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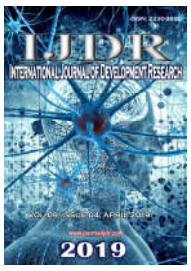

RESEARCH ARTICLE OPEN ACCESS

IL-10 PLAYS A KEY ROLE IN THE REGULATION OF TNF-alfa mRNA EXPRESSION DURING DNA FRAGMENTATION IN THE NEPHROTOXICITY GENERATED BY AMPHOTERICIN B AND CYCLOSPORINE

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ABSTRACT

Inflammation is considered the most important cause of tissue injury in organs subjected to nephrotoxicity induced by drugs. The mechanism that triggers inflammation and organ injury remains to be elucidated, although different causes have been postulated. Thus, this researchinvestigated whether nephrotoxicity generated by amphotericin B (AmB) and cyclosporine (CsA) depends on the balance of cytokines IL-10 and TNF-α, through the evaluation of DNA fragmentation and gene expression of these cytokines in renal cell lines.The results showed that LPS enhances TNF-α mRNA expression and that TNF-α mRNA significantly decreased in the presence of IL-10. The same profile was observed in response to incubation with AmB and CsA,in which both drugs increased the expression of TNF-α mRNA and IL-10 decreases this expression. Thus, IL-10 can reduce the effects generated by nephrotoxicity caused by AmB and CsAby reducing the production of TNF-α. In this context, these results suggest that therapeutic strategies that induce an increase in IL-10 gene expression may be an alternative to decrease the nephrotoxicity caused by AmB and CsA, as demonstrated by IL-10 that modulates the production and gene expression of the TNF-α mRNA and reduces the fragmentation of the DNA induced by drugs.

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INTRODUCTION

The kidney is an essential organ required by the body to perform several important functions including the maintenance of homeostasis, regulation of the extracellular environment, such as detoxification, and excretion of toxic metabolites and drugs. Therefore, the kidney can be considered a major target organ for exogenous toxicants and approximately 20% of nephrotoxicity is induced by drugs (Kim and Moon, 2012).

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Amphotericin B (AmB) has been the gold standard for treating invasive fungal diseases for many years. This drug combines with cell membrane sterols of host cells, forming pores that leak electrolytes in an antifungal action. This drug's mechanism of action can lead to systemic toxicity (Harmsen *et al*., 2011). Nephrotoxicity is the main treatment-limiting adverse effect of AmB (Kato *et al*, 2018). As the toxicity is generally related to biological disturbances at the cellular level, the knowledge of these and their impacts on cellular functions are essential(Van de Water *et al*., 2006). Cyclosporine A (CsA) is a very important immunosuppressive drug and greatly improves the survival rates of patients and

grafts after solid-organ transplantation (Tafazoli, 2015; Ziaei *et al*., 2016; Zimmermann *et al*., 2017). It is also increasingly being used to treat autoimmune diseases such as psoriasis and rheumatoid arthritis (Di Lernia *et al*., 2016; Kisiel *et al*., 2015). However, the chronic use of CsA is associated with high incidences of nephrotoxicity and the eventual development of chronic renal failure (Caires *et al*., 2017; Lai *et al*., 2017). The chronic CsA nephrotoxicity is usually characterized by tubular atrophy, inflammatory cell influx, striped tubule interstitial fibrosis, arteriolopathy and increased intrarenal immunogenicity (Yoon and Yang, 2009; Lee, 2010). Renal cell lines have been employed as alternative methods for the study of therapeutic products that cause nephrotoxicity (Pfallerand Gstraunthaler, 1998; Price *et al*., 2004; Lincopanand Mamizuka, 2005; Jung *et al*., 2009) and the use of *in vitro* techniques has enhanced the comprehension of molecular mechanisms of nephrotoxicity(Wilmes *et al*., 2009). 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; Servais *et al*., 2006; Yano *et al*., 2009; Yuan *et al*., 2011; Shin *et al*., 2010; Ramseyer *et al*., 2013).

