



Possible Prophylactic Effect of *Poria* Mushroom Extract on Chemically-Induced Chronic Kidney Disease *In Vitro* and *In Vivo*

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Abstract

Background: Hypothesizing that oxidative stress (OXS) could be a key pathogenic factor for the incidence of chronic kidney disease (CKD), we investigated if the *Poria* mushroom extract, PE, with possible antioxidant activity, would prevent the incidence of CKD in rats. **Materials and Methods:** Antioxidant activity of PE was examined against OXS induced by hydrogen peroxide (H₂O₂) in renal LLC-PK1 cells. Whether PE could prevent the development of CKD in the rat kidneys, mediated through adenine (ADN)-induced OXS, was also examined. After 2 weeks, blood and kidney specimens were collected from rats for blood, histopathologic, and biochemical analyses. **Results:** Although H₂O₂-induced OXS led to a significant cell viability reduction in LLC-PK1 cells, PE significantly diminished OXS and sustained high (~70%) cell viability. In rats, ADN-given rats showed typical renal dysfunction with palpable kidney damage; however, PE supplement improved renal function with better histology. A ~2.2-fold increased OXS level was also seen in ADN-given rats but it was reduced by ~27% with PE supplement. Moreover, analysis of kidney injury biomarkers further confirmed extended kidney damage by ADN. Nevertheless, PE effectively maintained the natural status of those markers, protecting the rat kidneys. **Conclusions:** OXS is indeed harmful to renal cells in vitro and could even lead to ADN-induced CKD in vivo. However, PE appears to have antioxidant activity capable of protecting renal cells and the rat kidneys from such detrimental OXS. Therefore, it is rather possible that PE could be a natural antioxidant with prophylactic effect against OXS-induced CKD.

Keywords: Oxidative stress, chronic kidney disease, antioxidant, *Poria* mushroom.

1. Introduction

Among various kidney diseases, chronic kidney disease (CKD) is a major public health problem worldwide, and it causes tremendous socioeconomic burdens on the patients, families, and societies as well.^[1] Some causes of CKD include diabetes, hypertension, glomerulonephritis, cardiovascular disease etc.^[2,3] CKD is also characterized as reduced glomerular filtration rate (GFR) but increased proteinuria, tubular atrophy, tubulointerstitial fibrosis, glomerulosclerosis, renal vasculopathy etc.^[4,5] Moreover, CKD is a progressive, irreversible disease,^[6,7] leading to permanent renal failure, which may require lifelong dialysis or kidney transplantation.^[1,5] Currently, the primary objective of treatment is to only slow down the disease progression, instead of curing it, and those patients have a high risk of death from stroke or heart attack as well.^[3] On the other hand, the incidence of CKD is steadily

increasing and we need to have a better understanding of CKD to reduce or halt such an increasing incidence. There must be something in common and finding such a common factor could be a key to a better control of this progressive and incurable disease.

An increasing number of reports now suggest that oxidative stress (OXS), i.e. generation of reactive oxygen species (ROS), is prevalent in CKD patients and may play a substantial role in the incidence/development of CKD.^[5-8] A variety of ROS can create a state of OXS, capable of attacking, damaging, mutating, or even killing all kinds of cells including renal cells. It is thus plausible that OXS could ultimately lead to renal dysfunction, renal failure, or CKD.

If OXS were the primary basis of CKD, theoretically certain *antioxidants* should be able to effectively reduce the incidence of CKD. In fact, antioxidants have been shown to have beneficial or protective effects on cellular injury/damage caused by OXS.^[9] We were particularly interested in the bioactive extract from *Poria*

mushroom, PE.^[10,11] This is not just another mushroom but one of well-established medicinal mushrooms, which has been used in Traditional Chinese Medicine (TCM) for 2,000 years.^[10] It has been well characterized and found to have chemical constituents, such as triterpenes, polysaccharides, and steroids.^[10] In addition, PE has been shown to have various biological properties including antioxidant, renoprotective, anticancer, immunomodulatory, anti-inflammatory, anti-hyperglycemic, antibacterial effects etc.^[11-16] Interestingly, it is possible that antioxidant and renoprotective activities of PE may help prevent CKD or even reduce its incidence. Moreover, PE has few side effects (documented in TCM) as it is a natural agent, implying its potential therapeutic utility.

