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RESEARCH ARTICLE

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Prevention of Calcium Oxalate Crystal Formation with Medicinal Mushroom Extract with Antioxidant Activity in a Rat Model

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ABSTRACT

Background: Oxidative stress (OXS) is believed to play a significant role in the development of nephrolithiasis. This possibility was tested by a medicinal mushroom extract, PE, with antioxidant activity capable of diminishing OXS. We examined whether PE would protect renal cells from OXS *in vitro* and prevent the calcium oxalate (CaOx) crystal formation in a rat model (*in vivo*).

Methods: Antioxidant activity of PE was assessed if it could reduce OXS exerted by hydrogen peroxide (H_2O_2), a typical OXS inducer, in renal epithelial MDCK cells. Whether PE may also prevent/reduce the CaOx crystal formation, which was chemically induced by orally giving the rats ethylene glycol (EG), was examined.

Results: The reduction in cell viability with elevated OXS by H_2O_2 was significantly prevented with PE in MDCK cells. Such elevated OXS was also diminished with PE, resulting in high cell viability. In the rat study, numerous CaOx crystals were found in the rats received EG only in 2 weeks, whereas PE significantly (~50%) reduced such EG-induced crystal deposits. Moreover, a ~1.2-fold increase in OXS with EG in the rat kidney was yet reduced by ~60% with PE, demonstrating antioxidant activity of PE.

Conclusions: The mushroom extract PE shows its antioxidant activity against H_2O_2-exerted OXS in MDCK cells. PE is also capable of preventing the CaOx crystal formation (~50%) in the rat kidneys, plausibly through its antioxidant activity. Therefore, PE could be a promising natural antioxidant, capable of protecting renal cells from OXS and potentially preventing the CaOx crystal formation.

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INTRODUCTION

With over $2.1-billion-dollar burden to the healthcare system for management of nephrolithiasis in the United States [1], its pathogenesis yet remains elusive and the improved preventative/therapeutic modalities must be urgently established. Particularly, calcium oxalate (CaOx) stone is the most common form (~74%) of nephrolithiasis and numerous therapies and preventative strategies have been targeted towards this incidence [2]. However, the overall outcomes of a diet plan and some preventative interventions [3] vary with individuals and are not generally satisfactory.

Hence, *besides* such physicochemical elements, we believe that a more specific and common factor must be acting on and unveiled.

A number of studies now suggest that *oxidative stress* (generation of reactive oxygen species) might play a primary role in the development of CaOx stone. Oxidative stress (OXS) can cause various renal cell injury/damage [4,5], which is considered a major risk factor for crystal deposition in the kidneys [6,7]. This is supported by the report that a binding of CaOx crystals to the renal tubular epithelium was required for the ultimate CaOx stone development [8]. Renal cell injury caused by OXS is also known to increase the binding affinity of CaOx crystals and facilitate the stone development [9,10]. In other words, no stones would develop unless CaOx crystals first bind to the renal tubular cells that have been injured/damaged.

Assuming that OXS is the critical factor for kidney stone formation, *antioxidants* could prevent/reduce

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the incidence of nephrolithiasis. In fact, antioxidants have been shown to have protective effects on cellular injury/damage caused by OXS [11]. For instance, our previous study also demonstrated that a potent antioxidant, N-acetylcysteine (NAC), had significantly (>70%) prevented chemically-induced CaOx crystal formation in a rat model [12].

Besides NAC, we recently came across the mushroom extract called "PE" isolated from *Poria* mushroom [13]. This is not a new mushroom but one of well-established medicinal mushrooms used for 2,000 years in Traditional Chinese Medicine. With the major chemical constituents including triterpenes, polysaccharides, and steroids [13], PE is found to have antioxidant, renoprotective, immunomodulatory, antitumor, antibacterial effects etc. [14-17]. We were particularly interested in its *antioxidant* and *renoprotective* activities, which might help reduce the incidence of kidney stones induced by OXS.

Accordingly, we investigated whether PE might have antioxidant activity and could prevent chemically-induced CaOx crystal formation in the rats. More details are described and the significant findings are also discussed herein.

