

## Prevention of amphotericin B nephrotoxicity through use of phytotherapeutic medication\*

Prevenção da nefrotoxicidade da anfotericina B por meio do uso de fitomedicamentos  
Prevención de la nefrotoxicidad por anfotericina B utilizando fito

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### ABSTRACT

**Objective:** To evaluate the effect of diosmin and hesperidin flavonoids in the prevention of amphotericin B nephrotoxicity, through an experimental model on rats. **Method:** Adult, male Wistar rats were distributed into the following groups: saline; diosmin hesperidin (animals that received 50 mg/kg of diosmin hesperidin, drinking water, for ten days); amphotericin B (animals that received 15 mg/kg/day of amphotericin B through intraperitoneal treatment for five days); amphotericin B+diosmin hesperidin. Renal function, fractional excretion of sodium; potassium and magnesium and oxidative metabolites were evaluated. **Results:** Treatment with amphotericin B reduced renal function, as shown by the clearance of creatinine, increased tubular function markers and fractional excretion of sodium, potassium, magnesium and oxidative metabolites. Pre-treatment with diosmin hesperidin ameliorated clearance of creatinine and reduced tubular and oxidative injury. **Conclusion:** Administration of amphotericin B resulted in reduction of renal function with tubular injury, and diosmin hesperidin showing an antioxidant protective effect on the kidneys.

### DESCRIPTORS

Antioxidants; Amphotericin B; Diosmin; Hesperidin; Nursing Care; Patient Safety.

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## INTRODUCTION

In 2004, the World Health organization launched the World Alliance for Patient Safety, with the objective of raising awareness and increasing political engagement for improving patient safety during healthcare<sup>(1)</sup>.

In Brazil, the governmental agency for patient safety, ANVISA (Agência Nacional de Vigilância Sanitária – the National Agency for Sanitary Care), promotes activities aimed at improving patient safety and the quality of health services. One of ANVISA's pillars is the safe use of injectable and immunobiological drugs, in which nurses are fully involved<sup>(2)</sup>.

Multiple pharmacological agents are used for health-care, treatment and diagnosis of hospitalized patients. Adverse effects are common<sup>(3)</sup>. An adverse reaction to drugs is understood to mean any negative or unwanted effect that arises after the administration of a normal dosage of the medication as used in clinical practice. This situation inserts nephrotoxicity induced by antimicrobial, chemotherapeutic, analgesic and immunosuppressive agents. This list should be added by diagnostic agents such as iodine and gadolinium radiocontrasts<sup>(3)</sup>.

This context stands out the use of amphotericin B (Amp-B). Amp-B is a polyenic antibiotic and antifungal, that is the first treatment option for fungemia in critical patients, particularly the candidemia, due to its broad spectrum of activity and low cost<sup>(4)</sup>. Although widely used, it is well known that the treatment should be carefully monitored due to the high incidence of adverse reactions including nausea, vomiting, fever, hypertension or hypotension, hypoxia and more serious issues such as acute kidney injury (AKI)<sup>(2)</sup>.

Nephrotoxicity caused by Amp-B is very common. This mechanism includes the accumulation of medication in renal tubules, once that 25% of cardiac output is dedicated to supporting renal blood flow, added the excretory capacity of the organ<sup>(3)</sup>. High dosage of Amp-B have been shown in a dose-dependent adverse effect. In addition, it has been reported that approximately 80% of patients with systemic fungal infections treated by Amp-B have shown an increase in serum creatinine levels, while between 40 and 60% of patients have doubled their serum creatinine levels and 15% of patients have developed renal dysfunction and requirement of renal replacement therapy (dialysis)<sup>(4)</sup>.