In this context, the development of nephrotoxicity was attributed to the propensity of AmB and CsAto induce proinflammatory cytokines (Chai *et al*., 2013; França *et al*., 2014a) and among the proinflammatory cytokines that were associated with the pathophysiology of nephrotoxicity we highlight the tumor necrosis factor alpha (TNF-α) (Streetz *et al*., 2001). TNF-α is a pleiotropic proinflammatory and immunoregulatory cytokine that acts as a mediator of tissue injury. In the kidney, infiltrating macrophages and endothelial, mesangial, glomerular, and tubular epithelial cells synthesize and release TNF-α. This cytokine is a key participant in the pathogenesis of kidney injury, triggering inflammation, apoptosis, and the accumulation of extracellular matrix, impairing glomerular blood flow and damaging the glomerular permeability barrier with the development of albuminuria (Sanchez-Niño *et al*., 2010). Interleukin 10 (IL-10) is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis. Dysregulation of IL-10 is associated with enhanced immunopathology in response to infection as well as increased risk for development of many autoimmune diseases. Thus, a fundamental understanding of IL-10 gene expression is critical for our comprehension of disease progression and resolution of host inflammatory response (Lyerand Cheng, 2012). However, there are no reports in the literature on the immunomodulatory role of IL-10 in the nephrotoxicity caused by AmB and CsA. Thus, in the present paper we have asked whether nephrotoxicity generated byAmB and CsA depends on the balance of cytokines IL-10 and TNF-αthrough the evaluation of DNA fragmentation and gene expression of these cytokines in renal cell lines.

MATERIALS AND METHODS

Drugs: AmB and CsAwere donated by Cristália (Produtos Químicos FarmacêuticosLtda- Itapira, SP, Brazil). A stock solution of 300 µg/mL of AmB in sterile buffer solution (PBS) was prepared and different volumes were added to the RPMI-1640 (Sigma St. Louis, MO, USA) to generate five different concentrations: 2, 4, 6, 8 and 10 µg/mL. A stock solution of 500 µM of CsA was prepared in PBS solution and different volumes were added to the RPMI-1640 medium to generate five different concentrations: 5, 10, 20, 25 and 30 µM. IL-10 and TNF-α cytokines (Sigma St. Louis, MO, USA) was reconstituted in 5 mM Sodium Phosphate, pH 7.2 to a concentration of 1.0 mg/mL, fractionated and stored at -20 °C. The concentrated solution was diluted immediately prior to use and the cells were pretreated with 1.0 ng/mL (Yin *et al*., 2015) of IL-10 or TNF-α for 30 minutes.

Cell culture: The LLC-PK1 (passages 20 to 30) and MDCK cells (passages 30 to 40) were obtained from the Cell Bank at Federal University of Rio de Janeiro (UFRJ). These were cultivated in an RPMI-1640 culture medium (Sigma St. Louis, MO, USA) and supplemented with 10% (v/v) bovine fetal serum (Invitrogen Co Ltda, Carlsbad, CA, USA), 100 IU penicillin/mL, and 100µg streptomycin/mL (Sigma St. Louis, MO, USA). Cells were cultivated in 75 cm^2 bottles and incubated at 37 °C humidified with 5% CO_2.

MTT assay: The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test was performed based on the studies from Mosmann (1983). The cells were placed on 96-well plates in a concentration of 5.0 x 10^3 cells/well containing 180 µL of medium and were then incubated with 20 µL of five different concentrations of the tested drugs for 24 hours at 37 °C in humidified air supplemented with 5% CO_2. Having completed these exposure times, the medium containing the drug was removed, 20 µL of MTT solution (5.0 mg/mL) was added, and the plates were incubated for one hour at 37 °C in humidified air supplemented with 5% CO_2. The MTT solution was then removed, and 100 µL of DMSO was added to each well. The absorbance was read at 570 nm (Thermo Plate model TP-READER) and the results were expressed as a percentage of the viability present in treated cells compared to control cells.

Effects of AmB and CsA on TNF-α and IL-10 production in renal cell lines: IL-10 and TNF-α levels in cell culture supernatants were performed in triplicate using commercially available high-sensitivity enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Inc, Plymouth Meeting, USA) according to the manufacturer's instructions. LLC-PK1 and MDCK cells were plated at 5.0 x 10^5 cells/well into 24-well plates. Twenty-four hours after cells were treated with AmB (4.0 µg/mL) and CsA (5.0µM). After 24 h, supernatants cells were obtained by centrifugation 1500 rpm, 10 min., and were stored at -80 °C. The sensitivities of each ELISA kit were 3.75 and 8.43 pg/mL for IL-10 and TNF-α respectively. To study the involvement in the IL-10 in TNF-α production, LLC-PK1 and MDCK cells were pretreated for 30 min with IL-10 (1.0 ng/mL) followed by LPS (1.0 ng/mL) and after 24 h TNF-α was quantified.

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 AmB (4.0 µg/mL) and cyclosporin (5.0 µM), 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 was indicative of nuclei with hypodiploid DNA content. Sub-diploid DNA content and cell viability were measured after 24 h to assess the cellular responses in the presence of the AmB and CsA. To study the involvement of

IL-10 and TNF-α in AmB and CsA induced cell death, LLC-PK1 and MDCK cells were pretreated for 30 minutes with IL-10 or TNF-α(1.0 ng/mL) followed by AmB (4.0 µg/mL) and CsA (5.0 µM) treatment. Sub-diploid DNA content and cell viability were measured after 24 h to assess the cellular responses in the presence of the IL-10 or TNF-α cytokines.