A rat model has been widely used for studying human CKD.^[17] Adenine (ADN) given orally to rats will be metabolized to 2,8-dihydroxyadenine (2,8-DHA), which then precipitates and forms tubular crystals causing palpable kidney damage similar to human CKD.^[17,18] Such kidney damage could be extended (70-80%) with fibrosis, enlarged granular appearance, apoptotic lesion etc.^[19,20] eventually leading to renal dysfunction with manifestation of CKD in rats. Hence, this is an appropriate experimental model for our study.

Accordingly, we first examined possible antioxidant activity of PE using cell culture *in vitro*. We then studied if PE might have prophylactic effect against ADN-mediated CKD (via OXS) in rats. Furthermore, the adverse effects of ADN-induced OXS on the kidneys were also assessed from physiologic (renal function) and biochemical (injury biomarkers) prospects. More details of the study are described and the interesting findings are also discussed herein.

2. Materials and Methods

2.1 Cell culture

The renal tubular epithelial LLC-PK₁ cells (American Type Culture Collection, Manassas, VA) were employed as our *in vitro* experimental model. They were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37 °C in a humidified incubator (with 95% air and 5% CO₂). For experiments, cells were seeded at the initial cell density of ~2 x 10⁵ cell/ml in the 6-well plates or T-75 flasks and treated with specified concentrations of hydrogen peroxide (H₂O₂; Sigma-Aldrich, St. Louis, MO) and/or *Poria* mushroom extract (PE; Mushroom Wisdom, Inc., East Rutherford, NJ). After 24-h incubation, cells were subjected to cell viability test (MTT assay) as described below.

2.2 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, St. Louis, MO). This will indicate what % of cells is yet *viable* under specific experimental conditions. MTT reagent (1 mg/ml) was added to the 6-well plate, which was incubated for 3 h at 37 °C. After MTT was discarded, dimethyl sulfoxide (DMSO) was added to the plate and the absorbance of formazan solution (purple color) was read in a microplate reader. Cell viability was then expressed by the % of sample readings relative to the controls (100%). All experiments were repeated three times separately to perform statistical analysis.

2.3 Lipid peroxidation (LPO) assay

The severity of oxidative stress (OXS) on LLC-PK₁ cells or the rat kidneys was assessed by LPO assay, measuring the amount of

malondialdehyde (MDA) formed due to OXS^[21] – *the more MDA formed, the greater OXS*. The detailed procedures were described in the vendor's protocol (Abcam, Cambridge, MA). Briefly, the reaction was initiated by mixing cell extracts with thiobarbituric acid (TBA) solution and incubated in a boiling water bath (~100 °C) for 1 h. Samples were read at A₅₃₂ on a microplate reader, and the amount of MDA formed was calculated and expressed by fold-increase relative to control's (1). All experiments were repeated three times separately for performing statistical analysis.

2.4 Animal study

Whether PE would protect/prevent the rat kidneys from ADN-mediated CKD was examined. CKD was chemically induced by orally giving the rats ADN (40 mg/ml) daily for 2 weeks as described elsewhere.^[8,11,22] Twenty male Sprague-Dawley rats (200-250 g), fed with standard chow diet and free access to water, were randomly divided into 4 groups (n=5 per group): *Sham* group; *ADN* group [Rats received 1 ml of ADN (40 mg/ml) daily]; *ADN/PE* group [Rats received 1 ml each of ADN (40 mg/ml) and PE (25 mg/ml) daily]; and *PE* group [Rats received only PE (25 mg/ml) daily]. At the end of 2 weeks, blood specimens were collected by retro-orbital bleeding and analyzed for blood urea nitrogen (BUN) and creatinine (Cr), while kidney specimens were surgically excised and subjected to histopathologic examination and biochemical analyses.