Renal toxicity mechanism of Amp-B is the result of vasoconstriction of afferent arterioles and the activation of tubuloglomerular feedback system, which induce reduction of renal blood flow, decline of the glomerular filtration rate and increased urea and creatinine serum levels<sup>(4-5)</sup>. Hypoxia and ischemia in endothelial and epithelial tubule cells lead to a rise in inflammatory mediators and generation of reactive oxygen species (ROS). The ROSs are represented by the superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH^{\cdot}$ ). Physiologically, the ROSs are continually formed as a product of the cell metabolism and they act as scavengers of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalysis and

glutathione peroxidase<sup>(6)</sup>. Nephrotoxicity mechanism of Amp-B leads to excessive production of ROSs and a reduction of the antioxidant capacity, leading to a predominance of free radicals that encourage lipid peroxidation in cell membrane, proteins and DNA oxidation, characterizing the process of oxidative injury and cell death<sup>(6)</sup>.

Several therapeutic procedures, including drugs with low or no toxicity, are used with the objective of restoring renal blood flow, and reducing inflammation and oxidative stress in the injuries caused by nephrotoxicity<sup>(7-9)</sup>. Since the 1930s, the flavonoids diosmin and hesperidin have been used to treat for vascular insufficiency<sup>(8)</sup>. Diosmin and hesperidin are also recognized for their antioxidant and anti-inflammatory properties<sup>(8-9)</sup>. The anti-inflammatory effect of diosmin and hesperidin is attributed to the suppression of  $E_2$  prostaglandins synthesis and inhibition of adhesive endothelial and leukocyte molecules. These flavonoids also act by ROS scavenging and chelants free iron molecule, confirming its antioxidant effect<sup>(8-9)</sup>.

The challenge of reducing the toxicity of drugs that have no clinical substitutes has given rise to a large variety of studies to identify procedural or pharmacological solutions. The incorporation of flavonoids, such as diosmin and hesperidin, has been explored by the scientific community as a preventive alternative therapy for AKI secondary to the use of Amp-B in order to contribute with its epidemiology. The hypothesis of this study is that diosmin and hesperidin demonstrate an antioxidant renoprotective effect in the nephrotoxic renal injury.

The high doses and long treatment period of Amp-B during care result in alarming rates of nephrotoxicity. The search for an alternative to the therapeutic intervention that shows promising results in terms of the development and intensification of AKI gives rise to opportunities for new studies that report data that will have an impact on improved care protocols, aimed at critical patients who use nephrotoxic medication. The study aims to evaluate the renoprotective effect of the flavonoids diosmin and hesperidin in the prevention of nephrotoxicity from Amp-B using an experimental model on rats.

## METHOD

The procedures developed in this study abide by the Ethical Principles in Animal Experimentation adopted by the Brazilian College for Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal). The study was approved by the Ethics Committee for Animal Use (CEUA – Comitê de Ética em Uso de Animais) at the Instituto de Ciências Biológicas of the Universidade de São Paulo (CEUA-ICB USP), under protocol number 61, sheet 129, book 02/12.

**Animals:** Male, Wistar rats were used, weighing between 250 and 350 g. The animals were housed in collective cages, with free access to water and feed, and temperature cycles alternating between day and night. The animals were distributed into the following groups: **Saline** (control, n=8): these animals received 3 ml/kg of 0.9% physiological solution through an intraperitoneal (IP) injection, once a day,

for five days; **diosmin hesperidin** (DH, n=9): the animals received 50 mg/kg of diosmin/hesperidin in drinking water for ten days; **amphotericin B** (Amp-B, n=6): the animals received 15 mg/kg of amphotericin B through IP once a day for five days; **amphotericin B and diosmin hesperidin** (Amp-B+DH, n=12): the animals were pre-medicated with a diosmin/hesperidin solution in their drinking water over a ten day period, and from the sixth day of the experiment, they also began to receive Amp-B through a single dose IP injection over a period of five days.

**Metabolic cage:** at the end of the period of experimentation, the animals were put inside individual metabolic cages for urine to be collected over a 24 hour period and for subsequent evaluation of renal function and oxidative metabolites.

**Total blood collection:** the total blood collection was carried out by puncturing the abdominal aorta, with the rats under a general anesthetic induced through 70-100 mg/kg of sodium thiopental (Thiopentax®, Cristália) through IP injection. The corpses of the animals that underwent euthanasia were wrapped and cooled in a freezer before being discarded.

