Original Article

Renoprotective effects of wine flavonoids in nephrotoxicity of the immunosuppressant Tacrolimus*

Efeito renoprotetor dos flavonoides do vinho na nefrotoxicidade do imunossupressor Tacrolimus

Efecto renoprotector de los flavonoides del vino en la nefrotoxicidad del inmunosupresor Tacrolimus

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ABSTRACT

Objective: To investigate the renoprotective effects of the extract of Vitis vinifera L in the Tacrolimus-induced nephrotoxicity in experimental studies with rats. **Methods:** Quantitative survey with an experimental model in which adult male, Wistar rats, weighing between 250g - 300g, were used. The rats were treated once a day for cincodias by gavage as follows: Saline (control, sodium chloride, 0.1 ml); Vitis (Vitis vinifera L 3mg/kg), FK (0.5 mg / kg); and, FK + Vitis (0.5 mg / kg + 3 mg / kg, respectively). Renal function was assessed by creatinine clearance (CrCL / 100g, Jaffe method) and lipid peroxidation by measurement of urinary peroxides (PU, FOX-2). **Results:** The administration of FK increased the excretion of peroxides and reduced creatinine clearance, and simultaneous administration with Vitis vinifera L protected the renal function in these parameters. **Conclusion:** These data confirm the injury induced by nephrotoxic Tacrolimus and demonstrated the renoprotective effect of Vitis vinifera L.

Keywords: Acute kidney injury/chemically induced; Immunosuppressive agents/toxicity; *Vitis vinifera L* ; Rats, Wistar

RESUMO

Objetivo: Verificar o efeito renoprotetor do extrato de *Vitis vinifera L* na nefrotoxicidade induzida pelo Tacrolimus em estudos experimentais com ratos. **Métodos:** Pesquisa quantitativa com modelo experimental, na qual foram utilizados ratos Wistar, machos, adultos, pesando entre 250g – 300g, tratados uma vez ao dia por cincodias, por gavagem, conforme segue: Salina (controle, cloreto de sódio 0,1ml); Vitis (*Vitis vinifera L* 3mg/kg), FK (0,5mg/kg) e FK+Vitis (0,5mg/kg + 3mg/kg, respectivamente) . A função renal foi avaliada por meio do clearance de creatinina (Clcr/ 100g, método Jaffé) e a peroxidação lipídica pela mensuração de peróxidos urinários (PU, FOX-2). **Resultados:** A administração de FK elevou a excreção de peróxidos e reduziu o clearance de creatinina, e a administração simultânea com *Vitis vinifera L* protegeu a função renal nesses parâmetros. **Conclusão:** Os dados confirmaram a lesão nefrotóxica induzida pelo Tacrolimus e demonstraram o efeito renoprotetor do *Vitis Vinifera L*.

Descritores: Lesão renal aguda/induzido quimicamente; Imunossupressores/toxicidade; *Vitis vinifera* L; Ratos Wistar

RESUMEN

Objetivo: Verificar el efecto renoprotector del extracto de *Vitis vinifera L* en la nefrotoxicidad inducida por el Tacrolimus en estudios experimentales con ratas. **Métodos:** Investigación cuantitativa con modelo experimental, en la cual fueron utilizadas ratas Wistar, machos, adultos, que pesaban entre 250g – 300g, tratados una vez al dia por cinco días, por alimentación forzada, conforme sigue: Salina (control, cloreto de sodio 0,1ml); Vitis (*Vitis vinifera L* 3mg/kg), FK (0,5mg/kg) e FK+Vitis (0,5mg/kg + 3mg/kg, respectivamente) . La función renal fue evaluada por medio del clearance de creatinina (Clcr/ 100g, método Jaffé) y la peroxidación lipídica por la mensuración de peróxidos urinarios (PU, FOX-2). **Resultados:** La administración de FK elevó la excreción de peróxidos y redujo el clearance de creatinina, y la administración simultánea con *Vitis vinifera L* protegió la función renal en esos parámetros. **Conclusión:** Los datos confirmaron la lesión nefrotóxica inducida por el Tacrolimus y demostraron el efecto renoprotector del *Vitis Vinifera L*.

Descriptores: Lesión renal aguda/inducido químicamente; Agentes inmunosupresores/toxicidad; *Vitis vinifera L*; Ratas Wistar

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INTRODUCTION

In recent years, the number of organ transplants has grown significantly, so that the targets proposed for 2009 exceeded expectations. The aim was to reach 4,000 kidney and 1,300 liver transplants. In this year, 4,259 kidney and 1,658 liver transplants were performed, these being the best results so far obtained in the country[1]. Despite this fact, there are a huge number of patients still on the waiting list. In the State of Sao Paulo alone, there are 12,260 waiting, of these 10,128 are awaiting a kidney transplant[2]. The increase in the number of transplant patients and the number of patients on the waiting list have further raised the interest in immunosuppressive therapies with better outcomes and fewer undesirable effects.

