Abstract

Purpose: Assuming that Oxidative Stress (OXS) could be a key pathogenic factor for the incidence of Chronic Kidney Disease (CKD), certain antioxidant(s) may effectively prevent it. Accordingly, we investigated whether the Poria mushroom extract, PE with possible antioxidant activity, might be able to protect the rat kidneys from developing to CKD.

Materials and Methods: A nephrotoxic agent, adenine (ADN), has been often used to chemically induce CKD in rats. Twenty rats were divided into four groups: Sham group; ADN (ADN only) group; ADN/PE (ADN with PE supplement) group; and PE (PE only) group. After ADN and/or PE were orally given to rats for 2 weeks, blood and kidney specimens were collected for histopathological examination and biochemical analyses.

Results: Compared to the Sham group, both blood urea nitrogen (BUN) and creatinine (Cr) levels were significantly elevated in the ADN group, indicating renal dysfunction, with palpable histological alterations. Such elevated BUN and Cr levels were reduced by ≥36% with PE supplement (the ADN/PE group), implying improved renal function and restoring better histology. The PE group (without ADN) also exhibited normal histology. Additionally, the ADN group notably showed the elevated OXS level with the inactivated antioxidant enzymes. Moreover, the expressions of four kidney injury biomarkers (neutrophil-gelatinase-associated lipocalin, kidney injury molecule 1, clusterin, and heat shock protein 70) were all enhanced in the ADN group, indicating apparent renal injury. However, PE effectively protected the kidneys and prevented all these adverse effects caused by ADN-triggered OXS.

Conclusions: This study shows that OXS plays a significant role in ADN-induced CKD in a rat model. However, PE with antioxidant activity is capable of protecting renal cells from such oxidative assault. Therefore, it is plausible that PE could be a natural antioxidant against CKD induced by ADN/OXS.

Keywords: Adenine; Chronic Kidney Disease; Mushroom Extract; Oxidative Stress; Rats

Introduction

Chronic Kidney Disease (CKD) is a major public health problem worldwide, which causes enormous socioeconomic burdens on the patients, families, and societies [1]. The various causes of CKD include diabetes, hypertension, glomerulonephritis, cardiovascular disease etc. [2,3]. Characteristics of CKD also include reduced glomerular filtration rate (GFR) but increased proteinuria, tubular atrophy, tubule interstitial fibrosis, glomerulosclerosis, renal vasculopathy, and reduced renal regenerative capability [4,5]. Those patients have a high risk of death from stroke or heart attack and CKD may further progress to permanent renal failure (i.e. end-stage renal disease, ESRD) that would require lifelong dialysis or kidney transplantation [1,5]. Unfortunately, CKD is a progressive, irreversible disease [6,7] and its therapeutic options are currently limited and unsatisfactory. The main objective of treatment is to only slow down the disease progression, not curing it. In the meantime, the incidence of CKD is steadily increasing, and a better understanding of CKD is the key to reduce the increasing incidence and delay its progression. It thus urgently demands for finding a more effective modality. Especially, it is crucial to seek and find a common factor for triggering CKD.
Increasing data suggest that oxidative stress (OXS), generation of reactive oxygen species (ROS), appears to be prevalent in CKD patients and may play a significant role in the incidence of CKD [5-9]. All ROS are known as highly reactive and harmful molecules, including oxygen free radicals (superoxide ion, singlet oxygen, hydroxyl radical etc.) and non-radical oxidants (hydrogen peroxide, nitric oxide, ozone etc.) [5]. They can create a state of OXS, capable of attacking, damaging, mutating, or even killing all kinds of cells including renal cells. It is thus possible that OXS could consequently lead to renal dysfunction, renal failure, or CKD.

Suppose OXS is the primary basis of CKD, it is plausible that certain antioxidants might be able to effectively reduce the incidence of CKD. In fact, antioxidants have been reported to have beneficial or protective effects on cellular injury/damage associated with OXS [10]. There are many antioxidants available such as vitamins (C/E), folic acid, β-carotene, reduced glutathione (GSH) etc., but the right antioxidant(s) must be found/used to be effective.