Gene expression using RT-PCR: Total mRNA was extracted from 1.0 x 10^6 of the renal cell lines using Trizol reagent according to the protocol provided by the manufacturer and resuspended in 30µL of RNase-free water. The concentration and purity of mRNA was estimated by A260 / A280 ratio in spectrophotometer. A total of 1µg of mRNA was converted to cDNA using an oligo (dT) and a High-Capacity DNA Reverse Transcription kit following manufacturer's recommendations. Real-time PCR was performed using a Power SYBR® Green PCR Master Mix reagent with a final volume of 12 µL. The reaction included 0.1 µg of cDNA and 0.5 µL of each primer (reverse and forward, 10µM). Primers used were as follows: IL-10 sense primer 5'-ATGCCCCAAGCTGAGAACCAAGA CCCA-Y (nt 323 - 349), IL-10 anti-sense primer 5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3' (nt 674 - 648) (Vieira *et al*., 1991), TNF-α sense primer 5'-AGAGGGAAGAGTTCCCCAGGGAC-Y (nt 310 - 333), TNF-α anti sense primer 5'-TGAGTCGGTCACCCTT CTCCAG-Y (nt 782 - 760) (Pennica *et al*., 1984), β-actin sense primer 5'-GTGGGGCGCCCCAGGCACCA-3' (nt 1 - 20), β-actin anti sense primer 5'-GTCCTTAATGTCACGC ACGATTTC-3' (nt 548 - 530) (Alonso *et al*., 1986). Total mRNA was isolated from cells using Trizol reagent according to the manufacturer's procedure (Life TM- Thermo Fisher Scientific, Waltham, MA) using the cell lines. Concentration of extracted mRNA was calculated using a nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was carried out with the IllustraTM Ready-to-GoTM Beads kit using 100 ng of mRNA and oligodT based on manufacturer's protocol (GE Healthcare, Chicago, IL). Relative expression level of the genes transcripts was compared to GAPDH as a housekeeping gene. PCR was performed on the Applied Biosystems Step One-Real Time PCR System at 50°C for 2 min, 95ºC for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Statistical analysis: All results were analyzed by ANOVA and Tukey post-test using GraphPad Prism version 5.00 for windows (San Diego, CA). $p<0.05$ was considered to indicate statistical significance.

RESULTS

Cytotoxic effects of AmB and CsAon cell lines: AmB and CsA proved to be cytotoxic for LLC-PK1 and MDCK cells. Significant reduction in cell viability could be observed in concentrations ≥ 4.0 µg/mL (AmB) and ≥ 5 µM (CsA) using MTT test in both cell lines (Figure 1).

Effect of AmB and CsA on the production of IL-10 and TNF-α: The results showed that AmB decreased IL-10 production in LLC-PK1 cells (46%) and MDCK cells (34%), as did CsA: LLC-PK1 (30%) and MDCK (61%) (Figure 2). These decreases were statistically significant ($p <0.05$). However, when cells were exposed to LPS, AmB and CsA, and then incubated with IL-10, there was a significant ($p <0.05$) reduction in TNF-α production when compared to the LPS-treated group alone (gold standard of TNF-α production by cells) (Figure 3).

