Red propolis ameliorates ischemic-reperfusion acute kidney injury

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Article history:
Received 11 November 2013
Revised 2 March 2015
Accepted 19 March 2015

Keywords:
Acute kidney injury
Red propolis
Heme-oxygenase

Abstract

Introduction: Acute kidney injury (AKI) remains a great problem in clinical practice. Renal ischemia/reperfusion (I/R) injury is a complex pathophysiological process. Propolis is a natural polyphenol-rich resinous substance collected by honeybees from a variety of plant sources that has anti-inflammatory and anti-oxidative properties. Red propolis (RP) protection in renal I/R injury was investigated.

Methods: Male Wistar rats underwent unilateral nephrectomy and contralateral renal I/R (60 min). Rats were divided into four groups: (1) sham group, (2) RP group (sham-operated rats treated with RP), 3) IR group (rats submitted to ischemia) and (4) IR-RP (rats treated with RP before ischemia). At 48 h after reperfusion, renal function was assessed and kidneys were removed for analysis.

Results: I/R increased plasma levels of creatinine and reduced creatinine clearance (CrCl), and RP provided protection against this renal injury. Red propolis significantly improves oxidative stress parameters when compared with the IR group. Semiquantitative assessment of the histological lesions showed marked structural damage in I/R rats compared with the IR-RP rats. RP attenuates I/R-induced endothelial nitric oxide synthase down regulation and increased heme-oxygenase expression in renal tissue.

Conclusion: Red propolis protects kidney against acute ischemic renal failure and this protection is associated with reduced oxidative stress and eNOS and heme-oxygenase up regulation.

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Introduction

Acute kidney injury (AKI) remains a great problem in clinical practice. It affects approximately 20% of hospitalized patients and half of critically-ill patients admitted to intensive care unit (Poukkkanen et al. 2013; Zeng et al. 2013; Uchino et al. 2005). Despite improved strategies for supporting vital organs during AKI recovery and in renal replacement therapy (dialysis), AKI mortality rates remain quite high (Leite et al. 2013). Also, renal I/R injury is a common cause of early allograft dysfunction in renal transplanted patients and represents an additional risk factor for late renal allograft failure (Ditonno et al. 2013). The prevention of kidney lesions and their progression continue to represent a great challenge. Although renal injuries are multifactorial in many patients, in the clinical scenario, animal models of renal ischemia/reperfusion (I/R) remain important to understand the pathophysiology and potential treatment options for AKI.

Renal I/R injury is a complex pathophysiological process involving oxidative and inflammatory damage, endothelium-mediated injury and apoptosis. Nitric oxide (NO) is involved in the pathophysiology of ischemic AKI. Increased expression of proinflammatory inducible nitric oxide synthase (iNOS) is considered a pivotal step in renal damage, whereas the reduced activity of endothelial nitric oxide synthase (eNOS) contributes to renal impairment resulting from endothelial dysfunction (Heemskerk et al. 2009).

Many molecules have intrinsic cytoprotective properties that include anti-apoptotic, anti-inflammatory and antioxidant actions. Heme-oxygenase (HO) 1 and 2 are the rate-limiting enzymes in the catabolism of heme, a reaction that yields equimolar amounts of biliverdin, Fe³⁺ and carbon monoxide. Expression of HO-1 is readily increased upon organ I/R injury, becoming the rate-limiting factor in the generation of biliverdin, Fe³⁺ and CO. Heme-oxygenase-1
provides protection against renal I/R injury through its antioxidant, anti-inflammatory and cytoprotective activities (Nath et al. 1992; Agarwal Nick 2000).

Propolis is a natural polyphenol-rich resinous substance collected by honeybees from a variety of plant sources. In recent years, propolis has gained popularity as a health drink, has been extensively used in food and beverages, and is thought to improve human health and prevent disease (Daleprane and Abdalla 2013). Beneficial health effects are largely attributed to its polyphenolic composition. Red propolis has been classified as a separate type based on its unique chemical composition, particularly rich in isoflavonoids (Righi et al. 2013). Anti-inflammatory and antioxidant properties have been attributed to red propolis (Bueno-Silva et al. 2013; Enis Yonar et al. 2012). In the present study, we aimed to evaluate the effects of red propolis extract on renal I/R injury.