2.5 BUN/Cr tests and histopathologic examination

Harvested blood and kidney specimens were sent to the commercial pathology laboratory for BUN/Cr tests and histopathologic examination, respectively. Histopathologic examination was performed by two independent veterinary pathologists and the pathology reports were sent to us separately. The reports for BUN/Cr tests were also received separately.

2.6 Western blot analysis

Effects of OXS on three kidney injury biomarkers were analyzed using Western blots. Briefly, cell extracts (10 µg), obtained from tissue homogenization, were subjected to SDS-polyacrylamide gel electrophoresis to resolve proteins and transferred to a nitrocellulose membrane. The blot (membrane) was incubated with primary antibodies against three biomarkers, neutrophil-gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (Kim-1), and clusterin (CLU)^[23-25] (Santa Cruz Biotechnology, Santa Cruz, CA) for 90 min, followed by 30-min incubation with appropriate secondary antibody conjugates. Specific immunoreactive protein bands were detected by chemiluminescence following manufacturer's protocol (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

2.7 Statistical analysis

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

3. Results

3.1 Cytotoxic effects of H₂O₂ but its prevention with PE

We examined if H₂O₂ would be cytotoxic to renal LLC-PK₁ cells but PE might protect cells from such cytotoxicity. How H₂O₂ would actually affect LLC-PK₁ cells in terms of cell viability was first examined. After cells were treated with H₂O₂ (0-100 µM) for 24 h, cell viability was determined by MTT assay. The results showed that cell viability was significantly reduced to ~25% as

H₂O₂ concentrations went up to 100 μM (Figure 1A). This demonstrates that H₂O₂ is indeed cytotoxic to LLC-PK₁ cells. Additionally, as the estimated IC₅₀ value for H₂O₂ was approximately 70 μM, the rest of study was carried out using this concentration.

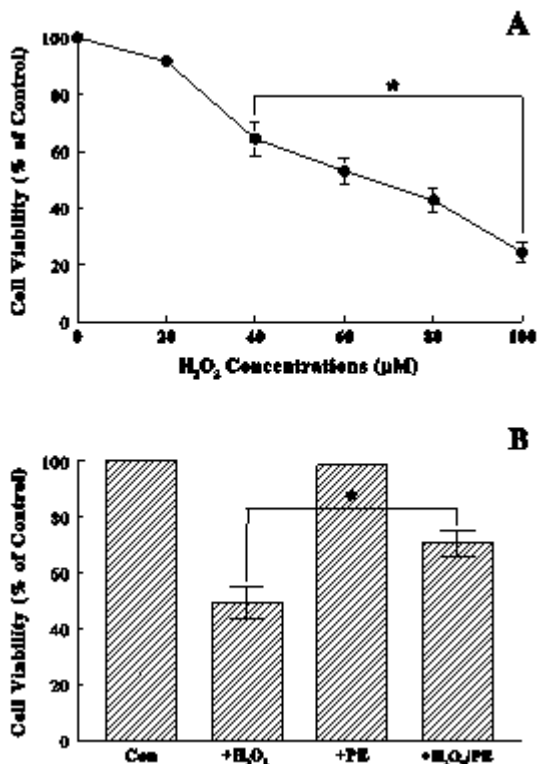


Figure 1: (A) Dose-dependent effects of H₂O₂ on LLC-PK₁ cell viability. After cells were exposed to varying concentrations of H₂O₂ (0-100 μM) for 24 h, cell viability was determined by MTT assay. (B) Protective effect of PE against H₂O₂. Cells exposed to H₂O₂ (70 μM), PE (50 μg/ml), or their combination (H₂O₂/PE) for 24 h were assayed for cell viability. Cell viability is 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).

We next examined if PE would protect LLC-PK₁ cells from H₂O₂ cytotoxicity, which could adversely affect cell viability. Cells were exposed to H₂O₂ (70 μM) alone, PE (50 μg/ml) alone, or combination of H₂O₂ and PE for 24 h. MTT assay showed that H₂O₂ led to a ~50% reduction in cell viability, which improved to a ~30% reduction (i.e. 20% higher cell viability) with PE (Figure 1B). Thus, PE appears to effectively protect renal cells from H₂O₂-induced cytotoxicity, implying its renoprotective effect.

