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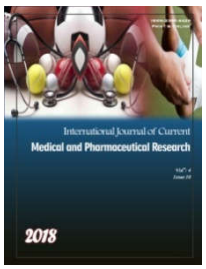

ROLE OF CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) AND CYCLIC GUANOSINE MONOPHOSPHATE (cGMP) IN OXIDATIVE METABOLISM, INFLAMMATION AND CELL DEATH: CASE AMPHOTERICIN B

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ABSTRACT

Amphotericin B (AmB) is an important antibiotic agent; however, nephrotoxicity is one of the main adverse effects. The purpose of this study was to evaluate the effect of cAMP and cGMP in nephrotoxicity caused by AmB from the assessment of cytokines, stress oxidative, nitric oxide production and cell death in MDCK cells line. Our results showed an increase of IL-6 production by AmB, and an inhibitory effect was observed when cells were incubated with AmB plus cAMP. Results with TNF-α showed that cAMP is able to produce TNF-α only up to the basal level similar to AmB. In contrast, cGMP was able to revert the production of TNF-α Also, AmB decreases IL-10 production but that incubation with cAMP and cGMP was able to reverse this inhibition. Both hydrogen peroxide and AmB were able to induced reactive oxygen species (ROS) generation and cAMP and cGMP were able to reduce ROS production. AmB decreases NO production and cAMP and cGMP increase NO production by reversing the effect generated by AmB. cAMP and cGMP were able to decrease the fragmentation of the DNA generated by AmB. Our findings open new perspectives in the clinical management of invasive fungal diseases. The alternative contributory intracellular mechanism of action of AmB should be considered in the rationale of combined therapy. Combinations of AmB and cAMP and cGMP can be based on that additional effect, as therapy can target both the membrane and the oxidative burst of the fungal cells.

INTRODUCTION

Amphotericin B is the gold standard for antifungal treatment for the most severe mycoses. However, adverse effects are common, with nephrotoxicity being the most serious, occurring early in the course of treatment, and usually being reversible in most patients. Tubular damage is a well-known problem associated with amphotericin B therapy but acute renal failure is the most serious complication (Fanos and Cataldi 2000).

Few studies have examined the chronic changes in renal hemodynamics and tubular transport that result from specific inflammatory mediators or cytokines. Renal inflammation occurs with the macrophage accumulation and infiltration of inflammatory cells. The contribution of inflammatory mediators to renal hemodynamic and tubular dysfunction depends on the pathological state, the inflammatory mediators, and the site of inflammation. This section will focus on renal inflammation and its impact on renal hemodynamic,

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glomerular, and renal tubular injury (Akchurin and Kaskel 2015, Mihai *et al.* 2018).

Regardless of whether innate or adaptive immunity is involved, or whether the renal disease is acute or chronic, it is clear that inflammatory cytokines (e.g. IL-6, TNF-α) have a central role as both mediators of immune function and initiators of renal injury. However, cytokines have immunomodulatory roles that can abrogate the development of renal disease as well. In addition, signaling pathways activated by many cytokines increase Nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) activation, a transcription factor that further promotes a proinflammatory phenotype (Sanz *et al.* 2008, Sanz *et al.* 2010). Indeed, NF-κB expression and/or activation are increased in the kidneys from patients with glomerulonephritis (Sakai *et al.* 2002, Zheng *et al.* 2006), diabetic nephropathy (Mezzano *et al.* 2004), and AKI (Loverre *et al.* 2004). One of the consequences of this inflammatory cycle is to drive the development of local oxidative stress (Reactive oxygen species, ROS, production) that enhances renal injury and impairs both renal tubular and renal hemodynamic function (Imig and Ryan 2013).