Methods

Animals and red propolis

The experimental protocol was approved by the Ethical Committee on Animal Research of Federal University of Ceará (no. 39/13). Wistar rats, weighing 250–300 g, were obtained from the Pharmacology Department of Federal University of Ceará and maintained under controlled temperature (21 ± 2 °C) and humidity conditions (60 ± 5%) with a 12:12-h light:dark cycle. A standard commercial diet and water were offered ad libitum.

Chemical characterization of red propolis

Red propolis was collected in the mangrove region in Marechal Deodoro (a city in the vicinity of Maceio, capital of Alagoas State, in the northeastern Brazil) (SL 09.40 and WL 35.41). Forty grams of propolis was macerated in 100 ml of 40% ethanol for 30 days, the solution was filtered, the filtrate evaporated to dryness. The residue, 12.5 g of dry extract (drug extract ratio of 3.2:1), was dissolved in 50 ml of ethanol to stock and transportation with a concentration of 0.25 g/ml. The botanical origin was Dalbergia ecastophyllum. The chromatographic analysis by high-performance liquid chromatography (HPLC) was performed. The assay was performed on Alliance – Waters 2695 (Milford, MA) chromatograph with a binary pump, auto-sampler, and photodiode–array detector (Waters–2996 PDA) at 268 nm. The separations were performed with an analytical reverse-phase column C18 (Waters, 250 mm × 4.6 mm, 5 μm) at 40 °C in a thermostatic oven. The mobile phase was made from water/acetic acid 0.1% (solvent A) and methanol (solvent B) in a gradient elution for 65 min (total run time), starting with 30% B (0–15 min), increasing to 90% B (15–60 min), held at 90% B and decreasing to 30% B (60–65 min) with a solvent flow rate of 1 ml/min. The solvents were previously degassed under vacuum by sonication during 5 min and filtered through Phenomenex nylon membrane (0.45 μm). The samples were dissolved in the initial mobile phase and filtered through a 0.45 μm filter unit (Millipore, USA) before injection (20 ml). The data was processed by Empower software (Waters, USA).

The identification of formononetin and biochanin A in RP by HPLC experiments were based on the retention time (tR) of external standards. The contents of the three flavonoids were calculated using calibration curves. The ranges of calibration curves were 0.04–0.12 mg/ml for formononetin and 0.005–0.013 mg/ml for biochanin A. The linear relationship was obtained correlating the concentration of flavonoids to the correspondent peak area.

For peak purity analysis, spectra in the range of 210–400 nm were recorded at a frequency of 1 Hz. Threshold was calculated employing noise and solvent angles. Reference spectra of formononetin and biochanin A standards were recorded in the Empower 2 software library for identification purposes.

The spectra search improves the identification of compounds in complex matrices since different substance can have identical retention times. Formononetin and Biochanin A were identified in propolis extract chromatogram through the comparison of peak apex spectrum against the results of reference standards solutions recorded previously in the software library. The peak height of biochanin A in propolis extract chromatogram is lower than formononetin (Fig. 1). The peak purity analysis provided by diode array detectors is essential to ensure reliability and accuracy of the chromatographic measurements of analytes in complex matrices. In the present work, the formononetin and biochanin A peaks were found pure since the purity angles were lower than the threshold angles and the threshold curves do not intersect the purity curves.

The chromatographic method shows linearity over the range evaluated and the correlation coefficients for and formononetin and biochanin A were 0.9915 and 0.9996, respectively. The concentrations (mean ± standard deviation for n = 12) of formononetin and biochanin A in the propolis extracted were 10.25 ± 0.21 and 0.50 ± 0.02 μg/mg, respectively. The amount of formononetin in the propolis extract is greater than 1% and was approximately fifteen times larger than biochanin A.