**Renal function:** renal function was evaluated through creatinine clearance. The Jaffe colorimetric method was used to determine the creatinine levels in blood and urine. The creatinine clearance was calculated using the formula: creatinine clearance=urinary creatinine x urinary flow in 24h/serum creatinine<sup>(10)</sup>.

**Fractional excretion of sodium:** The sodium values in blood and urine were determined by means of the ion-selective electrode method, using the Architect® CI8200 (Abbot) biochemical analyzer. The fractional excretion of sodium (FENa) was calculated using the formula: FENa=urinary sodium x serum creatinine/serum sodium x urinary creatinine x 100<sup>(11)</sup>.

**Fractional excretion of potassium:** the potassium values in urine and blood were determined by means of the ion-selective electrode method, using the Architect® CI8200 (Abbot) biochemical analyzer. The fractional ex-

cretion of potassium (FEK) was calculated using the formula: FEK=urinary potassium x serum creatinine/serum potassium x urinary creatinine x 100<sup>(11)</sup>.

**Fractional excretion of magnesium:** the magnesium values in urine and blood were determined by means of the ion-selective electrode method, using the Architect® CI8200 (Abbot) biochemical analyzer. The fractional excretion of magnesium (FEMg) was calculated using the formula: FEMg=urinary magnesium x serum creatinine/serum magnesium x urinary creatinine x 100<sup>(11)</sup>.

**Urinary peroxides:** these were measured using the FOX-2 method, with ferrous xylene orange which oxidizes with the ion Fe<sup>2+</sup> and produces a complex that is blue/purple in color ( $\alpha=4,3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ )<sup>(12)</sup>.

**Urinary TBARS:** urinary TBARS (thiobarbituric acid reactive substances) make it possible to evaluate the final products of the chain reaction of lipid peroxidation, which reacts in the presence of thiobarbituric acid in organic fluids ( $\alpha=1,56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ )<sup>(13)</sup>.

**Statistics:** the variance between groups was analyzed by means of the one-way analysis of variance (ANOVA) test, followed by the Kruskal-Wallis post-test for variance analysis, and then by the Steel-Dwass-Critchlow-Fligner test for comparison in pairs using the statistic program GraphPad Prism version-3 for Windows®. P values that were greater than 0.05 were considered significant.

## RESULTS

### RENAL FUNCTION

Table 1 shows that the rats treated with Amp-B showed a significant increase in urinary flow and serum creatinine, with a resulting reduction in urinary creatinine and creatinine clearance ( $p<0.05$ ). On the other hand, the pre-conditioning with diosmin and hesperidin in animals treated with Amp-B showed a significant reduction in the creatinine serum levels, resulting in increased clearance of creatinine ( $p<0.05$ ).

**Table 1** - Results referring to global renal function – Sao Paulo city, Brazil, 2015.

Groups	n	Urinary flow (ml/min)	Urinary Cr (mg/dL)	Serum Cr (mg/dL)	Crcl/100 g (ml/min)
Saline	8	0.01±0.002	70.65±18.87	0.25±0.08	0.85±0.08
DH	9	0.01±0.005	92.82±26.22	0.29±0.05	0.83±0.09
Amp-B	5	0.03±0.007 <sup>ab</sup>	24.72±10.78 <sup>ab</sup>	0.60±0.18 <sup>ab</sup>	0.22±0.07 <sup>ab</sup>
Amp-B+DH	12	0.03±0.007 <sup>ab</sup>	26.93±7.38 <sup>ab</sup>	0.42±0.11 <sup>abc</sup>	0.46±0.08 <sup>abc</sup>

Cr: creatinine; Crcl: creatinine clearance; DH: diosmin hesperidin; Amp-B: amphotericin B.

<sup>a</sup>p<0.05 vs Saline.

<sup>b</sup>p<0.05 vs DH.

<sup>c</sup>p<0.05 vs Amp-B.

Note: numbers are media +/- standard deviation.