The immunosuppressive medications more frequently used in renal transplantation are Cyclosporine or Tacrolimus (FK506), Azathioprine or Mycophenolate mofetil and Prednisone[3]. Because of its effectiveness, Tacrolimus was, in 2003, the calcineurin inhibitor more used in transplants performed in the United States of America, being prescribed in 67% of kidney transplants, 89% of liver, 81% of kidney/pancreas, 77% of pancreas, 65% of lung, 48% of heart/lung and 42% of heart transplants[4]. Tacrolimus is a macrolide antibiotic, its immunosuppressive effect occurs through the inhibition of calcineurin, preventing the activation of Th cells and the production of IL-2[5]. FK506 has emerged as an alternative to the use of Cyclosporine A. Comparing the two pharmaceuticals, FK506 demonstrates a lower rate of acute and chronic rejection, improved long term renal function after transplantation and lower rates of hyperlipidemia and hypertension[4].

The main undesirable effects of Tacrolimus are: tremors, diarrhea, constipation, headache, abdominal pain, hypertension, nausea and decreased renal function with creatinine increase, i.e. nephrotoxicity. Of these, the latter is the more significant, considering that the transplant is an alternative to the resumption of the impaired renal function compromised by various clinical causes. The nephrotoxicity of FK 506 is one of the causes of acute kidney injury after kidney transplantation. The pathophysiological mechanisms of this collateral effect are still not fully understood, however, they appear to be associated with an increased production of oxygen free radicals and a consequent redox imbalance, i.e. an imbalance between oxidants and endogenous antioxidants[6]. This mechanism of injury triggers a series of events related to the nephrotoxicity of FK506, among these, the isolated defects of the tubular function, interstitial leukocyte infiltration and glomerular vasoconstriction stand out. Depending on the dose and duration of therapy, the mechanisms of acute kidney injury (AKI) may determine the evolution to chronic kidney disease, with loss of the transplant[7].

Acute kidney injury is a syndrome characterized by the rapid decline of renal function, defined by an absolute increase in serum creatinine of at least 0.3 mg/dl, an increase of 50% (1.5 times) of the basal value or a reduction in urine flow, documented as oliguria or a flow of 0.5 ml/kg per hour for more than six hours[8]. Intrinsic AKI, which comprises injuries of nephrotoxic origin, is the second most common form. The mortality rate from AKI is alarming, remaining at 50%, despite numerous clinical initiatives[9]. Nephrotoxicity is an undesirable effect of several pharmaceuticals routinely used in the clinical practice. On this list, antibiotics, anti-inflammatories, antineoplastics, immunosuppressant medications and radiological contrasts are highlighted. Immunosuppressive medications have a separate chapter reserved for them in the study of nephrotoxicity due to the increase in the number of transplants. This study will focus on the investigation of medicinal agents, in particular, the phytomedicines, which may reduce the nephrotoxicity of Tacrolimus. In the case of FK 506, AKI is associated with decreased renal blood flow and glomerular filtration rate, possibly caused by vasoconstriction of the glomerular arterioles and mesangial cell contraction in response to the oxidation disturbances mentioned above[10]. The increased secretion of endothelin and the activation of the renin-angiotensin system, raise the belief that the decrease of the glomerular ultrafiltration coefficient (Kf) may be a participatory factor in the nephrotoxicity induced by this immunosuppressive agent[10].

Many studies have focused on the identification of renoprotective agents that are intended to reduce the nephrotoxicity of irreplaceable pharmaceuticals. However, sometimes, the very protective medication proves to be toxic, valorizing the use of agents with less potential for undesirable effects, such as the phytomedications. Phytomedicine, which consists of therapy characterized by the use of medicinal plants in their various pharmaceutical forms, has become increasingly popular. The application of phytomedicines in the therapeutic routine in different clinical situations may represent a significant and viable alternative, particularly, in Brazil where the flora is so diverse and plentiful. The dry extract of *Vitis vinifera L* is a phytomedication containing oligomeric proanthocyanidins, flavonoid species. These elements have the ability to neutralize oxygen and iron free radicals, being 15 to 20 times more potent than vitamin E[11]. Other studies have shown their effectiveness in ischemic AKI and as antihypertensives, acting on the inhibition of the angiotensin converting enzyme, by reducing levels of LDL (bad cholesterol) and increasing HDL (good cholesterol), through the increase of reverse cholesterol

transportation. There are no reports of collateral effects, nor toxicity related to *Vitis vinifera L*[12-14].