We are particularly interested in the bioactive extract from Poria mushroom, PE [11,12]. This is one of well-established medicinal mushrooms and has been used in Traditional Chinese Medicine (TCM) for 2,000 years [11]. It has been well characterized and its major chemical constituents, such as triterpenes, polysaccharides, and steroids, have been identified [11]. Additionally, a number of studies revealed that PE had antioxidant, renoprotective, anticancer, immunomodulatory, anti-inflammatory, antibacterial, anti-hyperglycemic effects etc. [12-17]. We are rather interested in its antioxidant and renoprotective activities, which may help prevent CKD or even reduce its incidence. Moreover, PE as being a natural agent has few side effects (documented in TCM), implying its potential clinical utility.

To study human CKD, rat models have been often used to chemically induce kidney damage similar to human CKD [18]. Oral administration of adenine (ADN) to rats will subsequently lead to the conversion of ADN to 2,8-dihydroxyadenine (2,8-DHA), which then precipitates and forms tubular crystals causing palpable kidney injury [19]. Such injury includes extended (70-80%) damage of renal tissue with fibrosis, enlarged granular appearance, apoptotic lesion etc. [20,21], which eventually results in renal dysfunction with manifestation of CKD. Additionally, cardiovascular changes, including impaired vascular responses, increased left ventricular stiffness and increased left ventricular mass, are all characteristics of human CKD [22]. Hence, this is an excellent experimental model for studying CKD.

Accordingly, we investigated if PE might have protective effect against ADN-induced CKD in the rats. We also explored the possible protective mechanism of PE, focusing on the status of physiological (renal function) and biochemical (injury biomarkers and antioxidant enzymes) parameters associated with CKD. More details are described, and the interesting findings are also discussed herein.

Materials and Methods

Animal Study

Whether PE may protect the rat kidneys from ADN-induced CKD was examined. CKD was chemically induced by orally giving the rats ADN (40 mg/ml) daily for 2 weeks as described elsewhere [9,12,23]. 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.

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.

Lipid Peroxidation (LPO) Assay

The severity of ADN-induced OXS on the rat kidneys was assessed by the LPO assay, measuring the amount of Malondialdehyde (MDA) formed due to OXS [24] – 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 (obtained from tissue homogenization of kidneys) with Thiobarbituric Acid (TBA) solution and incubated in a boiling water bath (~100 °C) for 1 h. Samples were read at A_{532} on a microplate reader, and the amount of MDA formed was calculated and expressed by nmol/mg protein.

Assays for Antioxidant Enzymes

Activities of two key antioxidant enzymes, catalase (CTL) and glutathione peroxidase (GPX) [25], were assessed by CTL and GPX Activity Colorimetric Assay Kit (Bio Vision, Milpitas, CA), respectively, following the manufacturer’s protocols. Cell extracts (containing CTL and GPX) were separately added to the respective reaction mixtures and read for CTL at 570 nm or GPX at 340 nm on a microplate reader. CTL and GPX activities (mU/ml) were then expressed by the % relative to the respective Sham reading (100%).
Western Blot Analysis

Effects of OXS on four kidney injury biomarkers were analyzed using Western blots. Briefly, cell extracts (10 µg), obtained from tissue homogenization, were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot (membrane) was incubated with primary antibodies against four biomarkers, neutrophil-gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (Kim-1), clusterin (CLU), and heat shock protein 70 (Hsp70) [26-29] (Santa Cruz Biotechnology, Santa Cruz, CA) for 90 minutes, followed by 30-minute incubation with appropriate secondary antibody conjugates. Specific immune reactive protein bands were detected by chemiluminescence following manufacturer’s protocol (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

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.

Results

Status of Renal Function Following ADN and/or PE Administration

To assess renal function, all rats in the four groups were subjected to the evaluation of BUN and Cr in blood specimens at the end of two weeks. Blood analysis (Figure 1) showed that the BUN and Cr levels were ~3.1 and ~4.6 times higher in the ADN group, respectively, than those in the Sham group. However, these elevated BUN and Cr levels were reduced by ≥36% with PE supplement (ADN/PE group). PE by itself had no effects on BUN and Cr (PE group). Thus, the elevated BUN/Cr levels by ADN indicate renal dysfunction but their significant (≥36%) reduction with PE implies improved renal function.