ROS are formed by incomplete reduction of molecular oxygen. They include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($1O_2$). ROS may induce different types of cell injury, particularly lipid peroxidation and membrane damage. ROS have been shown to play an essential role in the mechanisms of experimental models of several renal diseases: ischemic acute renal failure, renal graft rejection, acute glomerulonephritis, and toxic renal diseases. They are produced by the renal cells and also by the inflammatory bone marrow-derived cells invading the renal tissue. ROS, regardless of their origin, may degrade the glomerular basement membrane and alter the glomerular and tubular cell functions (Baud and Ardaillou 1986, Sedeek *et al.* 2013).

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).

Cyclic adenosine monophosphate (cAMP) is ubiquitous and regarded as an intracellular messenger responsible for multiple functions, including protection against cell death due to drug use in renal tissue (Qin *et al.* 2012). cAMP is produced by adenylate cyclase activity which is, in turn, activated by a membrane receptor responsive to an intracellular or membrane stimulus (Dodge-Kafka *et al.* 2005). The signal from cAMP can be carried through ion channels, exchange protein directly activated by cAMP (Epac), and protein kinase A (PKA) (Breckler *et al.* 2011). PKA is a ubiquitous protein kinase expressed throughout the renal tissue, which has a wide range of functions, including cell protection (Deb *et al.* 2017).

In this context, renal cell lines have been employed as alternative methods for the study of therapeutic products that cause nephrotoxicity (Pfaller and Gstraunthaler 1998, Price *et al.* 2004, Lincopan *et al.* 2005, Jung *et al.* 2009) and the use of *in vitro* techniques has enhanced the comprehension of molecular mechanisms of nephrotoxicity (Wilmes *et al.* 2011). MDCK cells (canine distal cells) are considered acceptable model to study drug nephrotoxicity (Yano *et al.* 2009, El Mouedden *et al.* 2000, Servais *et al.* 2006, Shin *et al.* 2010, Yuan *et al.* 2011, Ramseyer and Garvin 2013).

Thus, the objective of this work was evaluate if cAMP and cGMP could protect MDCK cell line from DNA fragmentation to amphotericin B exposure through evaluation of the cytokines balance, ROS production and NO release.

MATERIALS AND METHODS

Drugs

Amphotericin B was donated by Cristália (Produtos Químicos Farmacêuticos Ltda- Itapira, SP, Brazil (purity of 90%). A stock solution of 300 µg/mL of amphotericin B in sterile buffer solution (PBS) was prepared and different volumes were added to the RPMI-1640 (Sigma St. Louis, MO, USA) to generate eight different concentrations: 2, 4, 6, 8, 10, 15, 20 and 30 µg/mL. The choice of concentrations of amphotericin B was based on the work of Wasan *et al.* (1994).

The concentration of db-cAMP (N6,2'-O-dibutiriladenosina3',5'cyclic monophosphate-db-cAMP – Sigma, St, Louis, MO) use solution was 10-5 M. The concentration of 8Br-cGMP (2-amino-8-bromo-9-[(1S,6R,8R,9R)-3,9-dihydroxy-3-oxo-2,4,7-trioxa-3λ5 phosphabicyclo[4.3.0]nonan-8-yl]-3H-purin-6-one – Sigma, St, Louis, MO) use solution was 10-3 M (viability 85%) (Chaves et al. 2008). H_2O_2 10.5% v/v as the stimulus dose chosen (curve concentration response) for MDCK cells since it increases ROS production and the cells remained viable (80.12%).

Cell culture

MDCK (distal tubular cells from dogs – passages 5 to 15) were obtained from the Cell Bank at Federal University of Rio de Janeiro (UFRJ). It was grown 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.

Cytokines

IL-6, TNF-α and IL-10 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, PA, USA) according to the manufacturer's instructions, MDCK cells were plated at 5.0 x 10^5 cells per well into 24-weel plates. Twenty-four hours later cells were pretreated for 30 min with db-cAMP and 8Br-cGMP followed by amphotericin B (4.0 µg/mL) treatment. After 24h, supernatants cells were obtained by centrifugation 413 *g*, 10 min, and stored at -80 °C. The sensitivities of each ELISA kit were 1.01 pg/mL (IL-6), 8.43 pg/mL (TNF-α) and 7.81 pg/mL (IL-10).

