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CONTRAST MEDIA
ORIGINAL ARTICLE

In vivo nephroprotective efficacy of propolis against contrast-induced nephropathy

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PURPOSE
Contrast agents administered in diagnostic imaging or interventional procedures of clinical radiology may cause contrast-induced nephropathy (CIN). Preventive measures against CIN involve pharmaceutical pretreatments, such as N-acetylcystein (NAC) or calpain, but alternative medicines can also be helpful. This study aims to assess the prospects of a natural compound, propolis, as a potential nephroprotector against a specific contrast agent, diatrizoate.

METHODS
In vivo experiments were performed on 35 male rats in five groups: control, diatrizoate alone, and pretreatments with propolis, NAC, or calpain one hour before diatrizoate administration. Three days later, blood and renal tissue samples were collected and quantitatively processed for determining induced changes in critical biomarkers malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT), as well as serum creatinine and plasma urea.

RESULTS
Diatrizoate increased creatinine (113%), urea (400%), and MDA (162%) levels and decreased GSH (-71%), SOD (-69%), GSH-Px (-77%), and CAT (-73%) levels. Evaluating the response of each pretreatment provided sufficient evidence that propolis was as effective as either NAC or calpain, but consistently more prominent in restoring the MDA, GSH, SOD, and GSH-Px levels close to their normal range. This outcome demonstrated the nephroprotective effect of propolis against CIN.

CONCLUSION
Propolis protects renal tissue against toxicity, free radicals, and other adverse effects induced by diatrizoate. This function is most likely exerted through the antioxidant and antitoxic activities of propolis.

Contrast agents are often used in diagnostic imaging or interventional procedures in clinical radiology or nuclear medicine for the purpose of providing contrast enhancement in images. Additional contrast improves the accuracy of detecting pathologic abnormalities in the underlying biological system or the outcome of the intervention. Some of the contrast agents, however, expose the patient to toxic side effects. Diatrizoate is one of those radiocontrast agents used for imaging kidney and its related structures. It has a high-osmolality and contains iodine for absorption of X-rays and hence for producing localized hyperintensities in computed tomography images. In patients with kidney dysfunction, as measured by serum creatinine clearance level of less than 60 mL/min, diatrizoate may lead to kidney failure (1). This effect is called contrast-induced nephropathy (CIN) and constitutes one of the most common causes of hospital-acquired renal failures in clinical practice. Therefore, it is important to develop preventive and/or protective strategies for safeguarding the kidney to be exposed to diatrizoate.

Diatrizoate induces kidney damage through a combination of renal ischemia and direct toxic effect on renal tubular cells (2). The mechanism of pathogenesis includes factors such as impaired nitric oxide production, blockade of vasodilation and generation of reactive oxygen species, all promoting oxidative stress. For preventing kidney from acquiring CIN, past attempts were focused on interventions against toxicity and oxidative stress. In this regard, pharmaceuticals N-acetylcystein (NAC) and calpain inhibitor-1 were investigated and determined to be effective (3). As alternative to such man-made pharmaceuticals, natural compounds found in folk medicine have also gained popularity as viable options in health care. The natural product propolis has been explored on various fronts and its therapeutic

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potential was experimentally investigated in treating or managing various diseases and injury conditions (4). The effectiveness of this product is essentially associated with its antioxidant and antitoxic functions. Considering its broad range of activities, we hypothesized that propolis may play an instrumental role in countermeasuring the renal damage induced by diatrizoate. Such features would make propolis an ideal candidate for preventing CIN and thus, it is worth pursuing a confirmatory proof. Based on this premise, we tested the nephroprotective potential of propolis when administered in rats prior to being exposed to diatrizoate. Accordingly, we arranged groups of rats and designed timing and dose paradigms for propolis and diatrizoate deliveries consistent with real-time circumstances experienced in nephrology and radiology clinics at our research hospital. In addition, for a benchmark comparison of the pretreatment efficacy of propolis, we included two additional groups of rats pretreated with reference drugs NAC and calpain, both with proven records in CIN prevention. We measured the consequences of our experimental procedures using biochemical analysis on relevant biomarkers extracted from blood, and renal tissue samples collected from the rats in all groups.