Histopathologic Examination on Rat Kidneys

Effects of ADN and/or PE on the rat kidneys were examined histopathologically and the findings are displayed in (Figure 2). The Sham group shows a normal, undamaged kidney while the ADN group exhibits remarkable histological alterations with renal tubular degeneration, indicating typical kidney damage. However, such palpable kidney damage is less remarkable with PE supplement as seen 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 kidney. Thus, PE is capable of protecting the kidneys to a certain extent from ADN assault, thereby preventing extended kidney damage (CKD).

ADN-Induced OXS and PE

It was important to understand how ADN would induce CKD and how PE would protect the kidneys from it. Since we hypothesized that OXS (induced by ADN) might play a significant role in such ADN-induced CKD, this possibility was then tested. The LPO assay was performed on rat specimens to determine the severity of OXS. As shown in (Figure 3), ADN exerted ~2.6-fold greater OXS (on the kidneys) than the Sham group, whereas this elevated OXS level was significantly (~30%) reduced with PE supplement (ADN/PE group). As expected, PE alone had no effects (PE group). Thus, ADN-mediated toxicity appears to be primarily attributed to OXS, but PE has significantly diminished/reduced it, demonstrating its antioxidant activity.
Figure 3: OXS mediated through ADN. LPO assay was performed to assess the severity of OXS by determining the amount of MDA formed (nmol/mg protein). All data are mean ± SD from three specimens of each group (*p <0.05 compared with Sham).

Up-Regulation of Kidney Injury Biomarkers by OXS

To confirm if extended renal cell injury is induced by OXS, its possible effects on four kidney injury biomarkers, NGAL, Kim-1,CLU, and Hsp70, in the rat kidneys were examined. Western blots revealed that the expressions of all four biomarkers were enhanced or up-regulated by ADN, but this was apparently prevented with PE (ADN/PE group) as they remained similar to those in the Sham’s (Figure 4). It should be noted that the actual intensities of four 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). The up-regulation of these biomarkers is indicative of renal cell injury [26,27,30], but sustaining their natural/basal status with PE suggests that renal cells are well protected (from ADN attack) with PE and remain fairly intact.

Figure 4: Up-regulation of kidney injury biomarkers by ADN. Kidney specimens obtained from different experimental conditions were analyzed for four biomarkers using Western blots. Autoradiographs of NGAL, Kim-1,CLU, and Hsp70, are shown and β-actin was also run as a protein loading control.

Protection of Antioxidant Enzymes with PE

Lastly, we examined if extended kidney damage/injury could be also due to some adverse effects of OXS on antioxidant enzymes [25], which played a major defense role against OXS [10]. Kidney specimens were assayed for activities of two key antioxidant enzymes, catalase (CTL) and glutathione peroxidase (GPX). The results showed that CTL and GPX activities declined to ~46% (i.e. ~54% loss) and ~61% (i.e. ~39% loss) of those (100%) in the Sham group by OXS, respectively (Figure 5). This may account for further extended kidney damage by OXS. However, PE effectively protected these enzymes (from OXS), sustaining >80% of their activities (Figure 5).

Figure 5: Inactivation of antioxidant enzymes by ADN. Kidney specimens with different experimental conditions were assayed for activities of two antioxidant enzymes, CTL and GPX. The data are mean ± SD from three specimens of each group (*p <0.05 compared with ADN).

Discussion

We hypothesized that OXS could be a key pathogenic factor for CKD and certain antioxidants might be able to diminish OXS to control CKD. In fact, antioxidants have been documented to effectively protect (renal) cells from such OXS [10]. PE has been shown to have various pharmacological properties including antioxidant and renoprotective activities [11,12]. 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, ADN was found to adversely affect kidney function, indicated by the significantly increased levels of BUN and Cr (Figure 1). However, those elevated BUN and Cr levels have declined with PE supplement, implying the improvement in renal function. Nevertheless, ADN-mediated renal dysfunction is a sign of CKD and in fact, more detailed histologic examination revealed deposition of 2,8-DHA (brown pigmented crystals), tubular degeneration, interstitial mononuclear inflammation etc.
This possibility may deserve further exploration. Some other derivative(s) may have even better/stronger activity (capable of reducing OXS by ~30%) but it is yet possible that activities of side-chain branching [37]. In other words, actual antioxidant monosaccharide composition, glycosidic linkages, and extent of several structural parameters, such as solubility, molecular weight, instance, antioxidant activity of these derivatives is associated with being isolated with the diverse degrees of biological properties such as antioxidant, anticancer, immunological activities etc. [36]. For mushrooms, 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 (Figure 4), demonstrating its antioxidant activity to protect renal cells from ADN assault.