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), db-cAMP and 8Br-cGMP treatment and luminol for 30 minutes. The chemiluminescence measurements were performed in a luminometer 1250-101 (Lumat, LB 9501, EG&G Berthold – Germany). The experiments were performed in duplicate and carried out at 37 ^oC. The results were expressed in Relative Light Units/min (RLU/min). The control experiments were done simultaneously.

Nitric Oxide (NO) quantification

MDCK cells were incubated for 24 hours into 24-well plates (5.0 x 10^5 cells/well and after this time were treated with amphotericin B (4.0 µg/mL), db-cAMP and 8Br-cGMP. After 24 h supernatants were obtained by centrifugation 1500 rpm, 10 min. and NO production was measured by means the Griess reaction. This involved comparing 100 µL aliquots of culture supernatant with serial dilutions $NaNO_2$ (from 7.81 mM to 1000 mM). To this an equal volume of Griess reagent (N-1-naphthylethylenediamine 0.1% in H_2O + sulfanilamide 1% in 2,5% H_3PO_4) was added and then incubated at room temperature for 10 minutes and read at 540 nm (Green *et al.* 1982).

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, db-cAMP and 8Br-cGMP 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 1 h and analyzed in a FACScan flow cytometer (Becton Dickinson, Germany) for shifts in PI fluorescence that were indicative of nuclei with hypodiploid DNA content.

Statistical analysis

All results were analyzed by One-Way ANOVA and Tukey post-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

Effect of amphotericin B on the balance of pro and antiinflammatory cytokines: modulating effect of cAMP and cGMP

The results showed that amphotericin B increase IL-6 production in MDCK cell line (Figure 1.A). In contrast, a significant reduction in IL-6 production occurred in the same cells treated with amphotericin B and cAMP (Figure 1.A). The treatment with cGMP increases the IL-6 production similar to those found only with amphotericin B (Figure 1.A).

The release of TNF-α presented a different profile. Amphotericin B was not able to increase TNF-α production significantly in relation to the negative control. cAMP incubated with amphotericin B was also not able to alter TNF-α production by MDCK cells. However, when MDCK was incubated which cGMP plus amphotericin B, we achieved an increase in TNF-α production when compared to LPS (Figure 1.B).

Production of IL-10 was decreased when MDCK cells were incubated with amphotericin B (Figure 10.C). However, both incubation with cAMP and cGMP plus amphotericin B was able to increase IL-10 production by MDCK cells. However, this increase was most observed when MDCK was incubated with amphotericin B plus cGMP (Figure 1.C).