MATERIALS AND METHODS

Cell culture

The Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) were employed in the *in vitro* study. They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were kept in the CO_2 incubator at 37 °C.

Exertion of OXS by hydrogen peroxide (H_2O_2) on MDCK cells

Hydrogen peroxide (H_2O_2) (Sigma-Aldrich, St. Louis, MO) was used an agent to exert OXS. PE was a generous gift from the manufacturer (Mushroom Wisdom, Inc., East Rutherford, NJ). For experiments, MDCK cells (2 x 10^5 cells/ml) were seeded in the 6-well plates or flasks for 24 hours and treated with the specified concentrations of H_2O_2, PE or their combinations for another 24 hours. They were subjected to either cell viability test or lipid peroxidation assay as described below.

Cell viability test (MTT assay)

Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor's protocol (Sigma-Aldrich). MTT reagent (1 mg/ml) was added to the 6-well plate, which was incubated for 3 hours at 37 °C. Absorbance of all samples was read in a microplate reader and cell viability was expressed by the % of sample readings relative to the controls (100%).

Lipid peroxidation (LPO) assay

The severity of OXS was assessed by the LPO assay, by measuring the amount of malondialdehyde (MDA) formed from OXS [18]. The assay was performed using the LPO colorimetric assay kit (Abcam, Cambridge, MA), following the procedures described in the vendor's protocol. The amount of MDA formed (in each sample) was expressed by fold-increase relative to controls (1).

Animal study

CaOx crystals were chemically induced in the rats with ethylene glycol (EG) through oral gavage [19]. Twenty male Wistar rats (200-250 g) were randomly divided into 4 groups (n=5): (A) Sham group (Rats received no agents); (B) EG group (Rats received 1 ml of 12% EG); (C) EG/PE group [Rats received both 1 ml of EG and PE (25 mg/ml) daily]; and (D) EG/NAC group [Rats received both 1 ml of EG and NAC (2 mg/ml) daily]. NAC was used as a positive control, capable of preventing >70% crystal formation [12]. All rats were sacrificed after 2 weeks and their kidneys were collected: one of the paired kidneys was subjected to histopathologic examination to evaluate CaOx crystals/deposits, while the other one was subjected to the LPO assay.

Pathology examination and blood urea nitrogen (BUN) and creatinine (Cr) tests

Kidney and blood specimens collected were sent to the commercial pathology laboratory for histopathologic examination and BUN/Cr tests, respectively. Histopathologic examination was performed by two independent veterinary pathologists and their reports were sent to us separately.

Statistical analysis

All data are presented as mean ± SD (standard deviation), and statistical differences between groups were assessed with the unpaired Student's *t* test or one-way analysis of variance (ANOVA). Values of p <0.05 are considered to indicate statistical significance.

RESULTS

Protective effect of PE against cytotoxic H_2O_2 in MDCK cells

We first examined if PE might protect MDCK cells from OXS exerted by H_2O_2. Following 24-hour seeding, cells were treated with H_2O_2 (0-400 μM) for another 24 hours and cell viability was assessed by the MTT assay. H_2O_2 indeed led to a significant cell viability reduction with the estimated IC_{50} value of

300 μM, which was then used in the rest of our study (Fig. 1A). The next study was performed to assess if PE would prevent or reduce H_2O_2-induced cell viability reduction. MTT assay revealed that a ~50% cell viability reduction by H_2O_2 went up to ~85% cell viability (i.e. a ~35% increase) in the presence of 50 μg/ml of PE (Fig. 1B). Thus, PE appears to protect renal cells from H_2O_2 attack, sustaining high cell viability.