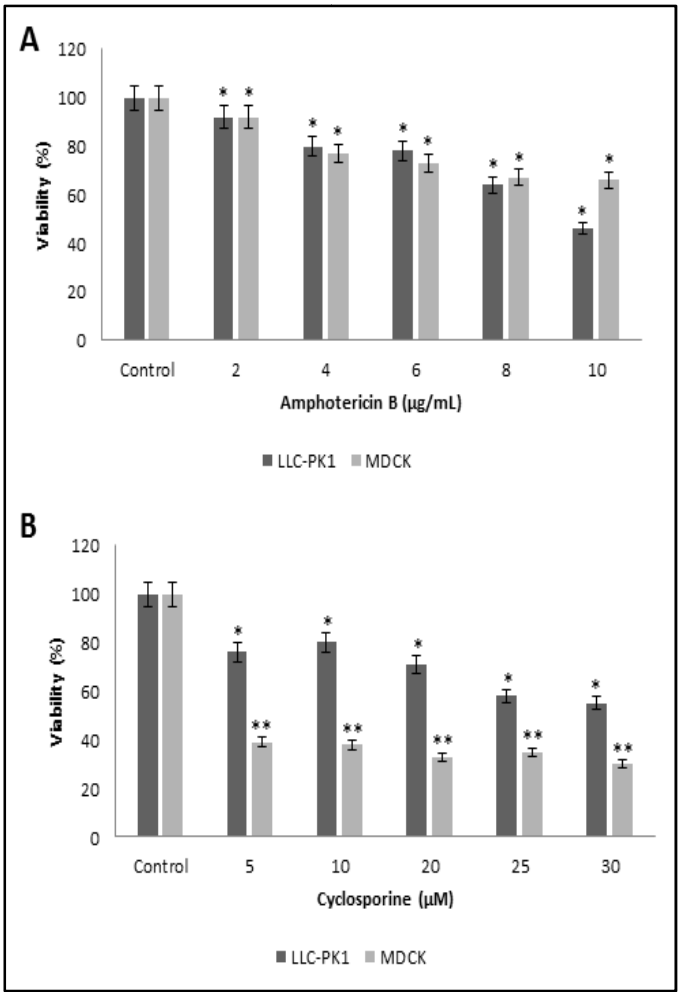

Figure 1. Cytotoxicity of cell lines exposed to amphotericin B and cyclosporine. The cultures were evaluated by MTT assay after 4 h of exposition to drugs. The average absorbance of each amphotericin B and cyclosporine concentration was compared to the control group (cells not exposed to the drug) and for this 100% viability was considered. For each time of incubation: n=5, and for each concentration 6 repetitions. *p< 0.05 and **p< 0.01 when compared with control group.

DNA fragmentation induced by AmB and CsA: An increase in the percentage of dead cells occurred 24 hours after treatment with AmB and CsA. These cells 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. The quantitative analysis of DNA fragmentation in two lineages is demonstrated in Figure 4. AmB caused 35.9% cell death in LLC-PK1 cells and 19% in MDCK cells, while CsA caused 9% cell death in LLC-PK1 cells and 16% in MDCK cells. When the cells were pre-treated with TNF-α and subsequently with the drugs there was a significant increase in DNA fragmentation compared to cells treated with the drugs alone. In LLC-PK1 cells the percentage of cell death was 57.25% for AmB and 37% for CsA. In MDCK cells the percentage of cell death was 36% for AmB and 29% for CsA. However, after cells were pretreated with IL-10 (Figure 5), there was a significant reduction in drug-induced DNA fragmentation. LLC-PK1 cells had 19.7% cell death after AmB treatment and 2.4% cell death after incubation with CsAIn MDCK cells the percentage of death was 10.3% for AmB and 5.25% for CsA, and these reductions were significant when compared to the groups treated only with the aforementioned drugs.

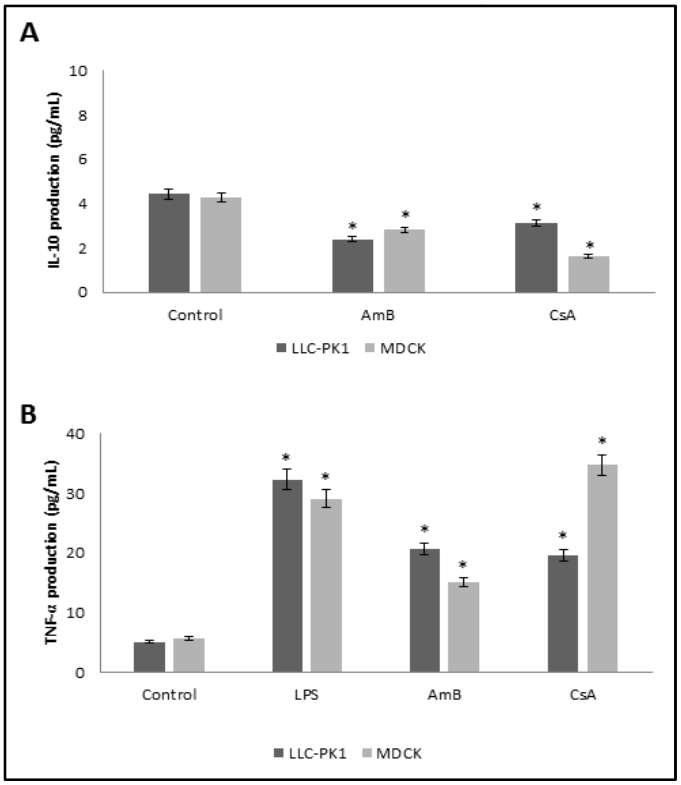

Figure 2. Effect of amphotericin B and cyclosporine on the production of IL-10 and TNF-α. IL-10 and TNF-α levels in cell culture supernatants were performed in triplicate using a commercially available high-sensitivity enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Inc, Plymouth Meeting, USA) according to the manufacturer's instructions. 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

LPS, AmB and CsA increased TNF-α production by renal cells. In LLC-PK1 the increase was 530%, 304% and 283%, respectively. In MDCK cells these increases were 407%, 165% and 506%, respectively

