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# Prooxidant Activity of *Poria* Mushroom Extract on Renal Cell Carcinoma: Potential Therapeutic Strategy

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## Abstract

**Background:** Establishing more effective therapeutic modalities for Renal Cell Carcinoma (RCC) has been urgently demanded. Oxidative Stress (OXS) has been known to exert adverse effects on a variety of cells, but it is currently considered as one of anti-cancer strategies, due to greater vulnerability of cancer cells (than normal cells) to it. A bioactive extract from *Poria* mushroom, PE, has been shown to have anticancer effect through possible prooxidant (exerting OXS) activity. Accordingly, we investigated if PE could be a potential therapeutic agent for RCC.

**Methods:** Human RCC cell line (ACHN), was treated with varying concentrations of PE and cell viability was assessed. Several biochemical parameters were examined for exploring the anticancer mechanism of PE.

**Results:** PE concentrations ≥100 μg/ml led to a significant cell viability reduction in ACHN cells. Compared to control cells, PE has exerted ~2.1 times severer OXS and antioxidant enzymes (catalase and glutathione peroxidase) significantly lost their activities as well. In the glycolytic pathway, hexokinase activity and ATP synthesis declined with PE, implying the impediment of glycolysis that could lead to the growth cessation and cell death. In fact, PE was found to ultimately induce apoptosis in ACHN cells.

**Conclusions:** The present study shows that PE is capable of exerting OXS on ACHN cells, resulting in the significant cell viability reduction. Such an anticancer mechanism involves inactivation of antioxidant enzymes, inhibition of glycolysis, and induction of apoptosis. Thus, PE is considered as an anticancer agent with prooxidant activity that could be used against RCC.

**Keywords:** Oxidative stress; *Poria* mushroom; Prooxidant; Renal cell carcinoma

## Introduction

Renal Cell Carcinoma (RCC) is the third most common genitourinary tumor with approximately 74,000 new cases and nearly 15,000 deaths (2019) in the United States [1], and nearly 30% of those patients with RCC would present with metastatic disease at the time of diagnosis [2]. Standard treatment for non-metastatic RCC is complete resection of the tumor by either a radical or partial nephrectomy [3]; however, 20-30% of patients will progress to a metastatic disease with the 5-year survival rate of <10% [4], which is devastating and dismal. Although several viable options, such as surgery, radiotherapy, chemotherapy, or immunotherapy [2,5], are currently available, no effective therapeutic modalities have been established yet. Among them, immunotherapy has some good outcomes but with a high dose, high cost, and considerable side effects [6]. Incidentally, combination of nephrectomy and immunotherapy, i.e. nephrectomy followed by interferon-$\alpha_{2b}$ or interleukin-2 infusion, has shown the improved outcomes with longer survival than immunotherapy alone [7]. However, more studies are yet demanded for establishing an improved therapeutic modality.

To find a safer and better treatment modality, we have been exploring natural agents/substances with anticancer activity. We came across the bioactive mushroom extract, PE isolated from *Poria* mushroom, which has been used in Traditional Chinese Medicine for 2,000 years [8]. It has been well characterized and found to have various properties, such as antioxidant, immunomodulatory, anticancer/antitumor, and renoprotective effects [9-11]. As PE is a natural agent, it may have few side effects and clinical implications in cancer treatment. Oxidative stress (OXS), i.e. generation of Reactive Oxygen Species (ROS), has been known to exert adverse effects on a variety of cells, injuring, damaging, and even killing them [12]. Hence, OXS is harmful or detrimental and was

never appreciated by any means. There is also the interesting fact that cancer cells are generally more vulnerable to OXS than normal counterparts [13], presumably due to the weakened or lack of antioxidant system. The exact reason remains elusive but at least it is believed to be the inherent difference in tissue-specific antioxidant enzymes. If we can exert threshold OXS, it could be severe or strong enough to kill only cancer cells but not severe enough to even injure normal cells. Taking advantage of this bizarre nature of cancer cells, OXS is currently used as one of anticancer strategies and the successful cases of OXS strategy have been reported in several cancers [14-16].