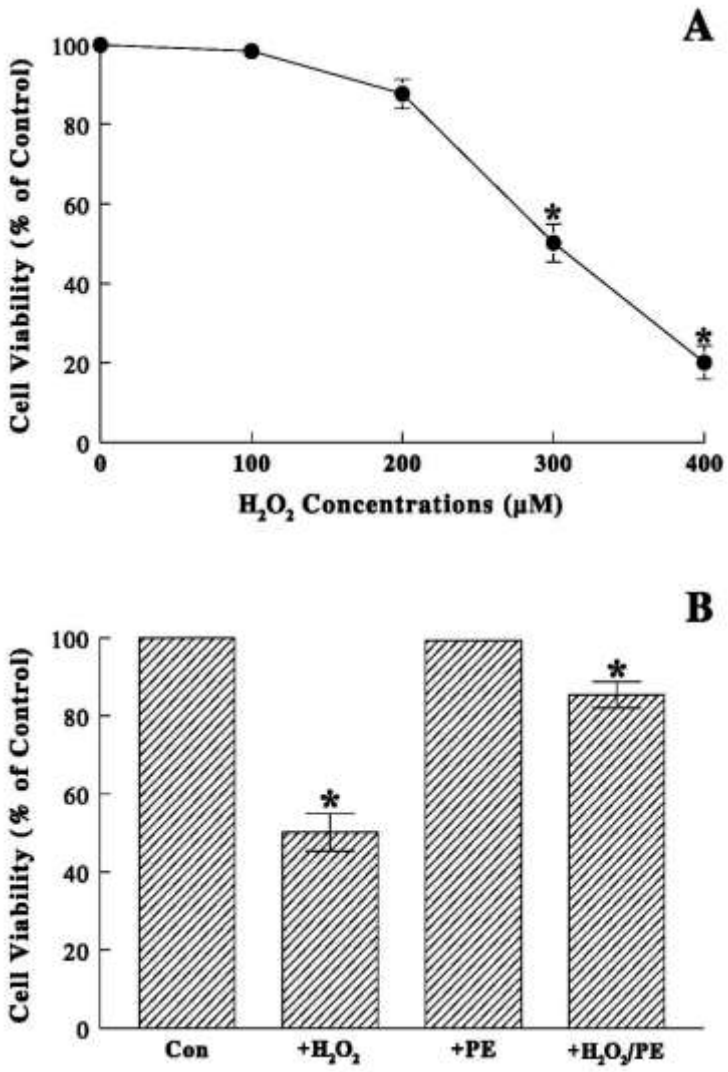

Figure 1: (A) Dose-dependent effects of H_2O_2 on MDCK cell viability. Cells were exposed to varying concentrations of H_2O_2 (0-400 μM) for 24 hours and cell viability was determined by MTT assay. (B) Protective effect of PE against H_2O_2. Cells exposed to H_2O_2 (300 μM), PE (50 μg/ml), or their combination (H_2O_2/PE) for 24 hours were assayed for cell viability. Cell viability was then expressed by the % of sample readings relative to the controls (100%). All data are mean ± SD (standard deviation) from three separate experiments (*p <0.05 compared with control).

Antioxidant activity of PE against OXS exerted by H_2O_2

The LPO assay was performed to assess the severity of OXS to address if a H_2O_2-induced cell viability reduction is primarily attributed to OXS. MDCK cells were exposed to H_2O_2, PE, or their combination (H_2O_2/PE) for only 6 hours, followed by the LPO assay. Figure 2 shows that the MDA level increased to ~2.7 times of controls by H_2O_2 but such an increase was reduced by ~47% with PE. Hence, H_2O_2 does exert severe OXS consistent with the fact that *the more MDA formed, the greater OXS*. Nevertheless, PE can yet significantly diminish such OXS, implying its antioxidant activity.