3.2 Antioxidant activity of PE against OXS

Now, the question is *how* H₂O₂ reduces cell viability or *where* its cytotoxicity comes from. As H₂O₂ is known to induce harmful oxidative stress (OXS), we tested such a possibility. Knowing that OXS usually takes place rapidly and abruptly, cells were exposed to H₂O₂ (70 μM) alone, PE (50 μg/ml) alone, or H₂O₂/PE combination for merely 6 h. The severity of OXS was assessed by LPO assay as described in Materials and Methods. Such results revealed that no apparent changes in the amount of MDA formed were seen in control or PE-exposed cells (Figure 2). However, H₂O₂ led to a ~2.6-fold increase (compared to controls) in the MDA amounts, which was yet significantly (~24%) reduced with PE (Figure 2). Therefore, H₂O₂ appears to exert severe OXS on

LLC-PK₁ cells, but PE is capable of effectively diminishing such oxidative assault, demonstrating its antioxidant activity.

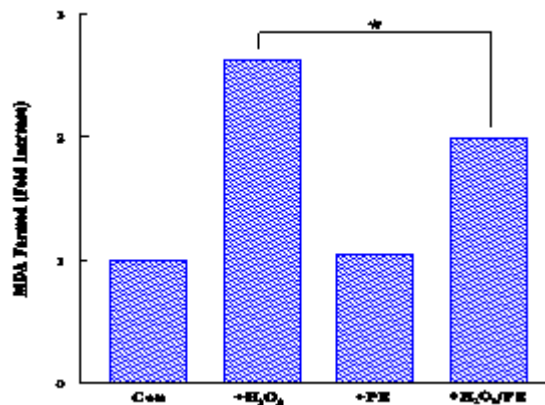


Figure 2: Severity of OXS. Cells were exposed to H₂O₂ (70 μM), PE (50 μg/ml) or their combination (H₂O₂/PE) for 6 h and LPO assay was performed to assess the severity of OXS. The amount of MDA formed was calculated (in triplicate) and expressed by fold-increase relative to controls (1). (*p < 0.05 compared between two groups).

3.3 Histopathologic examination on rat kidneys

Since PE is capable of protecting LLC-PK₁ cells from OXS mediated through H₂O₂, we further investigated if PE might prevent ADN-induced CKD in the rats, due to OXS. As described in Materials and Methods, 20 male rats were randomly divided into 4 groups: Sham group, ADN group (Rats received ADN daily), ADN/PE group (Rats received both ADN and PE daily), and PE group (Rats received only PE daily). After 2 weeks, all rats were sacrificed and blood and kidney specimens were collected for further analysis/examination. Effects of ADN and/or PE on the rat kidneys were examined histopathologically and the findings are displayed in Figure 3. Briefly, the Sham group shows normal, undamaged kidneys while the ADN group exhibits palpable histological alterations with renal tubular degeneration, indicating typical kidney damage. However, such kidney damage is less notable with PE supplement in the ADN/PE group. As expected, histology of the PE group looks quite similar to that of the Sham group with an undamaged, normal appearance. Thus, PE is capable of protecting the kidneys from ADN assault to a certain extent, thereby preventing extended kidney damage (CKD).