Methods

This study was approved by the institutional ethics committee and performed following the Principles of Laboratory Animal Care (NIH Publication). Propolis was prepared in-house, but diatrizoate (Urografin 76%, Bayer Schering Pharma), NAC (Asist 10% injectable form, Husnu Arsan Pharmaceuticals) and calpain ([N-Acetyl-Leu-Leu-norleucinal]^{R}, Roche Diagnostics) were acquired from commercial companies. Our research hospital is a regional service facility. Patients regularly arrive from far distances and after presentation at the clinics, promptly go through contrast-enhanced procedures such as intravenous pyelography or computed tomography scans with contrast agent, diatrizoate. For logistic reasons, rescheduling of scans is avoided, as much as possible. Considering the comfort, care, and management of the patient, especially those with kidney problems, any medication against CIN is required to be administered at least once and one hour prior to the scan. In routine practice, this time frame is long enough for the medication to dissolve in the body and exert its activities. To experimentally mimic this real life scenario practiced in clinics, the pretreatment agents propolis, NAC, and calpain were given to the rats only once and one hour prior to the diatrizoate injection. The administered doses were chosen from the literature demonstrating the effectiveness in experimental studies with rats or based on those used in the clinics, as adjusted from humans to rats. Pretreatments with either propolis (100 mg/kg/bodyweight), NAC (300 mg/kg/bodyweight) or calpain (10 mg/kg/bodyweight) were administered one hour before diatrizoate (6 mg/kg/ bodyweight IV).

Main points

- Contrast agent diatrizoate can cause contrast-induced nephropathy when administered in diagnostic imaging or interventional procedures.
- The natural product propolis is an effective therapeutic with its broad range of antioxidant and antitoxic activities.
- Propolis protects renal tissue from diatrizoate-induced toxicity.

Table 1. Chemical constituents of Turkish propolis used in this study

Retention time (min)	Constituents	% Total ion current
	Phenolic compounds	
27.93	4,5 dimethoxy-(2-propenyl) 2-phenol	1.25
31.06	Pinocembrin	14.75
34.12	Chrysin	7.67
34.84	Galangin	4.90
	Organic and fatty acids	
9.03	Decanoic acid	0.23
13.31	4-pentenoic acid	1.74
20.30	Cinnamic acid	0.29
20.73	3-hydroxy-4-methoxycinnamic acid	1.82
16.74	2-propenoic acid	2.70
20.91	3,4-dimethoxycinnamic acid	3.40
22.93	Coumaric acid	0.19
25.12	9-Octadecanoic acid	2.05
25.49	Octadecanoic acid	0.21
	Alcohols, ketones and terpenes	
8.02	2-propen-1-ol	0.21
34.38	5-3,3-dimethyl-cyclohexanone	1.36
24.49	2-Nonadecanone	0.66
15.22	Gamma-eudesmol	0.37
15.66	Beta-eudesmol	0.38
15.71	Alpha-eudesmol	0.59
16.26	Alpha-bisabolol	0.17
29.72	2-propen-1-one	15.30

Table 2. Experimental groups, treatments, and procedures

Control (n=7)	Normal controls injected with saline twice, with one-hour interval between the injections
CA (n=7)	Exposure to the contrast agent, diatrizoate 6 mg/kg/bodyweight IV
Propolis+CA (n=7)	Pretreatment with propolis 100 mg/kg IP, one hour prior to diatrizoate exposure (6 mg/kg/bodyweight IV)
NAC+CA (n=7)	Pretreatment with NAC 300 mg/kg IP, one hour prior to diatrizoate exposure (6 mg/kg/bodyweight IV)
Calpain+CA (n=7)	Pretreatment with calpain 10 mg/kg IP, one hour prior to diatrizoate exposure (6 mg/kg/bodyweight IV)

CA, contrast agent; NAC, N-acetylcystein; IV, intravenous; IP, intraperitoneal.

Propolis preperation

The composition of propolis varies with the geographical location from which it is collected in the world. Therefore, its origin matters as it would affect the treatment efficacy. This study was specifically performed with Turkish poplar from the honeybee colonies of *Apis mellifera caucasica*, located in Kayseri (Middle Anatolia region) by hand. The collected samples were stored in dark desiccators until its water-soluble propolis derivative is extracted using methods described earlier (5). The chemical composition of the resulting extract is listed in Table 1. The dose was specifically chosen as 100 mg/kg IP injection, as it was demonstrated to have optimum effectiveness in disease or injury intervention in rats.