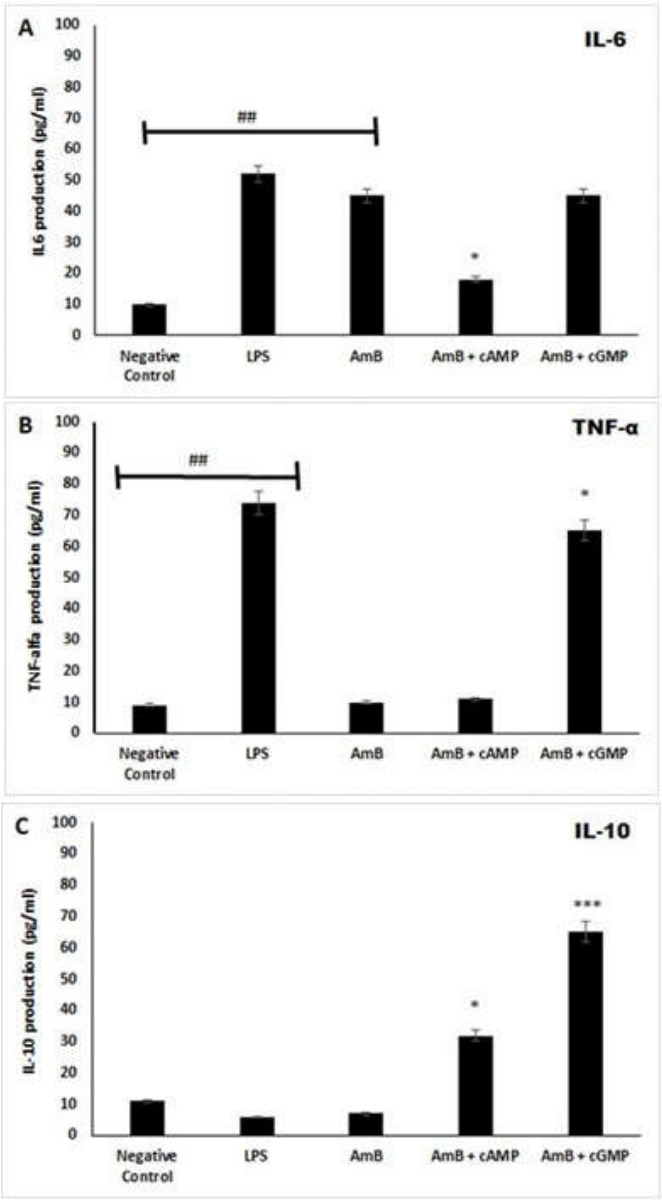

Figure 1 Effects of amphotericin B on cytokines production in MDCK cells line. The production of cytokines in the MDCK supernatants cultures was determined using a commercially available high-sensitivity enzyme-linked immunosorbent assay kit after 24 h incubation with amphotericin B (4.0 µg/mL), LPS (25 µg/mL), cAMP (10^{-3} M) and cGMP (10^{-3} M). The results represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. #$p<0.001$ when compared with the negative control group (untreated cells), *$p<0.01$ and ***$p<0.001$ when compared with AmB.

Pro-oxidant effect of the amphotericin B and the modulatory action of cAMP and cGMP

The ROS production expressed as RLU/min in MDCK cells in the experiment with amphotericin B is show in Figure 2. Amphotericin B generated a significant activation of ROS production in relation to negative control. The similar profile

was observed when the cells were incubated with hydrogen peroxide (H_2O_2), powerful activator of ROS production, showing a pro-oxidant profile (Figure 2). Incubation of the cell line with amphotericin B plus cAMP and cGMP significantly reduced ROS production. Our results also showed that this effect was more potentiated ($p<0.001$) by cGMP when compared to cAMP (Figure 2).

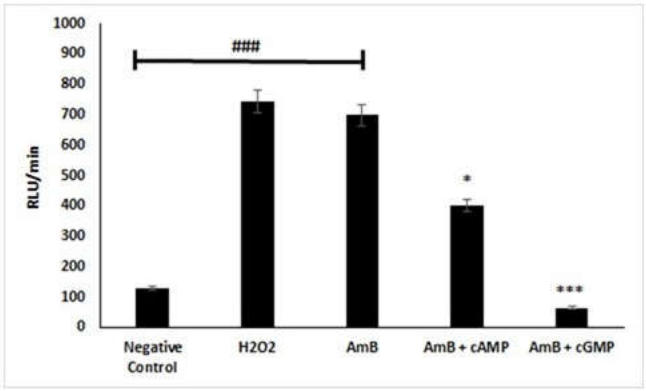

Figure 2 Effect of amphotericin B on reactive oxygen species (ROS) production in MDCK cells line. The production of ROS in the MDCK was performed in a luminol-dependent chemiluminescence assay incubated with H_2O_2 (10.5% v/v), amphotericin B (4.0 µg/mL), cAMP (10^{-3} M) and cGMP (10^{-3} M). The results represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. ###$p<0.001$ when compared with the negative control group (untreated cells), *$p<0.05$ and ***$p<0.001$ when compared with AmB.

