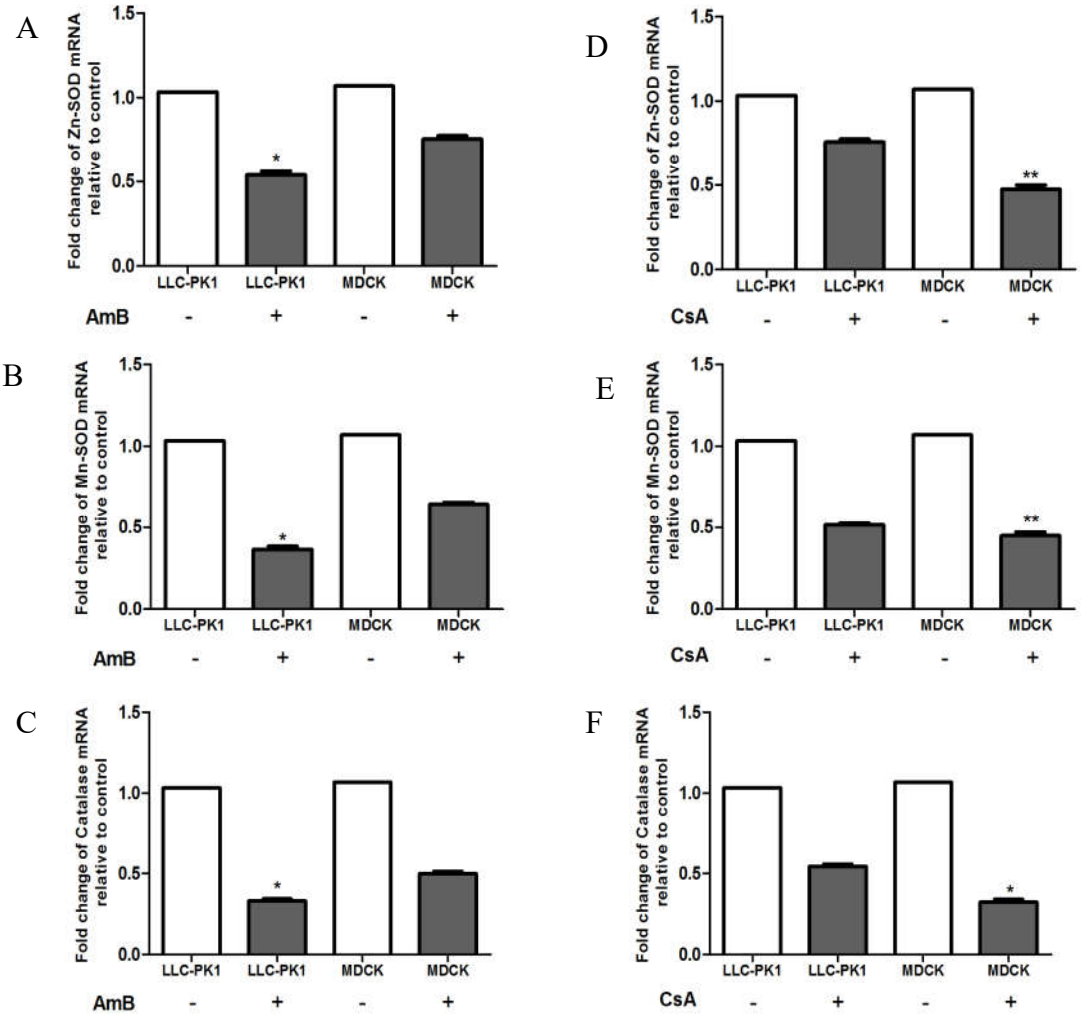

Figure 5. Effects of Amphotericin B (AmB) and Cyclosporine A (CsA) on mRNA expression of enzymes superoxide dismutase and catalase in LLC-PK1 and MDCK cells. (A) Catalase expression in cells treated with AmB (4.0 µg ml^{-1}), (B) Zn-SOD expression in cells treated with AmB; (C) Mn-SOD expression in cells treated with AmB; (D) Catalase expression in cells treated with CsA (250µM) ; (E) Zn-SOD expression in cells treated with CsA ; (F) Mn-SOD expression in cells treated with CsA. The data are presented as the mean ±SEM (n= 6). (*), (**) represents significantly difference (p<0,05), (p<0,001) respectively, relative to each control (cells without treatment)