Accordingly, we investigated if PE could be used as an anticancer agent, capable of exerting OXS on RCC *in vitro*, because it has antioxidant activity but may also have "prooxidant" activity (exerting OXS) like vitamin C (VC) [17]. We further explored how PE-induced OXS would adversely affect ACHN cells. We then examined whether OXS would negatively affect antioxidant enzymes and/or glycolysis that is the crucial metabolic process required for cellular activity, survival, and proliferation [18,19]. Possible effect on glycolysis was examined by how two key glycolytic parameters, Hexokinase (HK) [20] activity and cellular ATP synthesis, were affected by OXS. Lastly, we assessed whether cell death induced by OXS would be linked to apoptosis, which could primarily account for the significant reduction in cell viability. After all, these studies may help us address the OXS-mediated anticancer mechanism of PE. More details are described and the interesting findings are also discussed herein.

## Materials and Methods

### Cell Culture

The human renal cell carcinoma ACHN cells were employed as our *in vitro* model. PE was a generous gift from the manufacturer (Mushroom Wisdom, Inc., East Rutherford, NJ). For experiments, ACHN cells (2 x $10^5$ cell/ml) were seeded in 6-well plates or flasks and cultured with varying concentrations of PE. Cell viability was then assessed at 72 h by the MTT assay 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, St. Louis, MO). At the harvest time, MTT reagent (1 mg/ml) was added to cells in the 6-well plate. After 3-h incubation at 37 °C, dimethyl sulfoxide (DMSO) was added to the plate and absorbance was read in a microplate reader. Cell viability was then expressed by the % relative to the control reading (100%).

### Lipid Peroxidation (LPO) Assay

The severity of oxidative stress can be assessed by LPO assay, which measures the amount of Malondialdehyde (MDA) formed in the plasma membrane due to OXS [21]. It indicates that the more MDA formed, the greater oxidative stress. The detailed procedures are described in the vendor's protocol (Abcam, Cambridge, MA). The amount of MDA formed is determined from the MDA standards and expressed by μM.

### Assays for Antioxidant Enzymes

Activities of two antioxidant enzymes, Catalase (CTL) and Glutathione Peroxidase (GPX) [22], were assessed by CTL and GPX Activity Colorimetric Assay Kit (BioVision, Milpitas, CA), respectively, following the vendor's protocols. Cell lysates (containing CTL and GPX) of control or PE-treated cells 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 separately expressed by the % relative to the respective control reading (100%).

### Hexokinase (HK) Assay and Determination of Cellular ATP Level

HK activity and ATP level are determined by the HK or ATP Colorimetric Assay Kits (BioVision), respectively, following the manufacturer's protocol. The reaction was started by adding cell lysates to the respective reaction mixture, and absorbance changes at 340 nm for HK and at 570 nm for ATP were measured on a microplate reader. All readings were then calculated and normalized (with respective standards) and HK activity as well as ATP level was expressed by the % of sample activity relative to the controls (100%).

### Western Blot Analysis

An equal amount of cell lysates (10 μg) was first subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot (membrane) was incubated with primary antibodies against two apoptotic regulators, bcl-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA) for 90 min, followed by incubation with secondary antibody conjugates for 30 min. Specific immunoreactive protein bands were detected by chemiluminescence following manufacturer's protocol (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

### Statistical Analysis

All data are presented as mean ± SD (standard deviation), and statistical differences between groups are assessed with one-way analysis of variance (ANOVA). Values of $P < 0.05$ are considered to indicate statistical significance.

## Results

### Dose-Dependent Effect of PE on ACHN Cell Viability

ACHN cells were cultured with varying concentrations of PE (0-200 μg/ml) and cell viability was assessed in 72 h by MTT

assay. PE concentrations ≥100 µg/ml showed a ~50%, ~80%, and ~90% reduction in cell viability with 100, 150, and 200 µg/ml, respectively (Figure 1). Thus, PE is capable of significantly reducing ACHN cell viability. As PE (100 µg/ml) is proximate to its $IC_{50}$ (50% inhibitory concentration), this concentration was used in the rest of our study.