Effect of amphotericin B on the nitric oxide (NO) release: modulatory action of cAMP and cGMP

In the MDCK cells line, amphotericin B provoked a significant decrease in NO release (78%) (Figure 3). Incubation of these cells with cAMP and cGMP reversed the activating effect of amphotericin B (Figure 3). However, once more, our results showed that this effect was more potentiated ($p<0.001$) by cGMP when compared to cAMP (Figure 3).

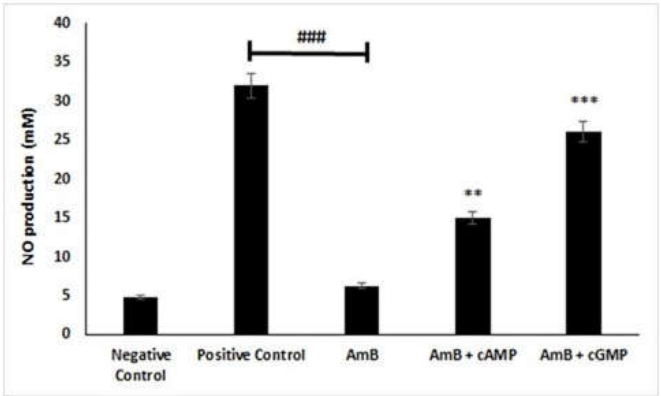

Figure 3 Effect of amphotericin B on nitric oxide (NO) production in MDCK cells line. The production of NO in the MDCK supernatants cultures was determined by Griess reaction after 24 h incubation with glyceryl trinitrate [(10^{-3} µM) positive control], amphotericin B (4.0 µg/mL), cAMP (10^{-3} M) and cGMP (10^{-3} M). The results represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. ### $p<0.001$ when compared with the negative control group (untreated cells), *$p<0.05$ and ***$p<0.001$ when compared with AmB.

DNA fragmentation induced by amphotericin B are reversed by cAMP and cGMP

An increase in the percentage of dead cells (DNA fragmentation) occurred 24 h after treatment with amphotericin B in the quantitative analysis of DNA fragmentation in MDCK cells line (Figure 4). 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 MDCK cell lines. MDCK cells presented 34% of dead after treatment with Amphotericin B (Figure 4). Our data also reported that cAMP and cGMP are able to revert the death (DNA fragmentation) profile generated by amphotericin B and again cGMP was most effective in this action (Figure 4).

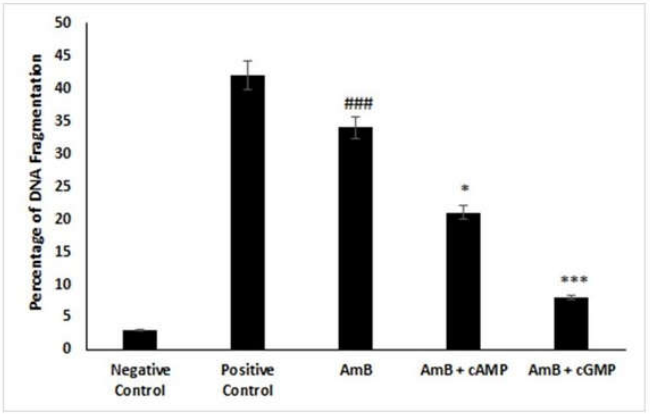

Figure 4 Effect of amphotericin B on DNA fragmentation in MDCK cells line. A flow cytometric DNA fragmentation assay was employed as a quantitative measure of cell death incubated with [Forskolin (10 µM) – Positive control], amphotericin B (4.0 µg/mL), cAMP (10^{-3} M) and cGMP (10^{-3} M). The results represent the mean ± standard deviation (SD) of the results of six independent experiments performed in sextuplicate. ### $p<0.001$ when compared with the negative control group (untreated cells), *$p<0.05$ and ***$p<0.001$ when compared with AmB.