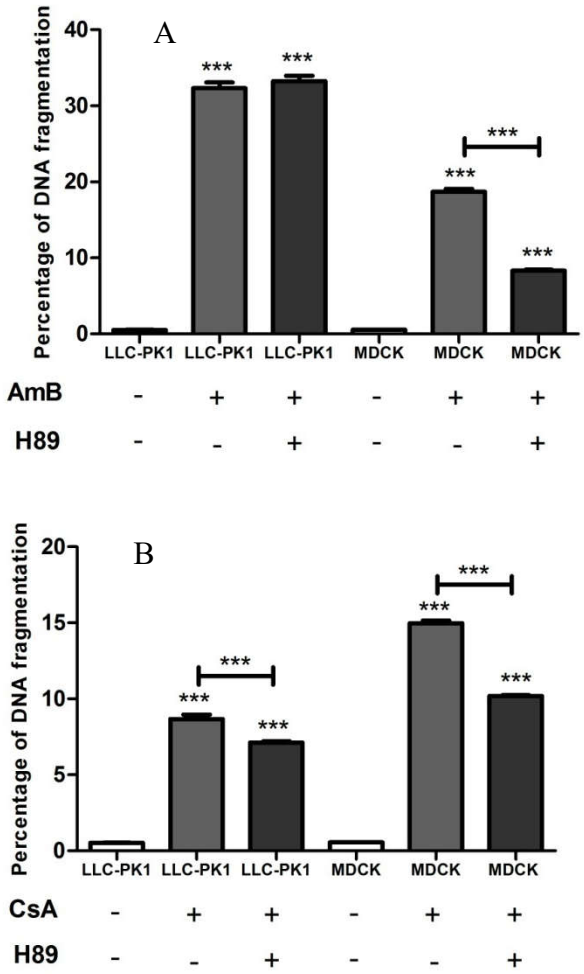

Figure 6. PKA influence in DNA Fragmentation in LLC-PK1 and MDCK cells when submitted to amphotericin B (AmB) - (Panel A) and Cyclosporin A (CsA) – (Panel B). Cells ($1x10^6$) were exposed to H89 (PKA inhibitor) for 30 minutes and then treated with AmB (4.0 µg ml^{-1}) or CsA (250µM for 24h). The data are presented as the mean ±SEM (n= 6). (***) represents significantly difference (p<0.0001)

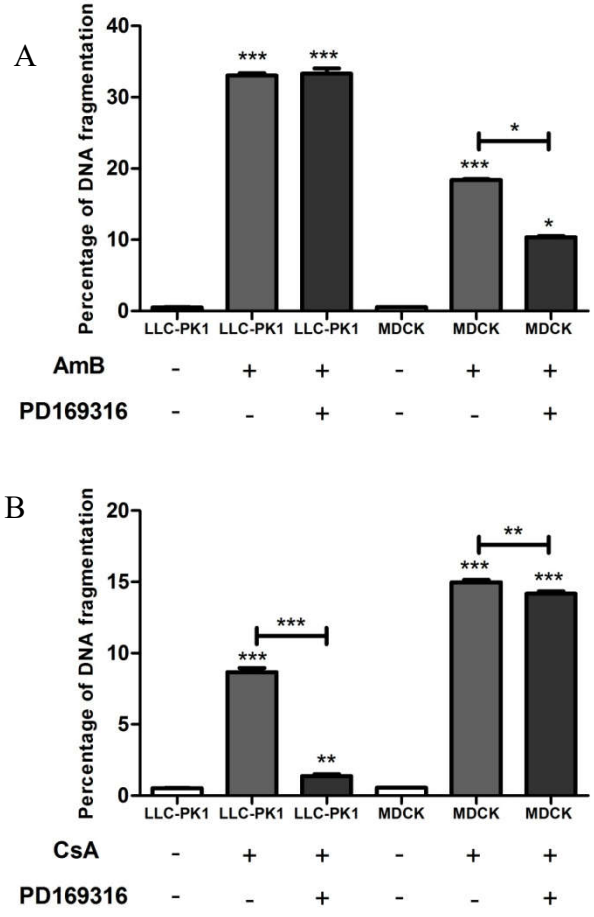

Figure 7. p38 MAPK influence in DNA Fragmentation in LLC-PK1 and MDCK cells when submitted to amphotericin B (AmB) - (Panel A) and Cyclosporine A (CsA) – (Panel B). Cells ($1x10^6$) were exposed to PD169316 (p38 MAPK inhibitor) for 30 minutes and then treated with AmB (4.0 µg ml^{-1}) or CsA (250µM for24h. The data are presented as the mean ±SD (n= 6). (*) and (***) represent significantly difference (p<0,05) and (p<0.0001) respectively