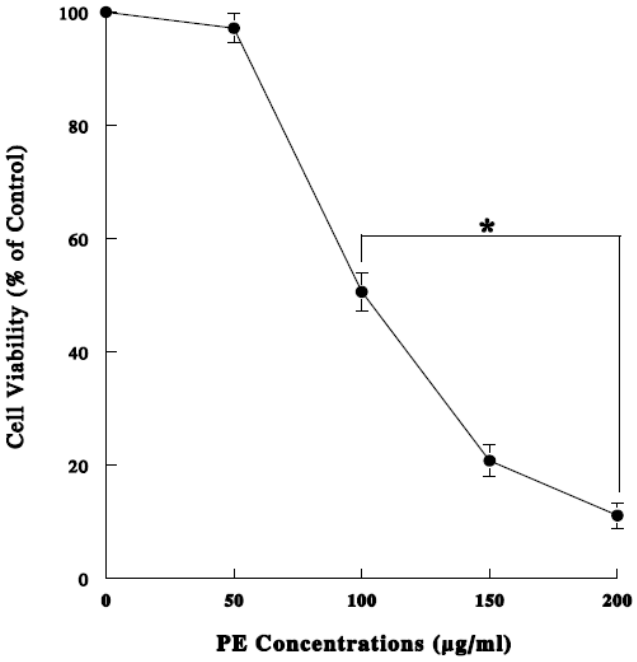


**Figure 1:** Dose-dependent effect of PE on cell viability. ACHN cells were cultured with varying concentrations of PE (0-200 µg/ml) and cell viability was assessed in 72 h by MTT assay. Cell viability was expressed by the percent (%) of viable cells relative to controls (100%). The data are mean ± SD (standard deviation) from three separate experiments (*P <0.05 versus control).

### Exertion of Oxidative Stress (OXS) by PE

Whether PE-induced cell viability reduction could be due to OXS was examined next. Hydrogen peroxide ($H_2O_2$, 50 µM), one of typical ROS [12], was used as a positive control for exerting OXS. After cells were exposed to PE (100 µg/ml) or $H_2O_2$ (50 µM) for 6 h (as OXS usually takes place at the early phase), they were subjected to LPO assay to determine the severity of OXS (relevant to the amount of MDA formed). Compared to controls, $H_2O_2$ and PE led to a ~3.2-fold and ~2.1-fold increase in the MDA amounts at 6 h, respectively, (Figure 2). Thus, PE can exert severe OXS on ACHN cells, eventually resulting in the significant cell viability reduction (Figure1).

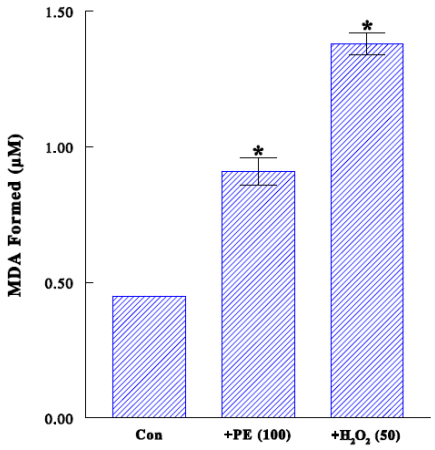


**Figure 2:** Assessment of severity of OXS. After cells were exposed to PE (100 µg/ml) or $H_2O_2$ (50 µM) for 6 h, the amount of MDA formed (µM) was assessed by LPO assay. All data are mean ± SD from three independent experiments (*P <0.05 versus control).

### Effects of OXS on Antioxidant Enzymes

It was important to address how OXS might affect two key antioxidant enzymes [22], Catalase (CTL) and Glutathione Peroxidase (GPX), which would diminish OXS to protect the cells. Cells were treated with or without PE (100 µg/ml) for 72 h and assayed for CTL and GPX activities. Enzymatic assays showed that activities of CTL and GPX have lost by ~60% and ~45%, respectively (Figure 3). Thus, it is plausible that the significant loss of these enzymatic activities by OXS would further weaken or collapse the cellular antioxidant (defense) system.