Surgical procedure

Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.). A midline laparotomy incision was performed, the right kidney was removed and left ischemic renal failure was induced by clamping the renal artery (with a nontraumatic clamp) for 60 min, followed by reperfusion. After 48 h, animals were sacrificed to obtain blood samples for biochemical tests. Additionally, the left kidneys were collected for histological and immunohistochemistry evaluation.

Experimental groups

To administration, the ethanol extract was filtered and then evaporated by using a vacuum evaporator. The gravimetric analysis was carried out in quintuplicate and disclosed a ratio of dry extract of 24.1 ± 0.09 (% m/m). Each 150 mg of dry extract contained approximately 6.38 mg of formononetin and 0.31 mg of biochanin A. The propolis samples were maintained in a dark environment, inside a deep freezer (kept at −20 °C). The dried extract was administrated according animal weight (150 mg/kg of body weight). The quantity of dried extract for each animal was suspended in tap water (5 ml) just before oral administration and the total volume (5 ml) was administered by gavage.

Rats were divided into the following groups (n = 8 in each group):

- Sham + tap water group (SHAM): Rats were submitted to identical surgical procedures, except for the nephrectomy and unilateral renal occlusion shock and were kept under anesthesia for the duration of the experiment.
- Sham + red propolis (RP): Identical to SHAM group, receiving red propolis (150 mg/kg/day) was administered by gastric gavage 3 days before the procedure and 1 h prior to surgical procedure.
- I/R + tap water group (IR): Rats were submitted to nephrectomy and unilateral renal occlusion (60 min) followed by reperfusion.
- I/R + red propolis group (IR-RP): Rats were submitted to the above mentioned surgical procedures and red propolis (150 mg/kg/day) was administered by gastric gavage 3 days before the procedure and 1 h prior to ischemia.

Measurement of biochemical parameters

Forty-eight hours after ischemia, rats were reanesthetized, and blood samples (1 ml) were collected via venipuncture. The samples were centrifuged (6,000 rpm for 3 min) to separate plasma. Plasma
and urine concentrations of urea (BUN) and creatinine (Cr) were measured as indicators of impaired glomerular function. Plasma and urine concentrations of sodium (Na\(^+\)) and potassium (K\(^+\)) were used as indicators of renal tubular injury. BUN and Cr levels were measured by means of colorimetric methods in a semi-automatic analyzer (LABQUEST\textsuperscript{R}) using diagnostic kits (Labtest\textsuperscript{R}, Brazil). The determination of sodium and potassium levels was made by means of ion-selective electrodes (Rapid Chem 744, Bayer Diagnostics).

Determination of MDA levels

Malondialdehyde (MDA) concentration in kidney tissue was determined as an indicator of lipid peroxidation, following a protocol previously described by Mihara et al. (1980). Briefly, the left kidney was removed and homogenized with KCl (1.15%) to make a homogenate. Then 3 ml of a solution containing phosphoric acid (1%) and 1 ml of thiorbituric acid (0.6%) were added to 0.5 ml of the homogenate in a tube. The mixture was heated in boiling water for 45 min. Then, 4 ml of a solution of n-butanol was added to the mixture, which was shaken vigorously. The absorbance was measured by spectrophotometry at 532 nm. The results were expressed in nmol/g tissue.

To determine the levels of MDA in urine samples, 0.4 ml of the urine sample was mixed with 0.6 ml of distilled water, 1 ml of TCA (trichloroacetic 10%) and 1 ml of thiorbituric acid (0.6% pH 2). All these solutions were kept on ice during this stage of the process. The solutions were homogenized and then placed in a water bath at 100 \(^\circ\)C for 20 min. After cooling and addition of 1 ml of TCA (70%), the final mixture was centrifuged for 15 min at 3000 rpm and the absorbance was measured by spectrophotometry at 534 nm.

Determination of GSH levels

Homogenate was prepared with EDTA (0.02 M). Then, 400 \(\mu\)l of the homogenate was removed and added to 320 \(\mu\)l of distilled water and 80 \(\mu\)l of trichloroacetic acid (50%). The material was centrifuged at 3000 rpm for 15 min. Then, 400 \(\mu\)l of the supernatant was collected and 800 \(\mu\)l of Tris--HCl buffer (0.4 M pH 8.9) and 20 \(\mu\)l of DTNB (0.01 M) were added. After 1 min of reaction, the absorbance was measured by spectrophotometry at 412 nm. The concentration of reduced glutathione was expressed in micrograms per gram of tissue.