Recent studies have demonstrated that treatment of HK2 cells with CsA significantly decreased the activities of SOD, GSHPx and Catalase, and that these effects of CsA were dose dependently (Huang *et al*., 2018). Hornedo *et al*., 2007 have shown that LLC-PK1 cells treated with CsA produce an increase in mitochondrial production of $O_2^{\cdot-}$ and this increase parallels the decrease in NADPH, an essential molecule in the maintenance of the reduced state of complex I of the mitochondrial respiratory chain, which decrease is related with different models of cellular death. Altuntas *et al*., 2014, did an experiment with renal tissues from mice and showed that catalase also is decreased after treatment with AmB, but SOD is increased. Our results also demonstrated that the treatment with AmB reduced the gene expression of Catalase, Mn-SOD and Zn-SOD in LLC-PK1 cells. It could explain the increased ROS and the higher percentage of DNA fragmentation observed in these cells compared to MDCK cells. França *et al*., 2014 (1,2) also show this difference, in DNA fragmentation, between these cells lines. So, LLC-PK1 is more sensitive to AmB than to CsA. Another profile was observed for MDCK cells, that presented decreased expression of Catalase, MnSOD and ZnSOD, and it is accordingly to our results, showing that MDCK is more susceptible to toxicity caused by CsA represented by ROS production, NADPH oxidase subunits expression and cell death (DNA fragmentation).

Numerous evidences demonstrated that overproduction of ROS and oxidative stress may play a crucial role in the pathogenesis of CsA induced renal impairment (Damiano *et al*., 2015). The mechanisms of CsA toxicity have been investigated and new possible factors implicated in its nephrotoxic effect have been demonstrated (Camilleri et al., 2016; Martin-Martin *et al*., 2010). However, rather studies explain the molecular mechanisms of AmB that causes nephrotoxicity (França *et al*, 2014 (1); Ferreira *et al*., 2015). In this study was possible to explain that PKA pathway and p38 MAPK are involved in the oxidative process and consequently in cell death in two different cell lines when they are treated with CsA. It is demonstrated by other studies, that the molecular mechanisms that underlie CsA nephrotoxicity involve a number of important signaling pathways. In particular, the p38, ERK, and JNK MAPK subfamilies all participate in CsA nephrotoxicity (Martin-Martin *et al*., 2010) and the crosstalk between MAPK and Smad regulates CsA-induced apoptosis in renal proximal tubular cells (Iwayama *et al*., 2011). França *et al*., 2014 (1) demonstrated that p38 MAPK is also involved in cell death caused by AmB in LLC-PK1 and MDCK cells. The ability of p38 MAPK to phosphorylate a wide range of downstream substrates, including several transcriptional factors and other protein kinases, makes it an important regulator of cell proliferation, survival and differentiation, affecting multiple physiological processes.MAPKAPK-2 (also