Attempting to alleviate the nephrotoxicity of Tacrolimus with medication that exhibits other toxicities may establish a synergistic relationship and incur more undesirable effects. The use of phytomedicines represents an alternative with less risk of toxicity and may contribute to increasing the life of the transplant. The absence of concrete data on the nephrotoxicity of Tacrolimus and the ineffectiveness of allopathic maneuvers to reduce this collateral effect reinforce the importance of studies that seek to elucidate these restrictions in the clinical practice. Considering the wide use of Tacrolimus in immunosuppressive therapies and emphasizing that its potential nephrotoxicity may lead to transplant loss, this study aimed to verify the renoprotective effects of the extract of *Vitis vinifera L* on Tacrolimus-induced nephrotoxicity in experimental studies with rats. The knowledge of nurses regarding the desirable and undesirable effects of medications, on which they base their routine practice, is the basic support mechanism of the professional practice and justifies the inclusion of this professional in experimental studies with animals, similar to their participation already established in clinical research.

METHODS

The procedures necessary for the realization of this study were consistent with the Ethical Principles of Animal Experimentation adopted by the Brazilian College of Animal Experimentation. The research project was approved by the Animal Experimentation Ethics Committee of the Institute of Biological Sciences IV, University of Sao Paulo, under Protocol No. 75/2009. This was a quantitative study with an experimental model, in which, adult male Wistar rats were included, weighing between 250g and 300g. The animals were provided by the bioterium of the Faculty of Medicine, University of Sao Paulo and only those considered fully healthy by the biological criteria of this institution were used. The rats were divided into the following groups:

Saline Group: four animals that received only solution of sodium chloride, 0.1 ml by gavage, for five days, once daily.

FK Group: seven animals that received Tacrolimus, 0.5 mg/kg by gavage, for five days, once daily.

Vitis Group: seven animals that received *Vitis vinifera L*, 3,0 mg/kg, by gavage, for five days, once daily.

FK + Vitis Group: seven animals that received Tacrolimus, (0.5 mg/kg by gavage, for five days, once daily) and *Vitis vinifera L* (3.0 mg/kg, by gavage, five days, once daily).

At the end of treatment, the animals were placed in metabolic cages for 24h to obtain urine and the subsequent measurements of creatinine and urinary peroxides were used as renal function and oxidative index markers. After obtaining the urine, the rats were anesthetized with sodium pentobarbital and underwent laparotomy for collection of blood, through puncture in the abdominal aorta, for subsequent dosage of serum creatinine. After the experiment, the animals were sacrificed according to the ethical standards for the handling of laboratory animals. The animals were maintained with free access to water and feed throughout the experiment and remained in appropriate thermal conditions and cycles alternating between day and night. The measurement of plasma and urinary creatinine was performed using Jaffe's method[15]. The renal function of the animals was analyzed by means of creatinine clearance, using the following formula:

$$\text{crCl} = \frac{\text{urinary creatinine (mg/dl)} \times \text{urine flow (ml/min)}}{\text{plasma creatinine (mg/dl)}}$$

The peroxides are considered potential indicators for the formation of, or resultant from, reactive oxygen species. The direct measurement of peroxides can be performed using the FOX-2 method, which consists of the use of iron-xylenol orange for the determination of peroxide levels. The peroxides, when diluted in an acid solution, oxidize the Fe^{2}+ ion to the Fe^{3}+ ion, as shown in the following reaction:

$Fe^{2} + ROOH \rightarrow Fe^{3} + OH^{-}$

Xylenol orange (3,3'-Bis[N,N-bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein tetrasodium salt) presents high selectivity for the Fe^{3}+ ion, producing a complex of blue-violet coloration ($\alpha = 4.3 \times 10^{4}\ M^{-1}\ cm^{-1}$)[16].

The solution FOX-2 was prepared using, in order, the following reagents:

90 ml methanol
10 ml distilled water
100 μM xylenol orange
4 mM BHT (2[6] - di-tert-butyl-p-cresol)
25mM sulfuric acid solution (H2SO4)
250 μM ferrous ammonium sulfate - (Vetec Química - RJ, Brazil)

In the next step 100μl of urine sample was added to 900μl of this solution. After homogenization the solution remained stationary at room temperature for 30 minutes. The solution was then centrifuged to remove the residues and kept on ice for approximately 10 minutes. The reading was performed through spectrophotometry at an absorbance of 560 nm. The values were stabilized by gram of urinary creatinine and expressed as nmol of peroxides/gram creatinine[17-18]. Data analysis was performed using the ANOVA method. When the

significance level was <0.05 it was considered that the effect of at least one of the groups was different from the others. The Bonferroni multiple comparison 2-2 tests were used to determine which groups differed or not from each other. The study was conducted from August 2008 to October 2010 in the Laboratory of Experimental Research with Animal Models of the School of Nursing, University of Sao Paulo.