Histological analyses

Renal tissue was removed and placed in a 10% solution of buffered formaldehyde. After 24 h, the tissue was transferred to an alcoholic solution (70%) and used for histological analysis. Kidney tissue was fixed using a 10% formalin solution and then paraffin-embedded. Slices of 5-\(\mu\)m thickness were obtained and then stained with hematoxylin and eosin (HE). Light microscopy sections were assessed for the presence of tubular cell necrosis, tubular dilation, inflammatory cell infiltration and cellular edema in the tubular interstitium. They were expressed according to the percentage of affected kidney samples using a semiquantitative scale: 1 – no abnormality, 2 – mild lesions affecting 10% of kidney samples, 3 – lesions affecting 25% of kidney samples.
Table 1
Effect of red propolis on renal function. Cr: creatinine; ClCr: creatinine clearance; FENa⁺: absolute excretion of sodium; FEK⁺: absolute excretion of potassium.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cr  Mean ± SD</th>
<th>Urea Mean ± SD</th>
<th>ClCr Mean ± SD</th>
<th>FENa⁺ Mean ± SD</th>
<th>FEK⁺ Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0.64 ± 0.05</td>
<td>36.36 ± 6.2</td>
<td>1.29 ± 0.46</td>
<td>0.23 ± 0.11</td>
<td>7.5 ± 2.9</td>
</tr>
<tr>
<td>RP</td>
<td>0.66 ± 0.13</td>
<td>40.17 ± 0.11</td>
<td>0.93 ± 0.24</td>
<td>0.19 ± 0.05</td>
<td>10.58 ± 6.4</td>
</tr>
<tr>
<td>IR</td>
<td>2.7 ± 0.99*</td>
<td>274.3 ± 91.81*</td>
<td>0.07 ± 0.04*</td>
<td>1.03 ± 0.39*</td>
<td>134.4 ± 54.9*</td>
</tr>
<tr>
<td>IRRP</td>
<td>1.82 ± 0.5#</td>
<td>181.1 ± 65.6#</td>
<td>0.41 ± 0.14#</td>
<td>0.58 ± 0.3#</td>
<td>64.7 ± 52.4#</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. SHAM.
# P < 0.05 vs. IRG.

Fig. 2. The effect of red propolis on tubular necrosis score after renal I/R. Values shown are mean ± SE. Mean of 8 rats for each group. *P < 0.05 vs. Sham, #P < 0.05 vs. IRG.

Fig. 3. Red propolis reduces malondialdehyde (MDA) levels induced by I/R injury. Values shown are mean ± SE of the MDA levels. (A) MDA levels of urine samples, *P < 0.05 vs. Sham, #P < 0.05 vs. IRG. (B) Kidney tissue MDA levels, *P < 0.05 vs. Sham, #P < 0.05 vs. IRG.
4 – lesions affecting 50% of kidney samples, and 5 – lesions affecting more than 75% of samples.

**Immunohistochemical localization of heme-oxygenase and eNos**

Immunohistochemistry for eNOS and heme-oxygenase was performed using the streptavidin-biotin-peroxidase method (Hsu and Raine 1981). After 48 h of reperfusion, the animals were sacrificed and the left kidney was removed and fixed in formaldehyde (10%) for 24 h, and subsequently submitted to treatment with EDTA (10%) for demineralization. Subsequently, the samples were suspended in sodium sulfate (5%), and then paraffin-embedded. After this procedure, serial 4-mm sections were obtained with an appropriate microtome and placed on L-polylysine slides, suitable for immunohistochemistry analysis. The sections were deparaffinized, hydrated in xylene and alcohol, and immersed in a citrate buffer (0.1 M; pH 6.0), by heating in a microwave oven for 15 min for antigen retrieval. After cooling to ambient temperature at 20 °C, washes were performed with phosphate buffered solution (PBS) and with an endogenous peroxidase blocking solution of H₂O₂ (3%) for 15 min. The sections were incubated “overnight” (at 4 °C) with primary rabbit anti-ENOS antibody diluted in PBS (1:200) and with primary goat polyclonal antibody against heme-oxygenase diluted in PBS (1:200). Then, samples were incubated with the secondary antibody for 30 min. After washing, the sections are incubated with the conjugated streptavidin peroxidase complex (ABC complex Vectastain®) for 30 min. After further washing with PBS, followed by staining with (DAB), samples were counterstained with Mayer’s hematoxylin. Finally, dehydration of samples was performed and they were mounted on slides. Negative controls were processed simultaneously as described above and the primary antibody was replaced by PBS–5% BSA.