known as MK2) is one of the p38 substrates, which has been implicated in signaling events affecting the regulation of stress and inflammatory responses (Trempolec *et al*., 2013). p38 MAPK is activated by a variety of cellular stresses and acts at early step prior to dysfunction of mitochondria and caspase activation in cell apoptosis (Choi *et al*., 2006). Some studies have demonstrated that the p38 MAPK kinase cascade is necessary during cell apoptosis by cellular stress (Choi *et al*., 2006). Both cell lines are extensively explored in studies that evaluate PKA pathway participation in various cellular process (Burgos *et al*., 2004; França *et al*., 2014 (1) suggest that nephrotoxicity of cyclosporine may well be related to decreased cell viability, DNA fragmentation, and that the inhibition of PKA signaling pathway can aid in decreasing this toxic effect. PKA is also involved in nephrotoxic mechanisms caused by treatment with AmB. It is know that the inhibition of PKA may have a beneficial effect in preventing nephrotoxicity of Amphotericin B through the vasodilator effect of NO in both cells lines, LLC-PK1 and MDCK (França *et al*., 2014)(1). The activated PKA can inhibit the NF-κB activity by modifying the C-terminal transactivation domain of p65 to reduce p65 nuclear translocation (Liberman *et al*., 2012) and then, the inhibited NF-κB induces apoptosis (Sen, 2006). The study from Xu *et al*., 2015, suggested that NF-κB regulated the transcription of apoptotic target genes by decreasing the ratio of Bcl-2/Bax and surviving mRNA expressions in lymphocytes. Some studies showed that the decreased ratio of Bcl-2/Bax and surviving expressions promoted apoptosis in cancer cell and lymphocytes (Liu *et al*., 2011). Bcl-2, Bax, and survivin are regulated by NF-κB(Sen, 2006). Bcl-2 and Bax are anti-apoptotic and pro-apoptotic protein, respectively. In this context, in our study we observed that when p38 MAPK and PKA were inhibited by PD169316 and H89, respectively in MDCK cells, the percentage of DNA fragmentation decreased, showing that when MDCK cells were treated with AmB or CsA, probably p38 MAPK and PKA were involved in cell death caused by these compounds (Figures 6 and 7). The cell death caused by AmB and CsA could be associated to the increase of ROS production and the decrease in antioxidant defenses in MDCK cells and this oxidative process could activate p38 MAPK and PKA pathways, leading cells to apoptosis as described previously. Despite we observed that AmB and CsA can increase ROS production, increased gene expression of subunits from NADPH oxidase and decreased antioxidant defenses in LLC-PK1 cells, p38 MAPK and PKA inhibition did not alter DNA fragmentation in these cells treated with AmB, showing that the nephrotoxicity caused by AmB in this cell line doesn´t involve these signaling pathways. Oxidative stress and apoptosis are important effects of exposure to AmB and CsA in pathological process of kidney injury. Different segments of the nephron have different response to drugs related to oxidative damage inducing cell death, as we observed in this study. The results presented showed that LLC-PK1 cells are more sensitive to AmB than MDCK cells and the opposite was observed in the treatment with CsA. Besides that we concluded that the p38 MAPK and PKA pathways participate in nephrotoxicity caused by AmB and CsA in MDCK cells, and CsA toxicity in LLC-PK1 can be mediated by both pathways as well.

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REFERENCES

Altuntas, A., Yilmaz, HR., Altunta, A., Uz, E., Demir, E., Gökçimen, A., Aksu, O. Bayram, DS. andSezer, MT. 2014. Caffeic Acid Phenethyl Ester Protects against Amphotericin B Induced Nephrotoxicity in Rat Model. HindawiPublishing Corporation Bio Med Research International, 8 pages.

Alvarez-Lerma, F., Soriano, MC., Rodrigue´z M., Catalán, M., Llorente, AM., Vidart, N., Garitacelaya, M., Maraví, E., Fernández, E., Alvarado, F., López, M., Alvarez-Sánchez, B., Espinosa, J., Quintana, E. 2012. Impact of liposomal amphotericin B on renal function in critically ill patients with renal function impairment. Rev EspQuimioter., 25: 206–215.

Badger, AM., Cook, MN., Lark, MW.,Newman-Tarr, TM., Swift, BA., Nelson, AH., Barone, FC., Kumar, S. 1998. SB 203580 inhibits p38 mitogen activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. J Immunol., 161: 467-73.

Bates, DW., Su, L., Yu, DT., Chertow, GM., Seger, DL., Gomes, DR., Platt, R. Correlates of acute renal failure in patients receiving parenteral amphotericin B. Kidney Int. 2001; 60(4): 1452-1459.

Bayram, DS. and Sezer, MT. 2014. Caffeic Acid Phenethyl Ester Protects against Amphotericin B Induced Nephrotoxicity in Rat Model. Hindawi Publishing Corporation BioMed Research International, 8 pages.

Bichet, DG. 2006. Lithium, cyclic AMP signaling, A-kinase anchoring proteins, and aquaporin-2. J Am Soc Nephrol., 17: 920–2.

Burgos, PV., Klattenhoff, C., Fuente, E., Rigotti, A., Gonzalez, A. Cholesterol depletion induces PKA-mediated basolateral-to-apical transcytosis of the scavenger receptor class B type I in MDCK cells. PNAS 2004; 101(11): 3845–3850.