DISCUSSION

Cyclic nucleotides are recognized as critical mediators of many renal functions, including solute transport, regulation of vascular tone, proliferation of parenchymal cells and inflammation (Cheng and Grande 2007). Activated cell surface receptors transduce their signals via intracellular second messengers such as cAMP and cGMP. Cyclic nucleotide signal transduction pathways are an emerging research field in kidney disease (Shen *et al.* 2016).

Nephrotoxicity is a poisonous effect of some substances, both toxic chemicals and medications on renal function. There are various forms, and some drugs may affect renal function in more than one way. Nephrotoxins are substances displaying nephrotoxicity (Barnett and Cummings 2018).

Amphotericin B is the gold standard for antifungal treatment for the most severe mycoses. However, adverse effects are common, with nephrotoxicity being the most serious, occurring early in the course of treatment, and usually being reversible in most patients. Tubular damage is a well-known problem associated with amphotericin B therapy but acute renal failure is the most serious complication (Baginski and Czub 2009, Laniado-Laborin and Cabrales-Vargas 2009).

Inflammation plays a central role in the pathogenesis of drug-induced kidney injury. Renal tubular epithelium is a major site of cell injury and death during nephrotoxicity. Studies have suggested that renal tubular epithelial cells play a proinflammatory role in nephrotoxicity (Kayama *et al.* 1997). In addition, amphotericin B stimulates both the transcription and production of pro-inflammatory cytokines, such as TNF-α (Mukherjee *et al.* 2010) and IL-6 (Chai *et al.* 2013).

Our results showed an increase of IL-6 production by amphotericin B (Figure 1.A). In particular, IL-6 has received

considerable attention as it appears at high concentrations to promote the progression of renal disease while at lower levels may be involved in regulating repair mechanisms. In the kidney, IL-6 is involved in mesangial proliferative nephritis (Horii *et al.* 1989), as well as renal tubular epithelial cell regeneration (Kayama *et al.* 1996).

The significative inhibitory effect was observed when cells were incubated with amphotericin B plus cAMP (Figure 1.A). Addition of cAMP elevating agents to immunologically-stimulated cells can have important regulatory effects on cytokine production. For example, elevation of cAMP down-regulates IL-6 production (Zitnik *et al.* 1993). Renewed interest in the use of cAMP-elevating compounds as a therapeutic approach to certain inflammatory disorders has arisen from studies demonstrating the ability of cAMP to downregulate IL-6 production in endotoxin-stimulated lung fibroblasts (Zitnik *et al.* 1993). Our results also showed that cGMP was unable to alter IL-6 production when compared to amphotericin B and LPS (Figure 1.A). TNF-α and LPS, but not IL-6, increased inducible nitric oxide synthase (iNOS) expression and cGMP production in vascular smooth muscle cells (Browner *et al.* 2004).

When we analyzed the results with TNF-α, we see a different profile. Our results showed that amphotericin B stimulates TNF-α production similar to basal conditions (MDCK plus DMEN) (Figure 1.B). cAMP is also able to produce TNF-α only up to the basal level similar to amphotericin B (Figure 1.B). Literature results showed that the production of IL-6 proved to be dependent on the PKA pathway and TNF-α was not influenced by the inhibition of the pathway (França *et al.* 2014). In contrast, cGMP was able to revert the production of TNF-α obtaining values similar to those found by LPS (Figure I.B). cGMP is an important second messenger that modulates various physiological functions, such as vasodilatation, inflammation and apoptosis, in diverse organ and cell types. TNF-α is largely implicated in renal inflammation and glomerular damage induced by immune complex deposition. TNF-α exacerbates proteinuria and accelerates the course of glomerular injury in experimental models of nephritis (Ernandez and Mayadas 2009).