When assessing immunohistochemical staining for eNOS and heme-oxygenase, each tubule interstitial grid field was graded semi-quantitatively and the mean score per kidney was calculated. Each score reflected mainly changes in the extent, rather than the intensity, of staining, based on the proportion of the grid field showing positive staining: 0, absent or <5%; I, 5–25%; II, 25–50%; III, 50–75%, and IV greater than 75%.

**Western blot analysis**

The protein expression of endothelial nitric oxide synthase (eNOS) and heme-oxygenase were assessed in the kidney samples that were

![Fig. 4. Red propolis improves glutathione (GSH) levels in animals with I/R injury. Values shown are mean ± SE of the GSH levels. *P < 0.05 vs. IRG.](image)

![Fig. 5. Red propolis restores the I/R-induced downregulation of eNOS. Values shown are mean ± SE. Eight rats for each group. *P < 0.05 vs. SHAM, #P < 0.001 vs. IRG.](image)
individually homogenized with a Polytron in K-HEPES buffer containing a mixture of protease inhibitors. After incubation at 4 °C for 15 min, the samples were centrifuged at 2000 g. The protein concentrations were quantified using the Bradford assay method and the 80 μg of protein from each sample was separated on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Subsequently, the membranes were probed with a primary rabbit monoclonal eNOS (1:200) or a primary goat polyclonal against heme-oxygenase (1:200) primary antibody, followed by anti-rabbit (1:1000) or anti-goat (1:5000) secondary antibody, respectively. The bands were visualized using a chemiluminescence substrate and analyzed by gel documentation Alience 4.7 Uvitec (Cambridge, Cambs, UK). The relative expression of NOS and heme-oxygenase proteins in each kidney were normalized using actin antibody and the values are expressed as a percentage of normal protein expression.

Statistical analyses

All continuous variables are shown as mean ± standard error. One-way analyses of variance with Newman–Keuls post hoc test was used for intergroup comparisons. All statistical analyses were performed using the GraphPad Prism 5.0® software. A P value less than 0.05 was considered significant.

Results

Red propolis attenuates I/R-induced functional impairment

Animals submitted to I/R injury had an increment in serum Cr (2.7 ± 0.9 vs. 0.6 ± 0.5 mg/dl, P < 0.05) and urea (274.3 ± 91.8 vs. 39.3 ± 6.2 mg/dl, P < 0.05), reflecting a marked reduction in CrCl (creatinine clearance) when compared to control animals (0.07 ± 0.04 vs. 1.29 ± 0.46 ml/min/100 g). This reduction in CrCl was associated with a higher absolute excretion of Na⁺ (1.03 ± 0.39 vs. 0.23 ± 0.01%, P < 0.05). While SHAM-operated animals receiving red propolis (RP) had no alteration in these parameters in relation to control group, red propolis attenuated the functional alterations induced by I/R injury. Animals in the IR-RP group had lower serum Cr and urea when compared with IR group. Also, there was attenuation in CrCl reduction (0.41 ± 0.14 vs. 0.07 ± 0.04 ml/min/100 g in the RP-IR and IR groups, respectively, P < 0.05). This improvement also was seen in the fractional excretion of Na⁺ (0.58 ± 0.30 vs. 1.03 ± 0.39, P < 0.05). Data are shown in Table 1.