RESULTS

As shown in the Table 1 data, the groups and Saline and Vitis groups, used as controls for this study, showed no significant variability regarding renal function and were confirmed as controls for the others. The administration of FK resulted in a significant reduction in creatinine clearance when compared to the control group (0.13±0.01 vs. 0.74±0.01). This finding confirmed the occurrence of nephrotoxic AKI ($p<0.001$). Regarding the excretion of urinary peroxides, used in this study as an indirect marker of the production of oxygen free radicals, an increase in this parameter was observed, comparing the Saline and FK groups (0.6±0.02 vs 4.2±0.26). In contrast, the animals that were treated simultaneously with FK and *Vitis vinifera L*, presented significantly better renal function than the animals that received only FK (0.28±0.01 vs. 0.13±0.01). This data highlights that the antioxidant effect beneficially affected the nephrotoxicity of FK. These findings confirmed the renoprotection of *Vitis vinifera L* in this toxicity model ($p<0.001$).

DISCUSSION

The number of kidney transplants has increased annually, from January to March 2010 a total of 1,160 kidney transplants were performed. Compared to the previous year, there was an increase of 8.4% in this type of transplant[19]. For the maintenance of the transplantation, immunosuppressive drugs are needed, with Tacrolimus being one of these. One of the undesirable effects of Tacrolimus is nephrotoxicity[20]. It is believed that one of the mechanisms associated with kidney damage caused by FK506 is the production of reactive oxygen species (ROS)[21]. In this context, studies with phytomedications such as *Vitis vinifera L*, aimed at preventing or reducing damage caused by FK506, may be promising, considering that the use of calcineurin inhibitors is, to date, indispensable in immunosuppressive therapy.

The flavonoids in wine have been highlighted for their antioxidant activity through free radical scavenging by modulating enzyme activities in support of the inhibition of lipid peroxidation[22-23]. This study confirmed that the use of FK506 significantly increased the production of urinary peroxides, which suggests, indirectly, the increased production of ROS. Together with this parameter, a decline of creatinine clearance was observed. The concomitant administration of FK506 and *Vitis vinifera L* promoted a significant reduction in the levels of urinary peroxides and improved renal function, observed by the increase of creatinine clearance. Another study showed the pro-oxidant action of calcineurin inhibitors through lipid peroxidation and confirmed the renoprotective effects of the use of vitamins A and C, both antioxidants[24]. The renoprotective effects afforded by *Vitis vinifera L* may be related to scavenging free radicals, inhibition of lipid peroxidation, or even its action in both pathways. It is a fact that the antioxidant effect of wine has been highlighted due to its beneficial implications in the control of endothelial inflammation and improvement of myocardial perfusion. The satisfactory result in myocardial homeostasis prompted the investigation of its effects on other organs, in situations of injury similar to those cited for myocardial ischemia: inflammation and redox imbalance. Acute kidney injury comprises this scenario and has shown to be reactive to treatment with this phytomedication.

In summary, the results presented in this study highlight the protective effect of *Vitis vinifera L* in the kidney function of rats subjected to treatment with Tacrolimus. The reduction of urinary peroxides confirmed the antioxidant effect of this medication. Certainly, other studies that detail the oxidative status and the intracellular mechanisms of nephrotoxic AKI

Table 1 - Global parameters of renal function and urinary excretion of peroxides in rats of the groups studied - Sao Paulo, 08/2008 to 10/2010.

Groups	Weight (g)	Urinary flow (ml/min)	crC1/100g (ml/min)	Urinary peroxides (nmol/g of creatinine)
Saline (n=4)	256+3	0.008+0.0002	0.74+0.01	0.6+0.02
Vitis (n=7)	262+4	0.011+0.0002	0.76+0.02	0.6+0.01
Fk (n=7)	271+5	0.011+0.0004	0.13+0.01[a]	4.2+0.26[a]
Fk+Vitis (n=7)	277±4	0.011±0.0004	0.28±0.01[b]	2.6±0.19[b]

[a] $p<0.001$ vs Saline, [b] $p<0.001$ vs FK
Results are expressed as mean ± standard deviation

caused by Tacrolimus and its treatment with *Vitis vinifera L* will bring more clarity to the data presented here. The research activities aimed at improving health and respecting ethical principles in man and animals are rights of all health researchers and should also be present in the quotidian of the nurse.

CONCLUSION

The data confirmed the nephrotoxic injury induced by Tacrolimus with reduced renal function evidenced though creatinine clearance. *Vitis vinifera L* showed significant renoprotective effects with clearance data higher than those of Tacrolimus. The administration of FK 506 increased the excretion of urinary peroxides and the concurrent administration with *Vitis vinifera L* decreased these parameters, which demonstrates the antioxidant effect of *Vitis vinifera L* and suggests the oxidative mechanism as being one of the types of nephrotoxic acute kidney injury induced by Tacrolimus.

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