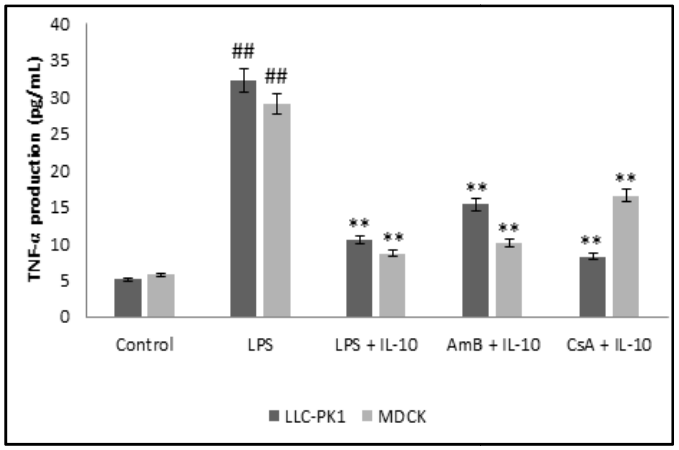

Figure 3. Modulating effect of IL-10 cytokine on the production of TNF-α generated by LPS, amphotericin B and cyclosporine. TNF-α levels in cell culture supernatants were performed in triplicate using a commercially available high-sensitivity enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Inc, Plymouth Meeting, USA) according to the manufacturer's instructions.The values represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. ##p<0.01 when compared with the negative control and **p<0.01 when compared with LPS.

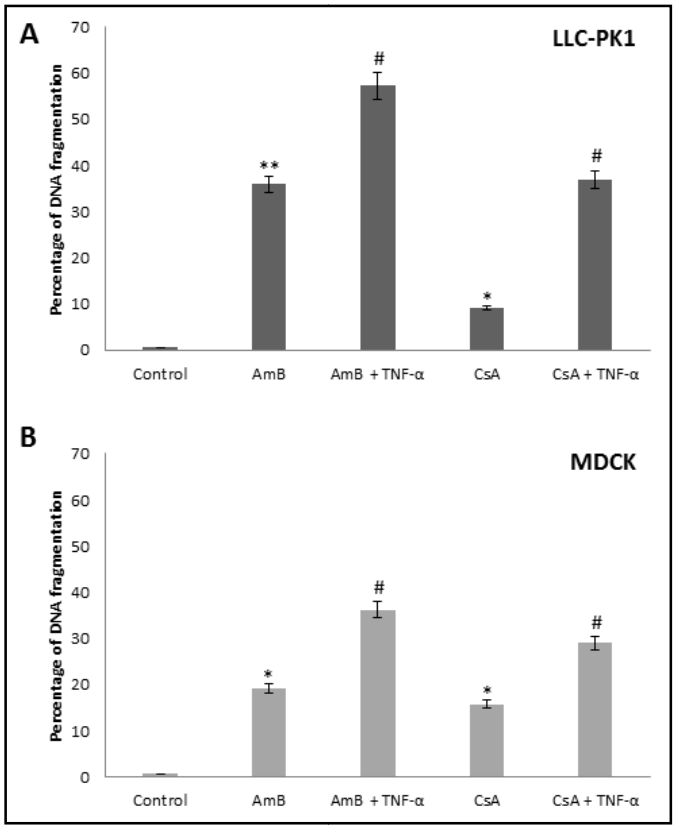

Figure 4. Effect of the treatment with TNF-α on DNA fragmentation induced by amphotericin B (AmB) and cyclosporine (CsA). The cells were placed at the density of 1.0 x 10^4 cells/well in a 24-well plate and were treated with AmB 4.0 µg/mL and CsA5.0 µM in triplicates. DNA fragmentation was analyzed after 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 (n=3) from three independent experiments. * and # mean significantly different from control or group treated with AmB or CsA (p<0.05), respectively.p<0.01 significantly different from control.**

Effect of treatment with IL-10 on kidney gene expression induced by AmBand CsA: Gene expression in renal cells was analyzed by real-time reverse-transcribed polymerase chain reaction (RT-PCR) (Figure 6). The results indicated that AmBand CsA reduced IL-10 mRNA expression (Figure 6A) and increased TNF-α mRNA expression (Figure 6B). When cells were treated with LPS, AmBand CsA this increase occurred, however when cells were pre-treated with IL-10 and subsequently with LPS, AmB and CsA, there was a significant reduction in TNF-α mRNA expression, which suggests the immunomodulatory role of IL-10 in drug-induced nephrotoxicity.