### FRACTIONAL EXCRETION OF SODIUM, POTASSIUM AND MAGNESIUM

Table 2 presents data for the fractional excretion of sodium, potassium and magnesium. The animals treated with Amp-B showed a significant increase in the fraction-

al excretion of sodium, potassium and magnesium, when compared with the Saline and DH groups ( $p<0.05$ ). In addition, the pre-conditioning with diosmin and hesperidin meant that it was possible to reduce the values for all excretion fractions ( $p<0.05$ ).

**Table 2** - Results of the fractional excretion of sodium, potassium and magnesium – Sao Paulo city, Brazil, 2015.

Groups	n	FENa (%)	FEK (%)	FEMg (%)
Saline	8	0.32±0.05	21.13±7.61	2.04±1.80
DH	9	0.30±0.07	21.32±7.44	3.02±2.62
Amp-B	5	0.64±0.35 <sup>ab</sup>	38.16±11.48 <sup>ab</sup>	11.14±3.45 <sup>ab</sup>
Amp-B+DH	12	0.34±0.19 <sup>c</sup>	22.09±18.26 <sup>c</sup>	7.03±3.92 <sup>abc</sup>

FENa: fractional excretion of sodium; FEK: fractional excretion of potassium; FEMg: fractional excretion of magnesium; DH: diosmin hesperidin; Amp-B: amphotericin B.

<sup>a</sup>p<0.05 vs Saline.

<sup>b</sup>p<0.05 vs DH.

<sup>c</sup>p<0.05 vs Amp-B.

Note: numbers are media +/- standard deviation.

## PEROXIDES AND URINARY TBARS

Table 3 shows the oxidative metabolic dosage of different groups. The Amp-B group showed increased excretion of urinary peroxides, compared with the saline and DH groups (p<0.05). In addition the Amp-B+DH group displayed a significant reduction in urinary peroxides in relation to the Amp-B group (p<0.05).

With regard to the urinary TBARS, the Amp-B group was found to have greater levels of this metabolite compared with the saline and DH groups (p<0.05). The pre-conditioning with diosmin and hesperidin significantly reduced the levels of TBARS in the rats treated with Amp-B, compared with the Amp-B group (p<0.05).

**Table 3** - Results relating to the oxidative metabolites – Sao Paulo city, Brazil, 2015.

Groups	n	Urinary peroxides (nmol/g de UCr)	Urinary TBARS (nmol/g de UCr)
Saline	8	7.1±3.6	0.009±0.003
DH	9	5.7±1.9	0.008±0.002
Amp-B	5	10.8±4.5 <sup>b</sup>	0.044±0.034 <sup>ab</sup>
Amp-B+DH	12	7.4±2.5 <sup>c</sup>	0.018±0.007 <sup>abc</sup>

UCr: urinary creatinine; TBARS: thiobarbituric acid reactive substances; DH: diosmin hesperidin; Amp-B: amphotericin B.

<sup>a</sup>p<0.05 vs Saline.

<sup>b</sup>p<0.05 vs DH.

<sup>c</sup>p<0.05 vs Amp-B.

Note: numbers are media +/- standard deviation.

## DISCUSSION

The search for strategies to reduce the harmful effects of medication has encouraged the development of studies that provide concrete evidence that can be included in clinical application and in healthcare protocols managed by the entire multi-professional team. In this context, the nurse is of particular importance since he or she is the staff member that is most responsible for the administration of medication. This study confirms the nephrotoxic effect of amphotericin B, and confirms that the simultaneous use of

a phytotherapeutic medicines with an antioxidant effect can act as a preventive alternative.

In this investigation, rats treated with Amp-B were found to show an increase in urinary flow and serum creatinine, which resulted in a reduction of creatinine clearance and tubular dysfunction, confirmed by the increase of the fractional excretion of sodium, potassium and magnesium. The analysis of oxidative metabolites, evaluated in order to understand their role in oxidation as a possible cause of injury, confirmed the increase in levels of urinary peroxides and the presence of lipid peroxidation, as demonstrated through urinary TBARS. The administration of diosmin and hesperidin in rats with AKI, induced through Amp-B, showed an increase in the glomerular filtration rate, as evidenced by the increase in creatinine clearance, as well as a reduction in tubular dysfunction and the antioxidant protective effective, confirmed by the reduction in oxidative metabolites.