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 ~2.6-fold higher/severer in the ADN group (than that in the Sham) (Figure 3). 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 four kidney injury biomarkers, NGAL, Kim-1, CLU, and Hsp70 [27-30], 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 [26,27,30]. 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 [31]. Its production is known as an indicator in response to OXS even before kidney dysfunction can be detected by other biomarkers [32]. 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 3) and maintaining the basal status of all four biomarkers (under OXS) (Figure 4), demonstrating its antioxidant activity to protect renal cells from OXS.

It would be also interesting to talk more about antioxidant activity of PE. Nowadays, many synthetic antioxidants are available in the market, but they could be potentially hazardous to your health [33]. 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 [34,35]. 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. [36]. For instance, antioxidant activity of these derivatives is associated with several structural parameters, such as solubility, molecular weight, monosaccharide composition, glycosidic linkages, and extent of side-chain branching [37]. In other words, actual antioxidant activities of Poria mushroom derivatives could vary with others. PE used/tested appears to have fairly good antioxidant activity (capable of reducing OXS by ~30%) but it is yet possible that some other derivative(s) may have even better/stronger activity. This possibility may deserve further exploration.

Another interesting finding was that the two key antioxidant enzymes, CTL and GPX [25], significantly (~40-55%) lost their enzymatic activities by OXS (Figure 5). This finding implies a further breakdown/collapse of the antioxidant defense system that makes renal cells even more vulnerable to OXS and leads to extended renal cell injury. Besides non-enzymatic or chemical antioxidants (e.g., PE, vitamins C/E etc.) [10], antioxidant enzymes are also an integral part of the key cellular defense system (against OXS etc.) that must be active and functional. These enzymes are present in our body (since a birth) but will become weaker or inactive as we get older. This may partially account for why old people are less healthy and more vulnerable to illness. OXS can indeed inactivate antioxidant enzymes in the kidneys as well as other organs, feasibly resulting in CKD and other serious diseases. Nevertheless, PE may also help protect a variety of organs from OXS-induced adverse and life-threatening conditions.

Furthermore, 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 phosphoribosyl transferase, it is usually kept at the significantly low level in blood and urine [38]. However, once ADN becomes excessive, it acts as a substrate for xanthine dehydrogenase, which could then oxidize ADN to 2,8-DHA [19,39]. Due to such a low solubility of 2,8-DHA, it readily precipitates in renal tubules and forms tubular crystals [21]. 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, ADN-induced CKD in the rats could be primarily attributed to accumulation of ADN crystals in the rat kidneys, which in turn exerts severe OXS on renal cells. OXS then causes renal cell damage/injury, accompanied by inactivation of antioxidant enzymes. Such renal cell injury would ultimately lead to CKD. However, PE is capable of protecting the kidneys from all these adverse effects induced by ADN/OXS. This finding is consistent with the early report describing that another extract of Poria mushroom prepared by different procedures was used for the prevention or treatment of CKD [40].

Conclusions

The present study demonstrates that oxidative stress mediated through adenine can eventually induce chronic kidney disease, a progressive and irreversible disease, in the rats. However, the Poria mushroom extract with antioxidant activity has protective effect against such an adenine-induced chronic kidney disease, preventing its development/progression. Therefore, the Poria
mushroom extract could be a natural antioxidant against chronic kidney disease and may have clinical implications with few side effects. Further studies are warranted.

**Acknowledgement:** We thank Ms. Donna Noonan (Mushroom Wisdom, Inc.) for generously providing us with PE and financial support.

**Conflicts of Interest:** The authors declare that there is no conflict of interest.

**References**