DISCUSSION

According to Figure 1, the renal toxicity caused by AmB and CsA is concentration-dependent. Many studies show that AmB causes lesions on renal cells when used in concentrations from 5 to 20 µg/mL (Yano *et al*., 2009), which is consistent with our study's findings, given that AmB, when applied in the range from 4 to 10 µg/mL, caused a significant loss of cell viability. Hence, one can infer that AmB promotes changes in mitochondrial metabolism, considering that the MTT evaluates the integrity of this organelle. It has been reported that nephrotoxicity appears after treatment with high-dose AmB

(1.5 mg/Kg/day), indicating that the plasma concentration is estimated to be approximately 4.0 µg/mL (Gates and Pinney, 1993). Therapeutic concentrations of CsA range from 0.1 to 1.6 µM (Hauser *et al*., 1998; Kovarik *et al*., 2003).

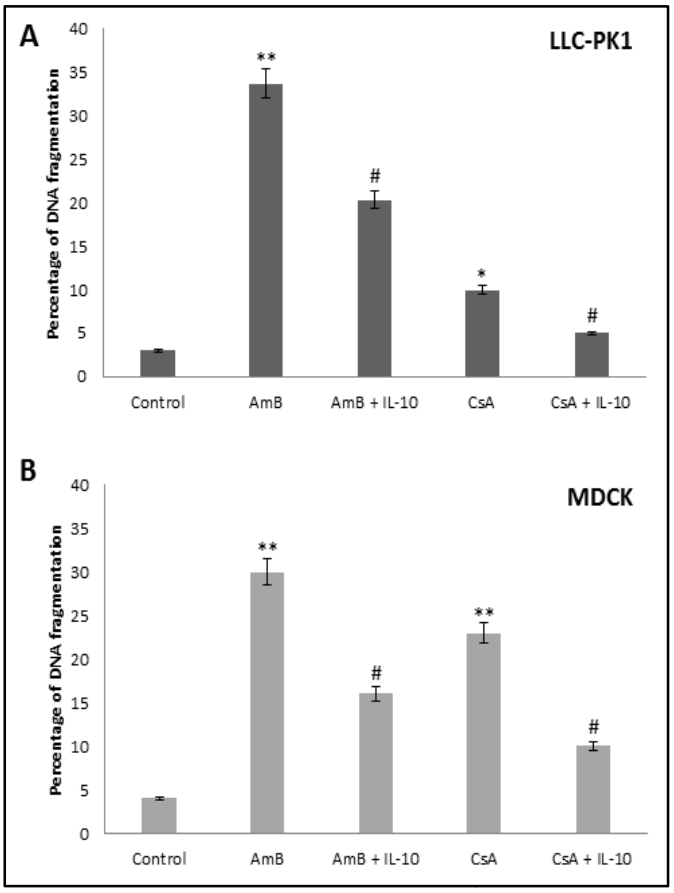

Figure 5. Effect of the treatment with IL-10 on DNA fragmentation induced by amphotericin B (AmB) and cyclosporine (CsA). The cells were placed at the density of 1.0 x 10^4 cells/well in a 24-well plate and were treated with AmB 4.0 µg/mL and CsA 5.0 µM in triplicates. DNA fragmentation was analyzed 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 (n=3) from three independent experiments. * and # mean significantly different from control or group treated with AmB or CsA (p<0.05), respectively.p<0.01 significantly different from control**

Jiang and Acosta (1993) also reported that due to its high lipophilicity, CsA mainly accumulates in the kidneys, with concentration levels reaching as much as 50 times that found in blood plasma. Therefore, the present study opted for the use of 4.0 µg/mL of AmB and 5.0 µM concentration of CsA in studies that evaluate immunomodulation involvement in nephrotoxicity. Inflammation plays a central role in the pathogenesis of drug induced kidney injury. CsA induces deranged renal functions and altered renal morphology through multiple mechanisms including hypoxia, generation of oxidative stress, inflammation (Chander *et al*., 2005; Ortega *et al*., 2011; França *et al*., 2014a) and expression of various inflammatory cytokines (Walker and Endre, 2008). This is mediated through the expression of cytokines upon activation of TLRs located on the renal parenchymal and tubulointerstitial cells (Chai *et al*., 2013).