Amp-B is an antifungal agent used conventionally and routinely. It is considered as the gold standard and it is very used in the treatment of many invasive fungal infections, principally due to its low cost and effectiveness in treatment. The main cause for reduced dosage or interrupting treatment with Amp-B is the occurrence of adverse effect, among which, one of the most significant is nephrotoxicity<sup>(4-5)</sup>. This study found that nephrotoxicity that had been induced by Amp-B through the administration of 15 mg/kg over five days with presumed endothelial and tubular dysfunction.

In the clinical context, the first signs of direct toxicity occur minutes after the Amp-B infusion, when a local increase in vasoconstrictor mediators such as endothelin, leukocytes and adenosine, that induce vasoconstriction with reduced renal blood flow in the endothelial cells in the renal microvasculature<sup>(14)</sup>.

The presence of Amp-B in renal tubules results in the formation of aqueous pores in the plasma membrane, which encourages an influx of protons to the inside of the cell and, as a result, tubular acidification occurs through a reduction in the pH<sup>(15)</sup>. The vasoconstriction mechanism, along with the tubular acidification, induces the tubular injury, which may be characterized through the transmigration of proteins located in the cell membrane, in particular the Na/KATPase located on the basolateral side, which changes its location to the apical side. This injury mechanism leads to the loss of polarity of the membrane and characterizes the tubular dysfunction with the induction of the distal tubular feedback, whereby the cells in the tubular distal lose their capacity to concentrate urine and reabsorption of electrolytes such as calcium and magnesium<sup>(3, 16)</sup>. In this way, the polyuria shown by the animals that receive Amp-B occurred as a response to the inhibition of the signalize to reabsorb water in the collector duct associated to the high concentration of electrolytes, which activated the tubuloglomerular feedback and intensified the vasoconstriction mechanism, leading to hypoxia and renal ischemia<sup>(3,17)</sup>.

The clinical signs that characterize nephrotoxicity from Amp-B are acidosis, hypocalcemia, depletion of magnesium and sodium, and polyuria<sup>(4,15)</sup>, which can be described as adverse effect related to the medication. According to the concepts of quality and safety in healthcare, adverse reactions to medication are described as most commonly result of errors in medication – and are also considered as preventable causes during the process of drugs administration<sup>(18)</sup>. Preventive care is recommended before, during and after the administration of therapeutic medication and diagnostic agents that may lead to nephrotoxicity. The care process begins with the identification of patients that are at risk (the elderly, those with chronic diseases such as diabetes, chronic kidney disease, heart failure and sepsis), which involves actions such as accompanying and monitoring serum creatinine, considering the kidney basal function at the start of and during therapy, adjustment of the dosage of medication to kidney function (the creatinine clearance estimate is calculated using the Modification of Diet in Renal Disease – MDRD, or the Cockcroft-Gault) and the reduction of association of nephrotoxic drugs whenever possible. Adequate hydration should also be considered for the maintenance of the kidney perfusion, with the aim of preventing renal toxicity caused by the medication<sup>(19)</sup>.

On the other hand, there are numerous agents that serve to protect kidneys and aim to prevent renal hypoperfusion, prolonged hypoxia and the loss of electrolytes in the kidney cells in the model of nephrotoxicity caused by medications. In this study, treatment with the flavonoids diosmin and hesperidin increased the glomerular filtration rate and encouraged a reduction in the fractional excretion of sodium, potassium and magnesium of the animals that experienced AKI due to Amp-B, showing the additional effect of diosmin and hesperidin in the prevention of electrolytic disorders. In addition to the electrolytic alterations and those effecting renal function,

the animals that were treated with Amp-B showed an increase in the levels of peroxides and urinary TBARS. The liberation and high concentration of oxidative metabolites damaged the plasmatic and mitochondrial membranes, affecting the protein function and inhibiting the proliferation of cell repair. The pre-conditioning of animals with diosmin and hesperidin showed a reduction in oxidative damage. Other studies have also confirmed the antioxidant protection of this flavonoid as one that scavengers free radicals in models of toxic hepatotoxicity and cerebral ischemia<sup>(20,21)</sup>.