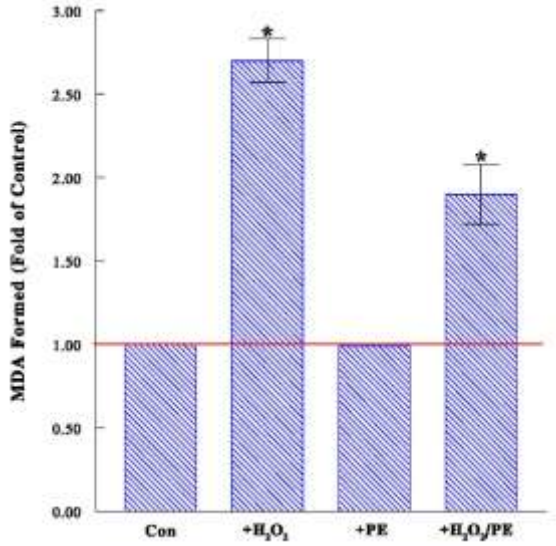

Figure 2: Severity of OXS. Cells were exposed to H_2O_2 (300 μM), PE (50 μg/ml) or their combination (H_2O_2/PE) for 6 hours and LPO assay was performed to assess severity of OXS. The amount of MDA formed was expressed by fold-increase relative to controls (1). All data are mean ± SD from three separate experiments (*p <0.05 compared with control).

Prevention of crystal formation with PE in rats

We next investigated if PE might also prevent/reduce CaOx crystal formation (triggered by OXS) in the rats. Twenty rats were divided into 4 groups and received appropriate agents (EG, PE or NAC) as described in Materials and Methods. The results of histopathologic examination (Fig. 3) show that specimens from the sham group (A) show no crystals but those from the EG group (B) exhibit numerous crystals. However, the significantly (~50%) reduced amount of crystals is seen in the EG/PE group (C) and the markedly (>80%) reduced crystal deposits in the EG/NAC group (D). Thus, PE is capable of significantly reducing the incidence of CaOx crystal formation in the rats.

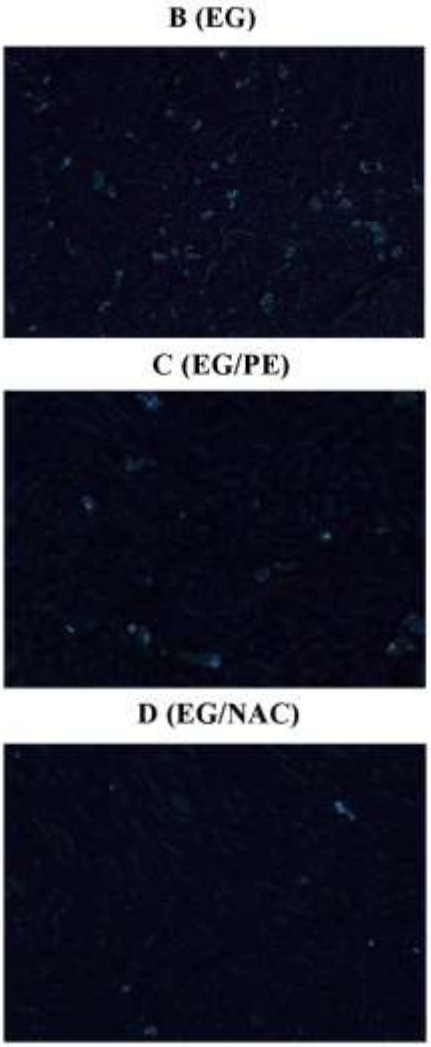

Figure 3. Histopathology of rat kidney specimens. Rat kidney specimens from the four groups were subjected to histopathologic examination to assess CaOx crystal deposits. Hematoxylin and eosin (H&E)-stained specimens were examined under polarized light microscopy (400X) that can highlight those crystals. The experimental groups shown here are A (Sham), B (EG), C (EG/PE), and D (EG/NAC).

The status of OXS in rat kidneys

Additionally, specimens from all 4 groups were subjected to LPO assay to assess the status/severity of OXS. Such results (Fig. 4) below showed that the EG group had a ~2.1-fold higher OXS (MDA formed) than the sham's, while the EG/PE and EG/NAC groups had ~60% and ~82% *lower* OXS than the EG group, respectively. Thus, EG can indeed exert OXS on the rat kidneys but it could be significantly diminished with PE (and NAC).