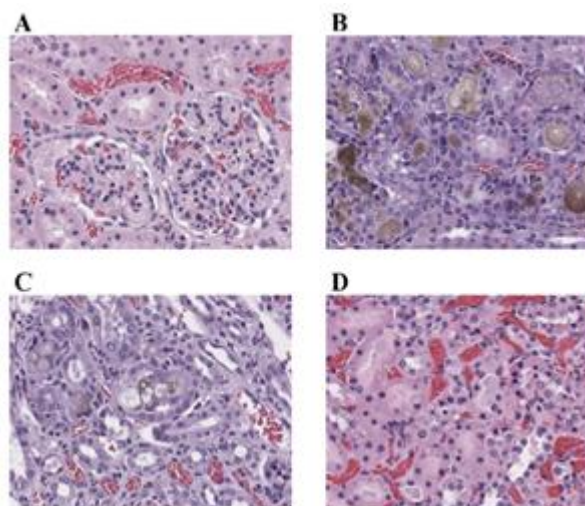


Figure 3: (A-D). Histopathology of kidney specimens (magnification, 400X). Kidney specimens of four experimental conditions were subjected to histopathologic examination. The

results shown are: Sham group (A); ADN group (B); ADN/PE group (C); and PE group (D).

3.4 ADN-induced OXS and PE

To understand *how* ADN actually induces CKD, we hypothesized that OXS (induced by ADN) might play a key role and this possibility was tested next. The LPO assay was performed on rat specimens to determine the severity of OXS. As shown in Figure 4, ADN exerted ~2.2-fold greater OXS (on the kidneys) than the Sham group, but this elevated OXS level was significantly (~27%) reduced with PE supplement (ADN/PE group). As expected, PE alone had no effects (PE group). Thus, although ADN-mediated cytotoxicity appears to be primarily attributed to OXS, PE can yet significantly diminish/reduce it, demonstrating its antioxidant activity.

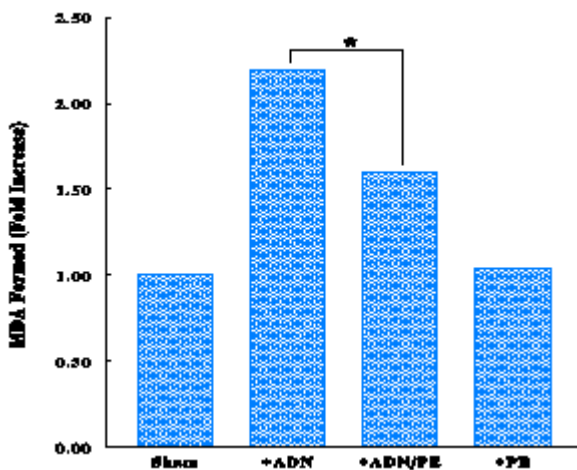


Figure 4: OXS mediated through ADN. The severity of OXS exerted on the rat kidneys was determined by LPO assay. The amount of MDA formed was calculated (in triplicate) and expressed by fold-increase relative to the Sham's (1). (*p <0.05 compared between two groups).

3.5 ADN-induced OXS and PE

To confirm if extended renal cell injury is linked to OXS, its effects on three kidney injury biomarkers, NGAL, Kim-1, and CLU in the rat kidneys were examined. Western blot analysis revealed that the expressions of all three biomarkers were *enhanced* or *up-regulated* by ADN, whereas these changes were yet prevented with PE (ADN/PE group) as they remained similar to those in the Sham's (Figure 5). It should be noted that the actual intensities of three biomarkers in the Sham and ADN/PE groups were quantitatively *similar* while those in the ADN group were significantly *different* from those in the Sham and ADN/PE groups, evidenced by densitometric analysis (data not shown). Thus, the *up-regulation* of these biomarkers is indicative of renal cell injury;^[23,24,26] however, sustaining their natural/basal status with PE also suggests that renal cells are well protected (from ADN attack) with PE.

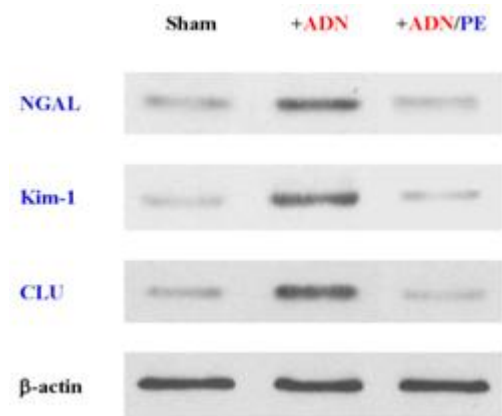


Figure 5: Up-regulation of kidney injury biomarkers by ADN. Kidney specimens obtained from different experimental conditions were analyzed for three biomarkers using Western blots. Autoradiographs of NGAL, Kim-1 and CLU are shown and β -actin was also run as a protein loading control.