Animals and treatment groups

A total of 35 male rats weighing between 180–220 g were randomly divided into five groups, each with an equal number of seven animals. The treatments and procedures applied to the rats in each group are summarized in Table 2. The animals were maintained in a 12 hour light/dark period, at 22°C–24°C and provided access to water *ad libitum* and food containing 2600 kcal/kg, 7% crude cellulose, and 23% crude protein. Seventy-two hours after diatrizoate administration, rats were sacrificed under general anesthesia (90 mg/kg ketamine and 4.5 mg/kg xyline coctail administered in 0.01 mL PBS IP), blood samples were collected and kidneys were removed and stored at –80°C until analysis.

Biochemical analysis

Serum creatinine and plasma urea levels were measured using a clinical chemistry analyzer (ILab 650, Diamond diagnostics) and expressed in mg/dL. For measurement of lipid peroxidation parameters, kidney samples (1/10, w/v) were homogenized in 1.15% KCl with a homogenizer (Glas-col LLC). Malondialdehyde (MDA) levels were measured directly in the homogenates. Tissue homogenates were centrifuged for glutathione (GSH), glutathione peroxidase (GSH-Px) and catalase (CAT) 15 minutes at 15.000 g and clear supernatant was removed for analysis using a UV-visible spectrophotometer (UV-1800, Shidmadzu). The obtained supernatant was centrifuged again at 25.000 g, +4°C for 30 min to determine superoxide dismutase (SOD) activities. The levels of MDA, GSH, GSH-Px as well as the activites of SOD and CAT were measured with biochemical assays as described (6).

MDA concentrations in tissue homogenates were spectrophotometrically measured at 532 nm after preparation according to the method of Placer et al. (7). CAT activity was estimated by measuring the breakdown of H_2O_2 at 240 nm (8). GSH concentration was measured by an assay using dithionitrobenzoic acid recycling and read at 412 nm on the spectrophotometer (9). GSH-Px presence was determined by absorbance at 340 nm after 5 min of recording. The activity was calculated from the slope of the line as µmol of NADPH oxidized per minute (10). SOD activity was measured at 560 nm using xanthine and xanthine oxidases to generate superoxide radicals to react with nitroblue tetrazolium (NBT) (11). One unit of SOD activity was defined as the amount of enzyme required to cause inhibition of NBT. Tissue protein contents were determined by the method of Lowry et al. (12). Their amounts were estimated by reading the absorbance at 750 nm of the end product of Folin reaction aganist a standard curve of a selected standard bovine serum albumin solution.

Statistical analysis

All biochemical analysis results were tabulated and statistically analyzed with nonparametric Kruskal Wallis Test and Mann-Whitney U test with Bonferroni correction using a software package (SPSS version 17.0 for Windows, SPSS Inc.). The level of statistical significance was set at $P < 0.05$.

Results

Rats in all groups tolerated the procedures and survived for three days without any visible or physiologic side effects or complications. Measurements from all groups are summarized with plots in Fig. Control group readings for each parameter were within the normal range reported in the literature (13). The rats injected with diatrizoate without pretreatment (group CA) had substantial changes in their biochemistry. Exposure to contrast agent has significantly increased serum creatinine (113%), plasma urea (400%) and MDA (162%) levels, but attenuated the GSH (-71%), SOD (-69%), GSH-Px (-77%) and CAT (-73%) levels considerably. Overall, pretreatment with each compound (propolis, NAC, and calpain) restored the biochemical readings closer to their normal values, and thus, appeared beneficial against CIN. However, in a few instances improvements in parameters were not as strong as the others and depended on the choice of the applied pretreatment.

Behavior of serum creatinine data was the only one consistent with an outcome expected from a successful treatment (Fig. a). Diatrizoate significantly increased the serum creatinine level, but pretreatment with propolis, NAC, or calpain was able to bring it down to its normal range. Serum creatinine differences between the control, propolis+-CA, NAC+CA, and calpain+CA groups were not statistically significant. These findings were encouraging and indicated that all compounds were equally capable and effective in maintaining the biological mechanisms responsible for preserving the serum creatinine status quo against diatrizoate toxicity.

Plasma urea and MDA data followed similar trend as serum creatinine, but pretreatments were not as effective in lowering the readings to normal levels (Fig. b, c). All pretreatments improved GSH levels, but failed to restore the readings to normal levels (Fig. d); nevertheless, NAC seemed to be the most effective drug in terms of GSH. Interestingly, propolis and calpain produced SOD readings in closer proximity to normal ranges than NAC (Fig. e). In two cases, pretreatment responses substantially exceeded the expectations. Specifically, NAC significantly overshot GSH-Px and CAT readings beyond their normal ranges measured in the control group (Fig. f, g).