To summarize, this study demonstrated the vulnerability of the renal system when it receives nephrotoxic medication such as Amp-B. In this way, several preventive measures are recommended with the objective of reducing adverse effect associated with Amp-B, among which different lipid formulations have already been described, along with hydration protocols or infusions over long periods<sup>(4,18-19)</sup>. Nonetheless, these strategies have not yet reduced the undesirable reality of nephrotoxicity through Amp-B. In this study, the effect of diosmin and hesperidin, a phytotherapeutic medication with an antioxidant effect and without related adverse effects, proved to be a safe pharmacological method that is a viable preventive measure against the toxic effect of Amp-B on the kidneys.

## CONCLUSION

The administration of Amp-B resulted in the decline of kidney function and tubular injury with the involvement of ROSs in the injury mechanism. The preconditioning with diosmin and hesperidin proved to have an antioxidant protective effect on the kidneys, reflected in the increased glomerular filtration rate, reduced tubular dysfunction and a reduction in the liberation of oxidative metabolites in the urine, which can be considered preventive measures against nephrotoxicity from Amp-B.

## RESUMO

**Objetivo:** Avaliar ação renoprotetora dos flavonoides diosmina e hesperidina na prevenção da nefrotoxicidade da anfotericina B em modelo experimental com ratos. **Método:** Ratos Wistar, adultos, machos foram distribuídos nos seguintes grupos: Salina; diosmina hesperidina (animais receberam 50 mg/kg de diosmina hesperidina em água de bebedouro por dez dias); Anfotericina B (animais receberam 15 mg/kg/dia de anfotericina B intraperitoneal por cinco dias); Anfotericina B+diosmina hesperidina. Foram avaliados função renal, fração de excreção de sódio, potássio e magnésio e os metabólitos oxidativos. **Resultados:** O tratamento com anfotericina B reduziu a função renal, vista pelo *clearance* de creatinina, elevou os marcadores de função tubular como a fração de excreção de sódio, potássio, magnésio e dos metabólitos oxidativos. O pré-condicionamento com diosmina hesperidina elevou o *clearance* de creatinina e atenuou da lesão tubular e oxidativa. **Conclusão:** A administração de anfotericina B resultou no declínio da função renal com lesão tubular e a diosmina hesperidina demonstrou efeito renoprotetor antioxidante.

## DESCRIPTORIOS

Antioxidantes; Anfotericina B; Diosmina; Hesperidina; Cuidados de Enfermagem; Segurança do Paciente.

## RESUMEN

**Objetivo:** Evaluar la acción renoprotetora de flavonoides, diosmina y hesperidina en la prevención de la nefrotoxicidad de Anfotericina B en un modelo experimental de animales. **Método:** Ratones Wistar, adultos y machos distribuidos en los grupos: Salina (control); Diosmina Hesperidina (50 mg/kg de diosmina hesperidina en agua, durante diez días); Anfotericina B (15 mg/kg de anfotericina B intraperitoneal durante cinco días); Anfotericina B+Diosmina Hesperidina. Función renal, la excreción fraccional de sodio, potasio en magnésio en los metabólitos oxidativos se realizaron. **Resultado:** El tratamiento con anfotericina B reduce el *clearance* de creatinina, aumento de la fracción de excreción de sodio, potasio, magnesio y metabólitos oxidativos. El pretratamiento

con hesperidina diosmina aumentó el aclaramiento de creatinina y la atenuación del daño tubular y oxidativa. **Conclusión:** La administración de anfotericina B dio como resultado la disminución de la función renal con lesión tubular y la diosmina hesperidina demostró efecto renoprotector antioxidante.

## DESCRIPTORES

Antioxidantes; Anfotericina B; Diosmina; Hesperidina; Atención de Enfermería; Seguridad del Paciente.

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