Caires, A., Fernandes, GS., Leme, AM., Castino, B., Pessoa, EA., Fernandes, S.M., Fonseca, C.D., Vattimo, MF., Schor, N., Borges, FT. 2018. Endothelin-1 receptor antagonists protect the kidney against the nephrotoxicity induced by cyclosporine-A in normotensive and hypertensive rats. Braz J Med Biol Res., 51 (2): e6373.

Camilleri, B., Bridson, JM., Halawa, A. 2016. Calcineurin inhibitor-sparing strategies in renal transplantation: where are we? a comprehensive review of the current evidence. Exp Clin Transplant, 14: 471–483.

Chaves, MM., Rocha-Vieira, E., Lima e Silva, R., Nogueira-Machado, JA. 2000. Increase of reactive oxygen (ROS) and nitrogen(RNS) species generated by phagocyting granulocytes relatedto age. Mech Ageing Dev.,119: 1 – 8.

Chen, S., Chen, QX., Li, QY., An, JX. Sun, P., Ma JB. and Gao, H. 2018. Chem Mater., 30: 1782–1790.

Choi, SY., Kim, MJ., Kang, CM.,Bae, S., Cho, CK., Soh, JW., Kim, JH., Kang, S., Chung, HY., Lee, YS., Lee, SJ. 2006. Activation of Bak and Bax through c-abl-protein kinase Cdelta-p38 MAPK signaling in response to ionizing radiation in human non-small cell lung cancer cells. J Biol Chem., 281: 7049-7059.

Cohen, EB. The role of signaling via aqueous pore formation in resistance responses to amphotericin B. Antimicrobial Agents and Chemotherapy 2016; 60(9): 5122–5129.

Damiano, S. Ciarcia, R. Montagnaro, S. Pagnini, U. Garofano, T. Capasso, G., Florio, S., Giordano, A. 2015. Prevention of nephrotoxicity induced by cyclosporine-A: role of antioxidants, J Cell Biochem.,116: 364–369.

Di Lernia, V., Stingeni, L., Boccaletti, V., CalzavaraPinton, PG., Guarneri, C., BelloniFortina, A., Panzone, M., Corazza, M., Neri, I., Cambiaghi, S., Lasagni, C., Ficarelli, E., Gisondi, P. Effectiveness and safety of cyclosporine in pediatric plaque psoriasis: a multicentric retrospective analysis. J Dermatol Treat. 2016; 27 (5): 395–398.

El-Bassossy, HM., Eid, BG. Cyclosporine A exhibits gender-specific nephrotoxicity in rats: effect on renal tissue inflammation. BiochemBiophys Res Commun. 2018; 495 (1): 468–472.

Fäldt, J., Ridell, M., Karlsson, A., Dahlgren, C. 1999. The phagocyte chemiluminescence paradox: luminol can act as an inhibitor of neutrophil NADPH-oxidase activity..Luminescence, 14: 153 – 160.

Fanos, V. and Cataldi, L. Renal transport of antibiotics and nephrotoxicity: a review. J Chemother.2001; 13(5): 461-72.

Ferreira, AF., França, FD., RossoniJr, JV., Viana, PHL., Moraes, KCM., Gomes, DA., Costa, DC., Tagliati, CA., Chaves, MM. Cyclicadenosinemonophosphateprotects renal celllinesagainstamphotericin B toxicity in a PKA-independentmanner, Drug and Chemical Toxicology2016; 39(1):28-34.

França, FD., Ferreira, AF., Lara, RC., Rossoni, JVJr., Costa, DC., Moraes, KC., Tagliati, CA., Chaves, MM. (1)Alteration in cellularviability, pro-inflammatorycytokines and nitric oxide production in nephrotoxicitygenerationby Amphotericin B: involvementof PKA pathwaysignaling. J ApplToxicol. 2014; 34(12): 1285-92.

França, FD., Ferreira, AF., Lara, RC., Rossoni, JVJr., Costa, DC., Moraes, KC., Tagliati, CA., Chaves, MM. (1)Alteration in cellularviability, pro-inflammatorycytokines and nitric oxide production in nephrotoxicitygenerationbyAmphotericin B: involvementof PKA pathwaysignaling. J ApplToxicol. 2014; 34(12): 1285-92.