In this context, when analyzing the inflammatory process, it is of fundamental importance to analyze the anti-inflammatory mechanisms. The net inflammatory response entails a coordinated balance between the proinflammatory and counter-inflammatory mediators; a relative exuberance of the former predisposes to tissue injury (Chai *et al.* 2013). Our results showed that amphotericin B decreases IL-10 production but that incubation with cAMP and cGMP was able to reverse this inhibition in MDCK cell lines (Figure 1.C). Findings from literature have shown that IL-10 gene expression and IL-10-induced signaling pathways have an important role in the regulation and maintenance of normal renal function. Moreover, accumulating evidence further demonstrates that abnormal IL-10 expression, whether transient or prolonged, as well as interactions with other growth factors as a response to diverse stimuli, is linked to the appearance and progression of a variety of kidney disorders. Thus, it has been suggested that the selective targeting of IL-10 expression and IL-10-related pathways may provide therapeutic approaches for many kidney diseases (Summers *et al.* 2011, Xu *et al.* 2011, Sinuani *et al.* 2013).

Works of literature have been reporting that IRF-1 is an early critical proinflammatory signal stimulated by ROS during ischemic injury *in vitro* and *in vivo* (Akcay *et al.* 2009, Wang *et al.* 2009). The antimicrobial and immunological modes of action of certain drugs involve cellular oxidative stress response. Thus, these types of drugs could be defined as oxidative stress drugs (Kim *et al.* 2012). Examples include amphotericin B (AmB) (Sokol-Anderson *et al.* 1986, Jukic *et al.* 2017). 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 B fungicidally.

Our results showed that both H_2O_2 and amphotericin B (AmB) were able to induced ROS generation in MDCK cells (Figure 2). 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). It has been shown that AmB induces the intracellular accumulation of ROS (Sokol *et al.* 1986, Phillips *et al.* 2003, Sangalli-Leite *et al.* 2011, Belenky *et al.* 2013). The addition of free radical scavengers, such as catalase and/or superoxide dismutase, protects *Candida albicans* protoplasts from AmB (Sokol *et al.* 1986). Furthermore, a genome-wide expression analysis confirmed that AmB not only affects the expression of genes involved in ergosterol synthesis but also promotes the expression of stress genes (Liu *et al.* 2005, Nett and Andes 2016). AmB has immunomodulatory properties in mammalian cells, which have been shown to be related to its toxicity and proinflammatory responses (Ben-Ami *et al.* 2008, Mesa-Arango *et al.* 2012).

cAMP and cGMP were able to reduce ROS production in MDCK cells. (Figure 2). MDCK cells treated with cAMP-derivative inhibits the upregulation of α-SMA via Epac. Therefore, Epac acts antifibrotically via inhibition of profibrotic TGFβ signaling (Insel *et al.* 2012). A more recent study demonstrated that Epac/Rap stimulation ROS production in the kidney (Stokman *et al.* 2014). Uncontrolled production of ROS mediated cellular injury and also occurred during renal fibrosis (Dendooven *et al.* 2011). Thus, activation of Epac/Rap signaling may protect against renal fibrosis. Studies from our laboratory have shown that cAMP protects MDCK cell line against amphotericin B toxicity in a PKA-independent manner (Ferreira *et al.* 2016). MDCK cells also show a greater reduction in the production of ROS in the presence of cGMP (Figure 2). Many studies report that enhanced cGMP levels have an effective antifibrotic benefit in various organs including the kidney (Wang *et al.* 2006, Yamaleyeva *et al.* 2012). Organic nitrates are used for the treatment of cardiovascular disease for the last centuries, as they increase NO availability and thereby support the NO/cGMP signaling pathway. However, this therapy option is limited due to formation of nitrate tolerance as well as accumulation of ROS (e.g., peroxynitrite) under oxidative stress conditions (Peters *et al.* 2000, 2003).