3.6 Status of renal function following ADN and/or PE administration

Lastly, as it was important to address the status of renal function following ADN or PE administration, all rats were subjected to the evaluation of BUN and creatinine (Cr) in blood specimens. Blood analysis (Table 1) showed that compared to the Sham group, the BUN and Cr levels were ~3.8 and ~4.6 times higher in the ADN group, respectively. However, these elevated BUN and Cr levels were reduced by 36-38% with PE supplement (ADN/PE group), while PE by itself had no such effects (PE group). Thus, the elevated BUN/Cr levels by ADN indicate renal dysfunction, which seems to be significantly improved with PE supplement.

Table 1: Assessment of Renal Function

Experimental Conditions	Sham	+ADN	+ADN/PE	+PE
BUN	17.6 ± 2.1	66.9 ± 4.6	43.1 ± 3.3*	17.3 ± 1.7
Creatinine	0.47 ± 0.1	2.16 ± 0.13	1.34 ± 0.1*	0.41 ± 0.1

*p <0.05 (compared between ADN and ADN/PE groups).

4. Discussion

To better understand the primary cause of CKD, a progressive and incurable disease, we hypothesized that OXS could be such a key pathogenic factor and certain antioxidants might diminish or reduce it. In fact, antioxidants have been documented to effectively protect (renal) cells from such OXS.^[9] PE has been shown to have various pharmacological properties including particularly antioxidant and renoprotective activities.^[10,11] Hence, we performed the *in vivo* study to address if PE might have protective effect against ADN to prevent or reduce the development of CKD mediated through OXS in a rat model.

First of all, our *in vitro* study showed that H₂O₂-induced cell viability reduction, due to OXS, was effectively prevented with PE (Figures 1 and 2), demonstrating renoprotective and antioxidant activities of PE. We then studied if PE would prevent or reduce the incidence of chemically-induced CKD in rats. ADN led to adverse histological changes with deposition of 2,8-DHA (brown pigmented crystals), tubular degeneration, interstitial mononuclear inflammation etc. in rat kidneys (Figure 3). These results thus suggest that accumulation of ADN crystals may trigger renal cell

inflammation, tubular degeneration, renal dysfunction, and ultimately result in CKD.

Nevertheless, our next question was how ADN crystals would induce such severe renal cell damage. We then looked into possible OXS induced by ADN and found that OXS was indeed elevated to ~2.2-fold higher/severer in the ADN group (than that in the Sham) (Figure 4). This finding suggests that ADN is capable of exerting severe OXS on renal cells, feasibly leading to renal cell injury and renal dysfunction. To confirm such OXS-induced renal cell injury, we also examined the status or expressions of three kidney injury biomarkers, NGAL, Kim-1, and CLU,^[23-25] in the rat kidneys. All biomarkers in the ADN group were *up-regulated* or *expressed more intensely* (compared to those in the Sham), indicating apparent renal cell injury.^[23,24,26] Particularly, as NGAL is a stable, small molecule (25 kDa) that is excreted and easily detected in urine, it has been validated as a useful biomarker for CKD progression.^[27] After all, these findings support the notion that ADN-induced OXS can lead to extended renal cell damage/injury, resulting in renal dysfunction and ultimate CKD. Despite severe ADN-induced renal injury through OXS, PE was capable of significantly diminishing OXS (Figure 4) and maintaining the basal status of all three biomarkers (under OXS) (Figure 5), demonstrating its antioxidant activity to protect renal cells from OXS.

After all, those histopathological alterations caused by ADN apparently led to renal dysfunction, indicated by the significantly increased levels of BUN and Cr (Table 1). However, such elevated BUN and Cr levels have declined with PE supplement, implying the improvement in renal function. Therefore, PE appears to physically and functionally protect the (rat) kidneys from ADN-mediated OXS.