França, FD., Ferreira, AF., Lara, RC., Rossoni, JVJr., Costa, DC., Moraes, KCM., Gomes, DA., Tagliati, CA., Chaves, MM. (2)Role of Protein Cinase A signaling pathway in cyclosporine nephrotoxicity. Toxicol Mech Methods 2014; 24 (6): 369-376.

Ghaznavi, R., Zahmatkesh, M., Kadkhodaee, M., Mahdavi-Mazdeh, M. Cyclosporine effects on the antioxidant capacity of rat renal tissues. Transplant Proc. 2007; 39(4): 866–867.

Groemping, Y. and Rittinger, K. 2005. Activation and assembly of the NADPH oxidase: a structural perspective. Biochem. J., 386: 401–416.

Hashimoto, K., Minagawa, H., Yanagi, Y. 2003. Caspase-dependent apoptosis in fulminant hepatic failure induced by herpes simplex virus in mice. J Hepatol., 39: 773–8.

Hornedo, JP., Arriba, G., Fernández, MC., Martínez, SB., Cid, TB. 2007. Cyclosporin A Causes Oxidative Stress And Mitochondrial Dysfunction In Renal Tubular Cells. Nefrologia, 27: 565-73.

Huang, J., Yao, X., Weng, G., Qi, H., Ye, X. 2018. Protective effect of curcumin against cyclosporine Ainduced rat nephrotoxicity. Molecular Medicine Reports, 17: 6038-6044.

Iwayama, H., Sakamoto, T., Nawa, A., Ueda, N. Crosstalk between Smad and mitogen-activated protein kinases for the regulation of apoptosis in cyclosporine Ainduced renal tubular injury. Nephron. Extra 2011; 1(1): 178–189.

Jung, SH., Lim, DH., Lee, JE., Jeong, KS., Seong, H., Shin, BC. 2009. Amphotericin Bentrapping lipid nanoparticles and their in vitro and in vivo characteristics. Eur J Pharm. Sci., 37: 313-320.

Kim, HM., Shin, JH., Cho, YB. and Roe, JH. Inverse regulation of Fe- and Ni-containing SOD genes by a Fur family regulator Nur through small RNA processed from UTR of the sodF mRNA. Nucleic Acids Res. 2014; 42(3): 2003–2014.

Le, T. Thwaites, G. and Wolbers, M. 2017. Itraconazole or Amphotericin B for Talaromycosis. N Engl J Med., 377: 1403–1403.

Lee, CH., Kim, S., Kang, CM., Kim, WY., Kim, J., Kim, GH. Altered expression of tight junction proteins in cyclosporine nephrotoxicity. Am J Nephrol. 2011; 33(1): 7–16.

Leon, C., Taylor, R., Bartlett, KH., Wasan, KM. Effect of heat-treatment and the role of Endoplasmic Reticulum Stress in the Rat Kidneys. ToxicolSci. 2007; 99(1): 346–353.

Liberman, AC., Refojo, D., Antunica-Noguerol, M., Holsboer, F., Arzt, E. 2012. Underlying mechanisms of cAMP-and glucocorticoid mediated inhibition of FasL expression in activation-induced cell death. Mol Immunol., 50: 220–235

Liu, N., Zheng, Y., Zhu, Y., Xiong, S., Chu, Y. 2011. Selective impairment of CD4+ CD25+ Foxp3+ regulatory T cells by paclitaxel is explained by Bcl-2/Bax mediated apoptosis. Int Immunopharmacol., 11:212–219.

Liu, X., Wall, Q., Taylor, L., Curthoys, NP. C/EBPb contributes to cAMP-activated transcription of phosphoenolpyruvate carboxykinase in LLC-PK1-F1 cells. Am J Physiol-Renal 2001; 281(4): 649-57.

Ma, JQ., Liu, CM. and Yang, W. 2018. Protective effect of rutin against carbon tetrachloride-induced oxidative stress, inflammation and apoptosis in mouse kidney associated with the ceramide, MAPKs, p53 and calpain activities. Chemico-Biological Interactions, 286: 26–33.