Effects of EG administration on renal function

Lastly, we examined if EG-induced crystal formation would also affect actual renal function. The results of blood analysis (Table 1) show that both BUN and Cr levels were significantly (p <0.05) elevated with EG administration (indicating renal

dysfunction), while PE supplement significantly decreased them to the levels comparable to control's (normal renal function)

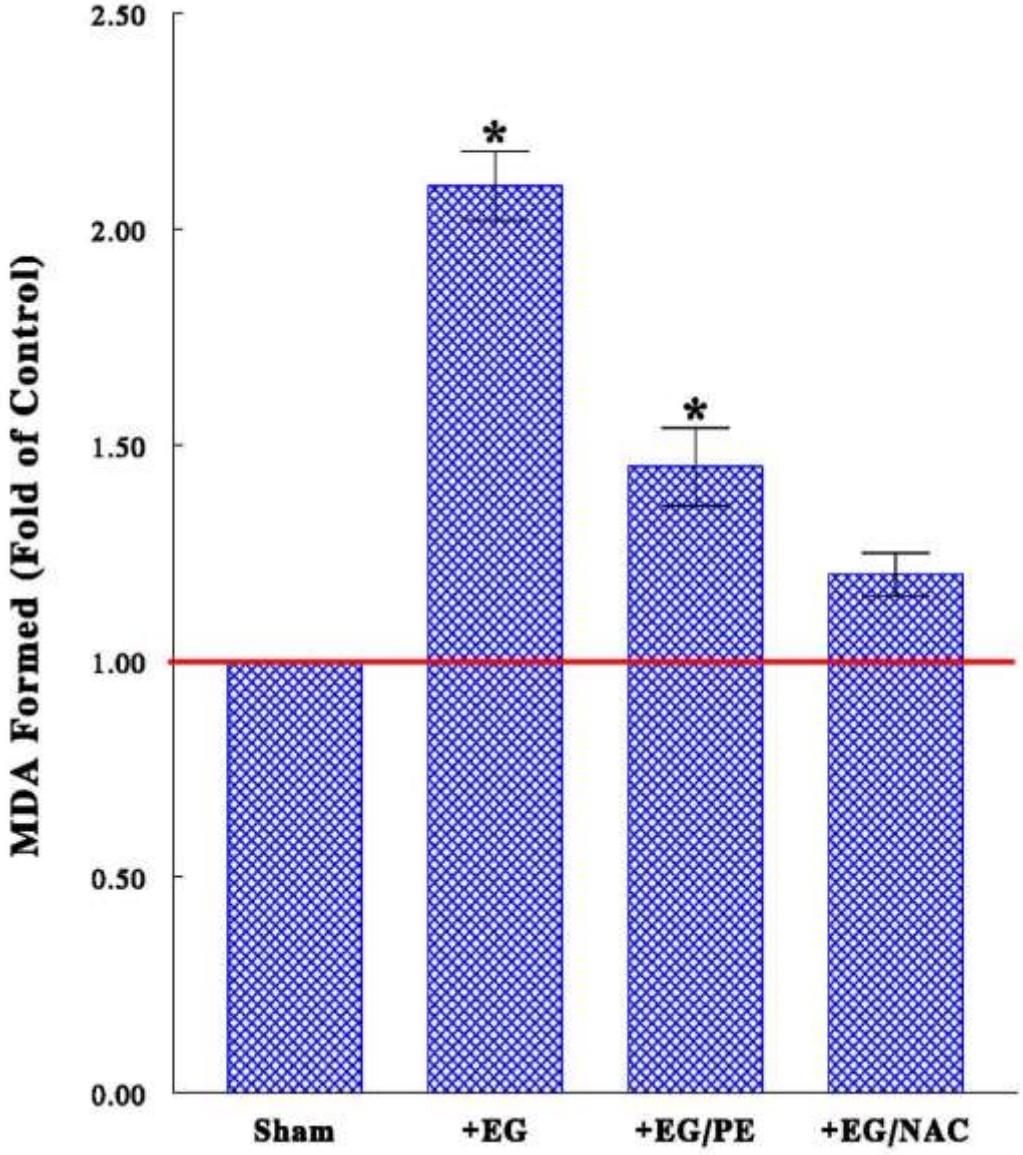

Figure 4. OXS exerted on rat kidneys. Cell extracts obtained from rat kidneys were subjected to LPO assay to assess severity of OXS. The amount of MDA formed was expressed by fold-increase relative to shams (1). All data are mean ± SD from three different specimens from each group (*p <0.05 compared with sham)

Table 1. Effects on BUN and Cr (assessment of renal function).