Discussion

Although nonionic contrast media with minimal nephrotoxicity are currently available, ionic contrast agents such as diatrizoate are still used in radiologic practice, as in our institute. The mechanism through which the vital organ kidney is affected by diatrizoate consists of both intrinsic and extrinsic pathways. The increase in the serum uric acid levels suggests the failure to excrete diatrizoate by the kidneys. Diatrizoate exposure results in excess production of oxygen free radicals and reduction of antioxidant enzyme activity in the rat kidney (14). The generation of free radicals constitutes one of the underlying mechanisms for intoxication (15). In addition to cytotoxic effects mediated by oxygen free radicals, renal toxicity can also be caused by direct effects on tubular cells (16). These events coupled with medullary ischemia from renal vasoconstriction, collectively contribute to CIN (17).

Changes in MDA and GSH levels and SOD, CAT, and GSH-Px activities develop due to

generation of free radicals as a reaction to diatrizoate. This proliferation also damages red blood cells and organ tissues, primarily renal. One possible explanation for the observed activities of these biochemical markers can be tied to induction, where free radicals are converted into less harmful or harmless metabolites. Another explanation is that diatrizoate promotes direct inhibitory or stimulatory pathways of the activity. For example, GSH controls redox status as a reducing agent or a major antioxidant within the cells. In this study, GSH level was decreased when compared with the control group and such depletion indicates apoptosis of renal cells. This supports the view that diatrizoate, which is among the most commonly used contrast agent, accelerates the renal cell death and thus leads to acute kidney injury (18).

Past studies have demonstrated that pretreatment with NAC and calpain maintains kidney's normal function (13, 19). Therefore, in this study, NAC and calpain were used as reference drugs for the novel pretreatment strategy with propolis. NAC and calpain data reported in Fig. were in agreement with those in the literature. This confirmed that our measurement protocols were correct.

Propolis includes flavonoids, aromatic acids, diterpenic acids and phenolic compounds and exhibits antifungal, antiviral, antiinflammatory, local anesthetic, antioxidant, immunostimulatory, and cytostatic effects (20). Studies on various organs indicated that compounds pinocembrin, chrysin and galangin found in propolis possess antioxidant activities (21–26). These compounds were also present in the extract used in this study (Table 1). Contrast administration caused an increase in the plasma urea level when compared to the control group. However, in the propolis+CA group, the plasma urea level demonstrated to be significantly decreased compared with the levels in the CA group. Eraslan et al. (27) have reported similar effects. In both groups administered with propolis, increase in uric acid levels may be related to either the increase in protein degradation, which is involved in uric acid formation, or the influence of propolis on the kidneys. On the other hand, the administration of propolis caused a decrease in the serum creatinine level compared with the serum creatinine level in the CA group, but this decrease was not significant. There was a significant difference in the antioxidant enzyme activities and MDA levels between the propolis+CA group and the CA group. Jasprica et al. (28) have shown a similar effect where propolis decreased the MDA levels and increased the activity of SOD.

Based on the overall data, propolis consistently improved the biochemical parameters, meaning that it played a protective role against CIN. Given that NAC improved GSH and had erratic effects on GSH-Px and CAT profiles, and calpain action was