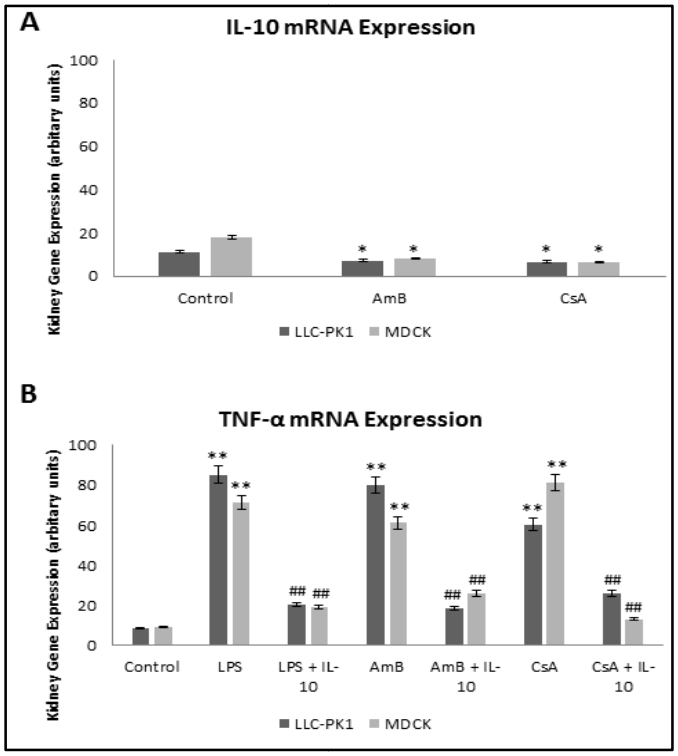

Figure 6. Effect of the treatment with IL-10 on Kidney Gene Expression induced by amphotericin B (AmB) and cyclosporine (CsA). The cells were placed at the density of 1.0 x 10^4 cells/well in a 24-well plate and were treated with AmB 4.0 µg/mL and CsA 5.0 µM in triplicates. DNA fragmentation was analyzed 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 (n=3) from three independent experiments. * and ## mean significantly different from control or group treated with LPS, AmB or CsA (p<0.05) and (p<0.01), respectively.p<0.01 significantly different from control**

Other studies in our group showed that the nephrotoxicity caused by AmB and CsA is related to increased production of the proinflammatory cytokineIL-6 and TNF-α (França *et al*., 2014a; França *et al*., 2014b; Ferreira *et al*., 2016). The proinflammatory response to AmB is mediated by TLR dependent mechanism and this ability to activate TLRs may be relate to the fact that it is a fermentation product of *Streptomyces nodosus* (Razonable *et al*., 2005). The development of drug-induced nephrotoxicity has been described as being immunologically mediated through and TLR4 signaling pathway was implicated in the pathogenesis of cisplatin-induced nephrotoxicity (Chai *et al*., 2013). Our results showed that both AmB and CsA significantly increased TNF-α production relative to control (Figure 2B). In contrast, IL-10 is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to drugs, thereby preventing damage to the host and maintaining normal tissue homeostasis (Lyerand Cheng, 2012). Our results showed that cells exposed to AmB and CsA decreased the IL-10 production (Figure 2A). Kumar *et al*. (2017) showed that the nephrotoxicity of another drug (cisplatin) decreased the level of IL-10. Thus, the reduction of IL-10 production by the cells exposed to drugs may contribute to nephrotoxicity, because IL-10 is a cytokine with anti-inflammatory action. While there are inflammatory conditions in the kidney, cytokines such as TNF-α and IL-1 are produced by both resident cells (including renal tubular cells) and infiltrating

monocytes/macrophages (Sedor, 1992; Sedor *et al*., 1993). TNF-α reduces renal blood flow and glomerular filtration rate by acting as a vasoconstrictor and can cause natriuresis by inhibiting renal epithelial sodium channel activity (Shahid *et al*., 2008; Shahid *et al*., 2010; Majid, 2011). TNF-α participates in the process of renal injury by recruiting and activating inflammatory cells (Egido *et al*., 1993). However, besides the production of proinflammatory cytokines, it is possible for an anti-inflammatory response to contain the inflammation and limit cellular destruction. A key cytokine that appears to restore the balance between proinflammatory and anti-inflammatory cytokines is IL-10 (Wang *et al*., 1995), which is a potent immunoregulatory cytokine produced by macrophages, T cells, B cells, epithelial cells, and mast cells. IL-10 has been shown to exert a protective effect against inflammation (Bean *et al*., 1993; Gérard *et al*., 1993) due to generalized downregulation of proinflammatory cytokines such as IL-1, TNF-α and IL-6 (Fiorentino *et al*., 1991). Though, the regulation of IL-10 in tubular cells (LLC-PK1 and MDCK) is not clearly described.