Analyses	Experimental Conditions			
	Sham	**+ EG**	**EG + PE**	**EG + NAC**
BUN (mg/dl)	34.5 ± 2.5	48.3 ± 1.2*	39.2 ± 2.3	33.4 ± 1.6
Cr (mg/dl)	0.63 ± 0.07	1.22 ± 0.12*	0.78 ± 0.08	0.61 ± 0.04

Values are expressed as mean ± SD (n=3).
BUN = blood urea nitrogen, Cr = creatinine, EG = ethylene glycol, PE = *Poria* mushroom extract, NAC = N-acetyl-cysteine.
*p <0.05 compared with Sham.

Similarly, NAC significantly reduced the elevated BUN and Cr levels as well. Thus, EG may adversely affect renal function but PE appears to restore proper renal function by normalizing the BUN and Cr levels.

DISCUSSION

As OXS is increasingly believed to play a significant role in nephrolithiasis [8,10], we studied if a medicinal mushroom extract, PE, with potential antioxidant activity could prevent the CaOx crystal formation in the rats.

We first examined if PE might have renoprotective and antioxidant effects on renal epithelial MDCK cells under OXS exerted by H_2O_2. Cell viability was significantly reduced by H_2O_2 (IC_{50} of 300 μM) and such a reduction was associated with severe OXS exerted on cells. However, PE was capable of *improving* cell viability reduced by H_2O_2 and *diminishing* the severity of H_2O_2-exerted OXS as well. Thus, PE has antioxidant activity against OXS, protecting renal cells from detrimental oxidative attack *in vitro*.

We next examined if PE could indeed have the prophylactic effect on chemically (EG)-induced CaOx

crystal formation in the rat kidneys (*in vivo*). Such study showed that PE led to the significant (~50%) reduction in crystal deposits induced by EG (Fig. 3). NAC also led to a >80% reduction in crystal formation as expected. Although PE may not be as potent as NAC, its ~50% reduction is yet significant to demonstrate the prophylactic effect. Now, the question was if this prophylactic effect of PE (or NAC) would be primarily attributed to its antioxidant activity against OXS exerted by EG. We found that EG did exert OXS on the rat kidneys; however, it was significantly (~60%) diminished with PE supplement (Fig. 4). Thus, OXS appears to substantially play a critical role in the CaOx crystal formation in the rats, which is yet prevented with PE.

Nevertheless, there is another significance of such renal cell injury caused by OXS. As the development of CaOx stone requires a "matrix", such as glycoproteins, glycosaminoglycans, or lipids [20], OXS may also provide this matrix as *membranous debris* created by renal cell injury. Hence, it is rather possible that OXS may further facilitate the ultimate CaOx stone development by injuring renal cells.

There was yet one more issue needed to be addressed – what might have happened to renal function under OXS? We saw the sign of renal dysfunction indicated by the increased BUN and Cr levels in the rats received EG (Table 1). However, PE supplement successfully maintained or lowered those elevated levels to restore normal renal function. Thus, EG-induced adverse effects could be effectively prevented with PE.

CONCLUSIONS

The present study shows that a *Poria* mushroom extract, PE, has antioxidant activity capable of protecting MDCK and rat kidney cells from oxidative stress exerted by H_2O_2 and ethylene glycol, respectively. Additionally, such oxidative stress would cause renal dysfunction in the rats, which was yet prevented with PE supplement. Therefore, PE might be considered as a promising prophylactic agent with antioxidant activity to prevent/reduce the development of calcium oxalate stone. Further studies are warranted.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR'S CONTRIBUTIONS

JW is a primary investigator who performed all experiments. NG and NP served as the essential assistants to JW. MC was in charge of administrative and financial matters. ME provided us his valuable advices and suggestions. SK is a senior author who designed the study, interpreted the data and primarily wrote the manuscript. All authors have read and approved the final manuscript.

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