In addition, it would be interesting to talk more about antioxidant activity of PE. Today, many *synthetic* antioxidants are available in the market but they could be potentially hazardous to your health.^[28] In contrast, PE is considered as a *natural* antioxidant with few side effects and its safety has been well documented in TCM. A number of mushrooms (including *Poria*) have been shown to exhibit strong activity of scavenging ROS and considered as potential natural antioxidants.^[29,30] Strictly speaking, various derivatives (such as PE) of *Poria* mushroom have been isolated with the diverse degrees of biological properties such as antioxidant, anticancer, immunological activities etc.^[31] For instance, antioxidant activity of these derivatives could vary with others, but PE we used/tested appears to have fairly good antioxidant activity (reducing OXS by ~30%). It is yet possible that some other derivative(s) could have even better/stronger activity. This possibility may deserve further exploration.

Moreover, it would be worthwhile mentioning that the amount of ADN crystals formed/deposited in the rat kidneys appears to be significantly *less* with PE supplement. This suggests that PE could somehow interfere with the metabolic pathway of ADN to 2,8-DHA conversion. In general, as ADN is efficiently salvaged by adenine phosphoribosyltransferase, it is usually kept at the significantly low level in blood and urine.^[32] However, once ADN becomes excessive, it acts as a substrate for xanthine dehydrogenase, which could then oxidize ADN to 2,8-DHA.^[18] Due to such a low solubility of 2,8-DHA, it readily precipitates in renal tubules and forms tubular crystals.^[20] Hence, it can be speculated that PE may disrupt the conversion of ADN to 2,8-DHA by inhibiting/inactivating xanthine dehydrogenase, although other unknown mechanisms cannot be ruled out. This deserves further investigations.

Taken together, chemically-induced CKD in the rats could be primarily attributed to severe OXS on renal cells. OXS can cause renal cell damage/injury and dysfunction, consequently resulting in CKD. However, PE is capable of protecting the kidneys from all these adverse effects induced by OXS. This finding is also consistent with the early report describing that *another* isolated extract of *Poria* mushroom has been used for the prevention or treatment of CKD.^[33]

5. Conclusions

The present study demonstrates that oxidative stress plays a key role in inducing chronic kidney disease *in vitro* and *in vivo*. However, the *Poria* mushroom extract with antioxidant activity has the preventative or prophylactic effect on the development of chronic kidney disease. Thus, this *Poria* mushroom extract could be a promising natural antioxidant against a progressive, incurable disease and may have clinical implications with few side effects. Further studies are certainly warranted.

Ethics approval and consent to participate

The protocol for our animal study has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the College. The IACUC approval number is #10-1-0216.

List of abbreviations

OXS: oxidative stress
CKD: chronic kidney disease
PE: *Poria* mushroom extract
ADN: adenine
2,8-DHA: 2, 8-dihydroxyadenine
H₂O₂: hydrogen peroxide
GFR: glomerular filtration rate
ROS: reactive oxygen species
TCM: Traditional Chinese Medicine
MTT: 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl-tetrazolium bromide
DMSO: dimethyl sulfoxide
IC₅₀: 50% inhibitory concentration
LPO: lipid peroxidation
MDA: malondialdehyde
TBA: thiobarbituric acid
BUN: blood urea nitrogen
Cr: creatinine
NGAL: neutrophil-gelatinase-associated lipocalin
Kim-1: kidney injury molecule 1
CLU: clusterin
kDa: kilodaltons
SD: standard deviation
ANOVA: analysis of variance

Data Availability

The data described in this article will be available upon request and shared by signing the data access agreement. For further information, contact a corresponding author.

Funding Statement

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Authors' contributions

JW performed all experiments and collected the data. KZ served as an essential assistant for JW. MC worked on administrative and financial matters. ME gave us his critical opinions and guidance. SK designed experiments, interpreted the data and primarily wrote this manuscript. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare to have no conflict of interest.

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