Our results showed that IL-10 was able to modulate the production of TNF-α in the proposed challenges: LPS, AmB and CsA. In all stimuli IL-10 was able to exert an anti-inflammatory stimulus, reducing ($p<0.01$) TNF-α production (Figure 3). Generally, a combination of physiologic and biochemical events contributes to the susceptibility of the kidney to several distinct classes of nephrotoxicity. Compared with other organs, the kidney is uniquely susceptible to chemical toxicity, partially because of its disproportionately high blood flow (25% of cardiac output), and due to its complexity, both anatomically and functionally. Kidneys also play an instrumental role in regulating overall blood pressure. Urine is the principal route by which most toxicants are excreted. As a result, the kidney concentrates toxicants in the filtrate, transports toxicants across the tubular cells and bio-activates certain toxicants. All these attributes make kidneys extremely vulnerable to a variety of adverse effects. A very recent study (Wirestam *et al*., 2017) suggests that CsA can also damage renal tubular cells by indirect mechanisms, inducing the production of osteopontin, which promotes inflammation that injures renal cells. The mechanism of renal damage has been the focal point of intense investigation for many years. Several studies advocate that inflammation, oxidative stress injury and apoptosis undoubtedly participate in renalimpairment (França *et al*., 2014a). Among these pathological changes, instigation of an inflammatory cascade is the most important issue, which is mediated by nuclear factor-κB (NF-κB) signal transduction pathway. Activation of NF-κB promotes proinflammatory cytokines and enzymes, including TNF-α, interleukins, nitric oxide (NO) and inducible nitric oxide synthase (iNOS), which may eventually cause renal damage (Kumar *et al*., 2017). Now it is well established that apoptosis is biochemically characterized by orderly DNA fragmentation, and morphologically characterized by sequential phases of chromatin margination and condensation, cell disintegration without an inflammatory response and phagocytosis by surrounding cells (Ray *et al*., 1996; Ray, 1999; Savilleand Fadok, 2000). Apoptosis has been observed in a wide variety of tissues, such as the liver (Ray *et al*., 1996; Ray, 1999), kidney (Strika *et al*., 1998; Hickey *et al*., 2001), heart (Sam *et al*., 2000) and intestine (Martin *et al*., 2000).Similarly, apoptosis being induced by a variety of drugs and chemicals in several tissues has also been demonstrated (Ray, 1999). 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). Renal cells are capable of producing intrinsic survival factors such as IGF-1, cyclooxygenase-2-derived prostaglandins, and eicosanoids, which can facilitate recovery from or even prevent toxic injury. It is conceivable that different cell types require different mediators to undergo apoptosis or to survive toxic injury (Varlam *et al*., 2001).

LLC-PK1 cells presented a higher percentage of cell death (DNA fragmentation), showing themselves to be more sensitive to the toxic effects of AmB.CsA also induces nephrotoxicity by directly inducing tubular cell apoptosis. Indeed, renal biopsy specimens from patients with CsA nephrotoxicity always exhibit apoptosis (Rao *et al*., 2007). In addition, CsA is directly toxic to LLC-PK1 and MDCK renal tubular cells and this effect associates with DNA synthesis inhibition and the induction of apoptosis that is mediated by the Fas antigen-ligand system (Kim *et al*., 2000). 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 the two studied cells lines, and these can be found in the late stages of apoptosis/necrosis (Figure 4). Our results also showed that TNF-α was able to potentiate the action of AmB and CsA in generating DNA fragmentation in the two strains studied (Figure 4). Other results showed that IL-10 was able to decrease the fragmentation of DNA generated by both AmB andCsA significantly (Figure 5). Another observation is that its inhibitory action was more pronounced in LLC-PK1 than in MDCK cells (Figure 5A).

According toLyer and Cheng (2012), the impaired IL-10 expression or signaling can enhances clearance of pathogens during an acute infection, but also exaggerates inflammatory response, resulting in exacerbated immunopathology and tissue damage. Our results showed that both AmB and CsA were able to inhibit IL-10 mRNA expression significantly (Figure 6A). Other results showed that LPS enhances TNF-α mRNA expression and that TNF-α mRNA significantly decreased in the presence of IL-10 (Figure 5B). We observed this same profile in response to incubation with AmB and CsA, in which both drugs increased the expression of TNF-α mRNA and IL-10 decreased this expression (Figure 6B). Thus, IL-10 can reduce the effects generated by nephrotoxicity caused by AmBand CsA by reducing the production of TNF-α. IL-10 production can inhibit proinflammatory response to a number of pathogens, including *Plasmodium spp*, *Leishmaniaspp*, *T. cruzi, Mycobacterium* and *Lymphocytic choriomeningitis virus*, to the extent that pathogens can escape immune control, resulting in either fulminant and rapidly fatal or chronic nonhealing infections (Lyer and Cheng, 2012). Our results suggest that therapeutic strategies that induce an increase in IL-10 gene expression may be an alternative to decrease the nephrotoxicity caused by AmBand CsA, as demonstrated by IL-10 that modulated the production and gene expression of the TNF-α mRNA, reducing DNA fragmentation induced by the drugs.

Conflict of interest

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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