Effects of red propolis on histologic injury

Light microscopy studies showed tubular necrosis, tubular dilation, inflammatory cell infiltration and cellular edema in the tubular

Fig. 6. eNOS expression represented graphically after actin housekeeping normalization; n = 6/group. Representative image of Western blot analysis of eNOS and actin in renal tissue according each group. *P < 0.001 vs. other groups; #P < 0.01 vs. SHAM and RPG groups.
interstitium of the renal cortex and outer medulla from animals that were killed 48 h after renal ischemia. These lesions were less intense in rats that were treated with red propolis when compared with untreated animals. Tubular necrosis scores can be seen in Fig. 2.

Red propolis reduces I/R-induced oxidative stress

As shown in Fig. 3, kidney tissue malondialdehyde (MDA) levels were found to be significantly higher in the IR group when compared with the Sham group (133.9 ± 23.36 vs. 68.10 ± 3.71, P < 0.05). After red propolis treatment, there was a significant decrease in MDA levels after I/R injury (90.22 ± 20.82, P < 0.05). The same results were found in urine samples, as MDA levels in the urine of animals from the IR group were significantly higher than in the urine of animals from Sham group (271.4 ± 145.6 vs. 81.37 ± 10.67, P < 0.05). MDA levels in the urine of the animals who received red propolis treatment were significantly lower after I/R injury (161.4 ± 81.01, P < 0.05).

However, the values of glutathione (GSH) were significantly lower when compared with those of the control group (1267 ± 229.5 vs. 1659 ± 107.9, P > 0.05), whereas the values of GSH were significantly improved by the red propolis treatment (1784 ± 297.4 vs. 1267 ± 229.5, P < 0.05) as shown in Fig. 4.

Red propolis restores the I/R-induced downregulation of eNOS

At 48 h after the surgical procedures, the IR group rats showed markedly lower eNOS protein expression when compared with the control rats at the semi-quantitative analysis of the area on immunohistochemistry (0.6 ± 0.5 vs. 1.8 ± 0.4, P = 0.003). It is noteworthy that red propolis administration attenuated the down regulation of eNOS expression (IRRPG: 2.2 ± 0.4; IR: 0.6 ± 0.5, P < 0.001), as can be seen in Fig. 5. All these results were confirmed by western-blot analysis (Fig. 6).

Heme oxygenase-1 upregulation by red propolis

Heme oxygenase-1 immunostaining was weakly present in tubules of both SHAM and RP groups (Fig. 7). Stronger positive staining of HO-1 was observed in the IR group (IR: 1.4 ± 0.5 vs. SHAM: 0.6 ± 0.54, P = 0.005). Heme oxygenase-1 immunostaining in the IR-RP group was significantly increased when compared with IR group (2.6 ± 0.5 vs. 1.4 ± 0.5, P < 0.001). There was no difference in HO-1 between animals that received red propolis, but were not exposed to renal ischemia. Although the western-blot analysis disclosed similar HO-1 expression in SHAM and IRG groups, it confirmed the increased expression in the IRRPG group (see Fig. 8).

Discussion

In the present study, previous treatment with an alcoholic extract of red propolis was protective against renal I/R injury. Also, we have demonstrated that this protection was associated with a reduction in oxidative stress and up regulation of eNOS and heme-oxygenase.
Acute kidney injury is a multifaceted entity that evolves through different stages, culminating in organ failure. Its pathogenesis is complex and involves apoptosis, endothelial damage, ROS and inflammation (Basile et al. 2012). Moreover, in the past decade it has become increasingly clear that the cytoprotective response prompted by an injury is crucial for determining the final functional outcome of an injured organ (Bonventre 2007). In this study, we focused on determining the protective mechanisms provided by red propolis treatment in a model of renal injury, mainly through anti-oxidant system, eNOS and HO-1.