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 NO 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). Our results showed that amphotericin B decreases significantly NO production in MDCK cell line (Figure 3). cAMP and cGMP increase NO production by reversing the effect generated by amphotericin B (Figure 3). NO effects were mediated principally through cGMP-related mechanisms (Ignarro 1991, Murad 1994) either through activation of cGMP-specific PDE or of protein kinase G and/or of phosphatases (Méry *et al.* 1991, Browning *et al.* 1999). However, this concept has subsequently been challenged, since it was unlikely to explain by a common mechanism the multiplicity of NO actions. In other tissues (cardiac myocytes and kidney), NO causes an elevation of the intracellular levels of cAMP due to a cGMP-dependent inhibition of cAMP hydrolysis by cGMP-inhibited PDE. These observations do not rule out the possibility of cAMP being increased by a direct modulation of the adenylyl cyclase/cAMP-dependent PK-A pathway by NO, as recently demonstrated in Human spermatozoa (Quinchia and Maya 2017).

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, NO and derivatives, endotoxin-induced inflammation, photodynamic therapy, ultraviolet-A and ionizing radiations and cigarette smoke (Ryter *et al.* 2007).

Given these results our next step was to verify whether the generation of inflammation and oxidative stress (ROS/RNS balance) could be contributing to the generation of DNA fragmentation caused by amphotericin B and the influence of cyclic nucleotides (cAMP and cGMP) in this process.

The increased fragmentation of DNA observed in flow cytometry (Figure 4) can be interpreted as cell death (Nicoletti *et al.* 1991). Therefore, it can be concluded the nephrotoxic drugs caused cell death in the MDCK cell line, 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).

MDCK cells presented a higher percentage of cell death (DNA fragmentation) and showed themselves to be sensitive to the toxic effects of amphotericin B (Figure 4). Oxidative DNA damage can be induced by oxygen radicals, various oxidizing agents, photo-oxidation and ionizing radiations. ROS induced DNA damage results in generation of apurinic/apyrimidinic (AP) sites, single/double strand breaks, thymine glycol and oxidatively generated clustered DNA lesions (Cadet *et al.* 2012). Oxidatively induced lesions in DNA (possibly due to ionizing radiations) results in chromosome breakage leading to mutation and genomic instability, nephropathies and cancer (Hemnani and Parihar 1998). In contrast, inflammation to be important in both, initial and further promoted cell alteration by inflammation related release of ROS, chemokines and pro-angiogenetic factors (Seifart *et al.* 2005).

Our results showed that both cAMP and cGMP were able to decrease the fragmentation of the DNA generated by amphotericin B. NO reduces pulmonary vascular resistance by stimulating soluble guanylyl cyclase (sGC), thereby increasing cGMP levels and stimulating cGMP-dependent protein kinase (PKG) in pulmonary vascular smooth muscle, and several therapies have emerged that target this pathway (Runo and Loyd 2003, Michelakis 2003). In addition to regulating vascular tone, NO and cGMP signaling can also inhibit proliferation and induce apoptosis in vascular smooth muscle cells (SMCs) (Garg and Hassid 1989, Lee *et al.* 1996, Pollman *et al.* 1996, Chiche *et al.* 1998). The mechanisms involved are still unclear; however, some reports have suggested that the antiproliferative responses to NO and cGMP in systemic vascular SMCs are dependent on cross-talk with the cAMP signaling pathway (Wharton *et al.* 2005).

CONCLUSION

Our findings open new perspectives in the clinical management of invasive fungal diseases. The alternative contributory intracellular mechanism of action of AmB should be considered in the rationale of combined therapy. Combinations of AmB and cAMP and cGMP can be based on that additional effect, as therapy can target both the membrane and the oxidative burst of the fungal cells. In addition, the induction of oxidative stress and the accumulation of free radicals are universal effects of AmB, which provides an explanation of the near absence of secondary resistance to this antifungal.

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