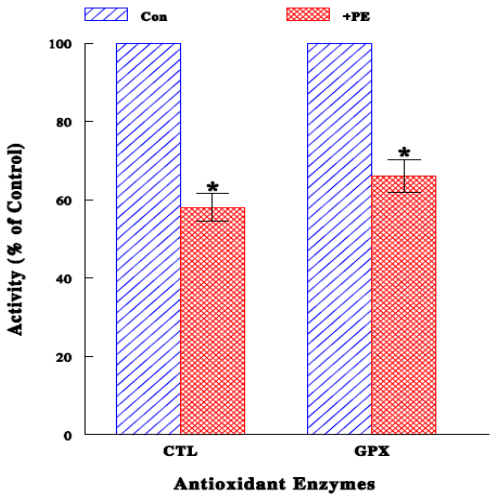


**Figure 3:** Effects of OXS on antioxidant enzymes. Cells treated with or without PE (100 µg/ml) for 72 h were assayed for their activities. All data are mean ± SD from three separate experiments (*P <0.05 versus control).

### Inhibitory Effect of PE on Glycolysis

To have an insight into how PE-exerted OXS would induce a significant cell viability reduction, we examined if glycolysis could be affected/inhibited by PE because it is the vital metabolic process required for cell proliferation and survival [18,19]. We specifically assessed the status of one of key glycolytic enzymes, HK, that was involved in the irreversible committed step in glycolysis [20]. Cells were treated with PE (100 µg/ml) for 72 h and subjected to HK assay. Compared to controls (100%), HK activity declined to ~60% (i.e. a ~40% activity loss) with PE treatment (Figure 4). As a disruption of glycolytic pathway could lead to the reduced yield of the end product (i.e. ATP), the status of cellular ATP level was also assessed. The ATP level was indeed down to ~45% (i.e. a ~55% reduction), presumably due to HK inactivation by PE (Figure 4). Thus, the reduction in HK and ATP levels by PE indicates the inhibition of glycolysis, eventually resulting in the growth cessation and the cell viability reduction.

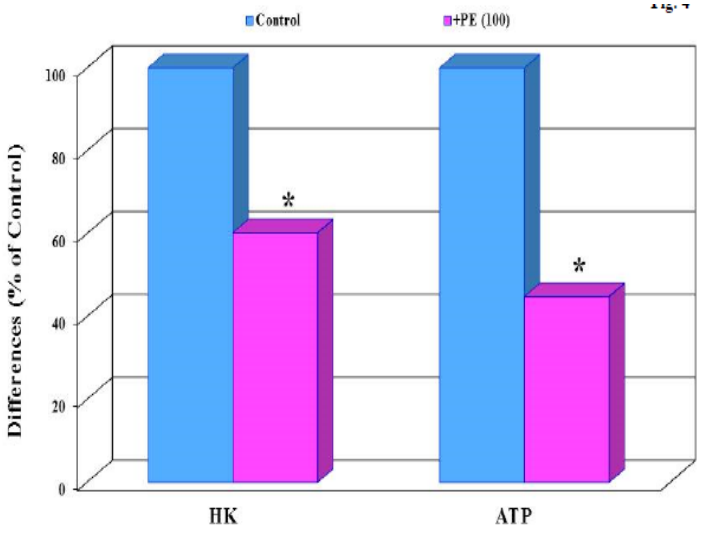


**Figure 4:** Inhibition of glycolysis by PE. After cells were treated with PE (100 µg/ml) for 72 h and subjected to HK and ATP assays separately. HK activity and ATP level are expressed by the % relative to respective controls (100%). The data are mean from three separate experiments (*P <0.05 versus control).

### Induction of Apoptosis by PE

Now, PE-induced cell death appears to be primarily due to OXS and such cell death has been reported to consequently lead to apoptosis [23]. To test this possibility, cells were treated with PE for 72 h and the status of two key apoptosis regulators, bcl-2 and Bax, were analyzed on Western blots. We found that PE treatment led to the down-regulation (reduced expression) of anti-apoptotic bcl-2 and the up-regulation (elevated expression) of pro-apoptotic Bax (Figure 5). The specific modulations of these regulators by PE will favor and indicate induction of apopotosis [24]. Thus, PE may ultimately induce apoptosis in ACHN cells, accounting for the cell viability reduction.