Propolis is a complex honeybee product with a resinous aspect, containing plant exudates and beeswax. In a previous study, red propolis was characterized as being especially rich in isoflavonoids (Silva et al. 2008). Several studies have demonstrated the anti-oxidative and anti-inflammatory activity of isoflavonoids (Kupeli et al. 2006; Bhandary et al. 2012), but their effects on renal I/R injury was never studied. In our study, animals were treated on the day before the procedure with a fixed dose of red propolis extract, resulting in a significant attenuation of GFR drop. Although creatinine is limited as a marker of GFR, the protective effect of red propolis was confirmed by a reduced FENa in IR-RP group, a marker of functional tubular viability and by lower tubular necrosis index in the histopathological analysis.

MDA and GSH were measured in renal tissue to assess oxidative stress. Reactive oxygen species can result in further renal damage by inducing apoptosis, inflammation and mitochondrial cell damage (Zou et al. 2013; Ye et al. 2010). Reduced oxidative stress induced by renal I/R in animals receiving red propolis was associated with an increase in eNOS expression. Earlier studies demonstrated that the oxidative stress status that is enhanced during I/R is increased in NO deficiency (Kuçük et al. 2012; Tajes et al. 2013). It is possible that reduced oxidative stress is at least partially mediated by increased eNOS expression.

While inducible NOS (iNOS) is fundamentally involved in the process of kidney damage, inducing inflammation and apoptosis, and the inhibition of its activity (or the absence of iNOS itself in KO-mice) improves renal I/R damage in vivo, the other NOS isofrom, eNOS, has protective effects on I/R injury (de Souza et al. 2012; Kato et al. 2009).

The effects of isoflavones have been demonstrated on endothelial production of NO. It is capable of increasing the expression of eNOS in endothelial cell culture and in the myocardium (Maulik et al. 2012; Joy et al. 2006). In the present animals, I/R injury induced a down-regulation of eNOS expression after 48 h. This down-regulation was completely prevented by previous administration of red propolis. In the kidney, this effect of isoflavones on eNOS has shown to be effective against diabetic nephropathy, fructose and lead-induced nephrotoxicity, but no study had evaluated I/R injury (Arya et al. 2011; Palanisamy and Venkataraman 2013; Liu et al. 2012).

In recent years, investigators have been able to show that the expression of HO-1 is enhanced in conditions associated with oxidative stress and that the end products of heme degradation, including biliverdin, bilirubin, and carbon monoxide, provide protection against renal IRI through their antioxidant, anti-inflammatory and cytoprotective activities (Correa-Costa et al. 2012). Many experimental investigations have demonstrated that HO-1 induction plays an important role in the protection against renal I/R injury (Ferenbach et al. 2010). Astaxanthin, the most abundant isoflavone in propolis, induce HO-1 expression through ERK MAPK cascade
(Wang et al. 2010). We investigated whether HO-1 participates in renal protection induced by red propolis. Similarly to other studies, I/R injury itself stimulated HO-1 expression (Ferenbach et al. 2010) and previous treatment with red propolis increased the renal capacity to increase I/R injury-induced HO-1. In addition to reducing oxidative stress, HO-1 can protect against I/R injury by generating CO gas as a byproduct of the breakdown of heme. Several studies have demonstrated the protective role of CO itself in limiting renal damage in ischemia-induced acute kidney injury (Hou et al. 2013).

Our study has several limitations that must be explored in future studies. First, although the main protective component of propolis is isoflavones, future studies with isolated fractions of RP are warranted. Second, other studies must be performed to investigate mechanistic pathways regarding red propolis protection in renal injury, mainly focusing on inflammatory pathway.

In conclusion, this study provides strong evidence of the beneficial effects of red propolis on renal I/R injury, which were evaluated for the first time. Red propolis, given at a dose of 150 mg/kg (gastric gavage) before the ischemic and reperfusion period, improved kidney damage. The beneficial changes in biochemical parameters, including antioxidant status, were also associated with parallel beneficial changes in the histopathological appearance of renal tissue and immunohistochemical evidence.

In summary, our results strongly suggest potential clinical benefits of red propolis use to protect kidneys against acute ischemic renal failure and this protection is associated with reduced oxidative stress and eNOS and heme-oxygenase up regulation.

Conflict of interest

Alexandre Braga Libório and Alice Maria Costa Martins are recipients of a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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