Mandal, SM, Chakraborty, A., Hossain, M., Mahata, D., Porto, WF., Chakraborty, R., Mukhopadhyay, CK., Franco, OL., Hazra, TK., Basak, A. Amphotericin B and anidulafungin directly interact with DNA and induce oxidative damage in the mammalian genome. Mol Biosyst. 2015; 11(9): 2551-9.

Maritim, AC., Sanders, RA., Watkins, JB. 2003. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol.,17: 24 – 38.

Martin-Martin, N., Ryan, G., McMorrow, T., Ryan, M.P. Sirolimus and cyclosporine A alter barrier function in renal proximal tubular cells through stimulation of ERK1/2 signaling and claudin-1 expression. Am J PhysiolRenPhysiol. 2010; 298 (3): 672–682.

Mesa-Arango, AC, Trevijano-Contador, N., Román, E., Sánchez-Fresneda, R., Casas, C., Herrero, E., Argüelles, JC., Pla, J., Cuenca-Estrella, M., Zaragoza, O. The Production of Reactive Oxygen Species Is a Universal

Action Mechanism of Amphotericin B against Pathogenic Yeasts andContributes to the Fungicidal Effect of This Drug. Antimicrobial Agents and Chemotherapy 2014; 58(11): 6627–6638.

Nicoletti, I., Migliorati, G., Pagliacci, MC., Grignani, F., Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods, 139: 271–9.

Oura, M., Sternberg, TH., Wright. ET. 1955. A new antifungal antibiotic, amphotericin B. Antibiot. Annu., 3: 566-573.

Radeke, HH., Cross, AR., Hancock, JT., Jones, OT., Nakamura M., Kaever, V., Resch, K. Functional expression of NADPH oxidase components (alpha- and beta-subunits of cytochrome b558 and 45-kDa flavoprotein) by intrinsic human glomerular mesangial cells. J Biol Chem. 1991; 266(31): 21025-9.

Sen, R. 2006. Control of B lymphocyte apoptosis by the transcription factor NF-kappaB. Immunity, 25: 871–883.

Trempolec, N., Dave-Coll, N. and Nebreda, AR. SnapShot: p38 MAPK substrates. Cell2013; 152(4): 924–924.

Uchimura, K., Nagasaka, A., Hayashi, R., Makino, M., Nagata, M., Kakizawa, H., Kobayashi T, Fujiwara K, Kato T, Iwase K, Shinohara R, Kato K, Itoh M.1999. Changes in superoxide dismutase activitiesand concentrations and myeloperoxidase activities in leukocytesfrom patients with diabetes mellitus. J Diabetes Complications, 13: 264 – 270.

Urso, ML. and Clarkson, PM. Oxidative stress, exercise, and antioxidant supplementation. Toxicology 2003; 189(1-2): 41–54.

Vinolo, MAR., Hatanaka, E., Lambertucci, RH., Newsholme, P., Curi, R. 2009. Effects of short chain fatty acids on effector mechanisms of neutrophils. Cell BiochemFunct., 27: 48 – 55.

Wilcox, CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? Am J PhysiolRegulIntegr Comp Physiol. 2005; 289(4): R913-35

Wilmes, A., Crean, D., Aydin, S., Pfaller,W., Jennings, P. Leonard, M.O. 2011. Identification and dissection of the Nrf2 mediated oxidative stress pathway in human renal proximal tubule toxicity. Toxicol in Vitro, 25: 613–622.

Xiong, Q., Xie, P., Li, H., Hao, L., Li, G., Qiu, T., Liu, Y. 2010. Acute effects of microcystins exposure on the transcription of antioxidant enzyme genes in three organs (liver, kidney, and testis) of male Wistar rats. J Biochem Mol Toxicol.,; 24: 361 – 367.

Xu, F., Wang, J., Cao, Z., Song, M., Fu, Y., Zhu, Y., Li, Y. cAMP/PKA Signaling Pathway Induces Apoptosis by Inhibited NF-κB in Aluminum Chloride-Treated Lymphocytes In Vitro. Biol Trace Elem Res. 2016; 170(2): 424-31.

Zimmermann, S., Sekula, P., Venhoff, M., Motschall, E., Knaus, J., Schumacher, M., Mockenhaupt, M. Systemic immunomodulating therapies for Stevens-Johnson syndrome and toxic epidermal necrolysis: a systematic review and meta-analysis. JAMA. Dermatol. 2017; 153 (6): 514–522.

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