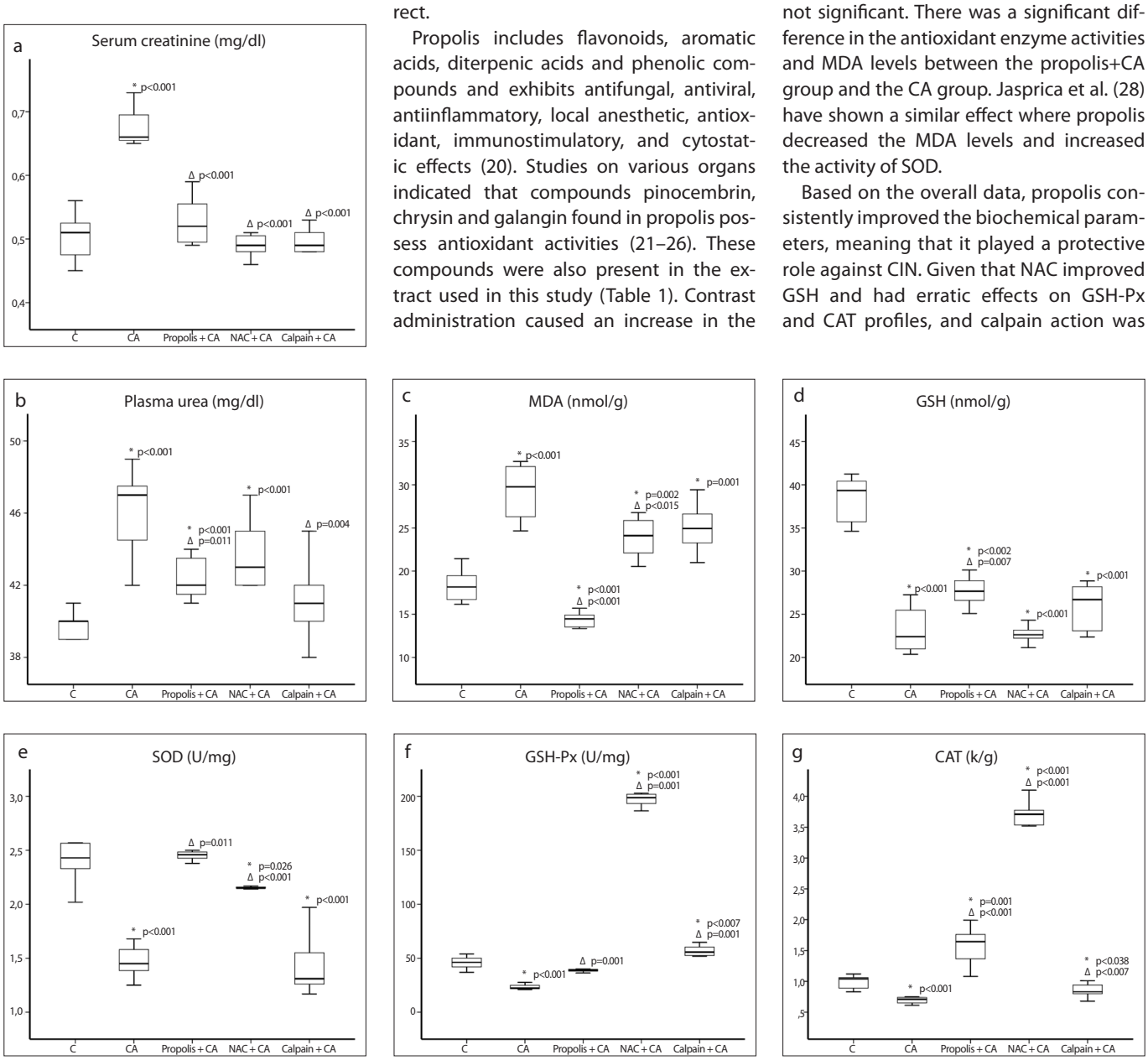

Figure. a–g. Graphical representations of plasma and tissue-based parameters as measured from the experimental groups (each with n=7). Panels show the following biochemical markers: **(a)**, serum creatinine (mg/dL); **(b)**, plasma urea (mg/dL); **(c)**, malonyldialdehyde; **(d)**, glutathione; **(e)**, superoxide dismutase; **(f)**, glutathione peroxidase; and **(g)**, catalase. *, Statistically significant difference against group C; Δ, Statistically significant difference against group CA.

not emphasized in SOD, propolis can be considered to be a robust alternative to NAC or calpain as a pretreatment against CIN. We recognize that propolis cannot be administered IV or IP; however, its active compound(s) may be isolated and pharmaceutically developed into a drug that can be for oral intake or IV/IP delivery in the future. At this time, clinical relevance of our finding is that propolis can be consumed orally as a supplement prior to a radiologic examination involving diatrizoate. At the least, pretreatment with propolis can be beneficial for increasing the body's antioxidant activity and thus providing nephroprotection against CIN.

The current study presents certain limitations. One time administration of the treatment prior to contrast agent exposure realistically mimics the clinical practice, but multiple administrations at different time points may be more useful in unequivocally proving the nephroprotective effect of the treatment. Such data would have been further strengthened with histopathologic evaluation of the kidney samples. In addition, other organs could be evaluated in terms of contrast agent-induced damage and the effects of pretreatment. We also note that the above results were obtained for ionic contrast media. Contemporary less toxic nonionic contrast media may produce a different outcome.

In conclusion, based on the findings from the current experimental study, propolis, is a powerful antioxidant that can be considered as a potential nephroprotective agent against the toxicity caused by the contrast media diatrizoate. Clinical practice aims at preventing renal cell damage and the consequent CIN in humans. Future studies should focus on exploring delivery strategies and dose paradigms for achieving optimal performance in pretreatment of patients with propolis.

Conflict of interest disclosure

The authors declared no conflicts of interest.

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