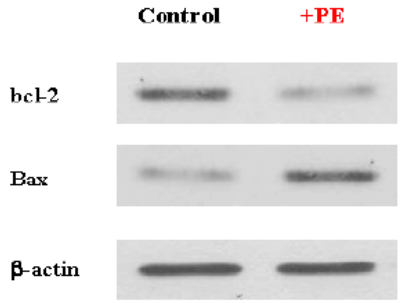


**Figure 5:** Induction of apoptosis. After cells were cultured with or without PE (100 µg/ml) for 72 h, the expressions of two apoptotic regulators, bcl-2 and Bax, were analyzed on Western blots. Autoradiographs of these regulators in control and PE-treated cells are shown for comparison. Beta-actin was also run as an internal protein loading control.

### Enhanced Anticancer Effect of PE Combined with VC

Lastly, as VC is a well-known antioxidant but can also act as prooxidant [17], we tested if prooxidant activity of PE could be further enhanced when combined with VC. Cells were cultured with an ineffective concentration of PE (50 µg/ml, See Figure 1), VC (100 µM), or combination of PE and VC (PE/VC), and cell viability was determined in 24 h. The results show that PE and VC alone had little effects (~100% cell viability) but cell viability declined to ~20% (i.e. a ~80% reduction) with PE/VC combination (Figure 6). Thus, this finding suggests that PE could be *synergistically* potentiated with VC, becoming highly cytotoxic to ACHN cells.

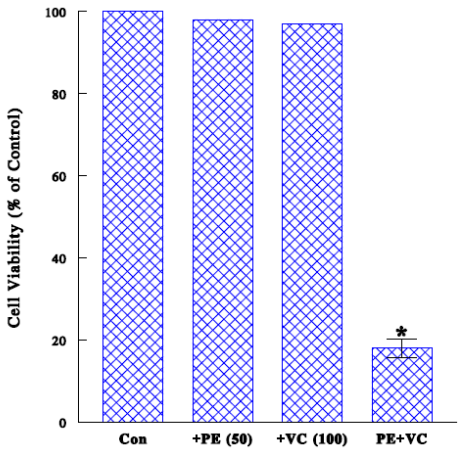


**Figure 6:** Synergistic effect of PE and VC combination. Cells were treated with PE (50 µg/ml), VC (100 µM), or combination of PE and VC (PE+VC), and cell viability was determined in 24 h. The data are mean from three independent experiments (*P <0.01 versus control).

## Discussion

It has been long overdue to establish the minimally invasive therapeutic modalities for improving prognosis and survival rate of metastatic RCC. Particularly, finding a more effective oral agent should be the primary target. In this study, we investigated the potential anticancer effect of *Poria* mushroom extract, PE, on human renal carcinoma ACHN cells *in vitro*. We found that PE indeed had a potent anticancer effect with the $IC_{50}$ of ~100 μg/ml. To explore its anticancer mechanism, we first examined if PE might exert Oxidative Stress (OXS) on ACHN cells. LPO assay showed that PE did exert severe OXS on ACHN cells as indicated by a ~2-fold increase in MDA formation (Figure 2). This finding suggests that anticancer mechanism of PE could be primarily attributed to OXS.

Actually, OXS has been always considered as an adverse or hostile cellular event because of its destructive nature. Especially, OXS has been often shown to injure and even kill normal cells but hardly mentioned to kill cancer cells as well. More interestingly, it has been documented that generally cancer cells were more vulnerable to OXS than normal cells [13]. For instance, CTL and GPX have been shown to be often deficient or to have significantly lower activities in cancer tissues than in normal [25]. Additionally, the reduced expressions of CTL and superoxide dismutase have been also found in prostate cancer specimens, compared to those in normal or benign prostate hyperplasia specimens [26]. It is thus plausible that at least a difference in antioxidant enzyme activities between normal and cancer cells may account for their different vulnerability to OXS. Based on this greater vulnerability of cancer cells to OXS, it is now considered as one of anticancer strategies and the successful outcomes have been reported in several cancer cases [14-16]. In our case, PE may act as a prooxidant, generating ROS, and its severity could be high enough to kill cancer (ACHN) cells (due to low enzyme activity) but would not be high enough to even harm normal counterparts (due to high enzyme activity). This supports the notion that anticancer mechanism of PE would be primarily attributed to OXS. Thus, PE could be used as an OXS-mediated anticancer strategy for RCC treatment.

As it was of our interest to examine if OXS would somehow affect antioxidant enzymes, we tested this possibility. Two key enzymes tested, CTL and GPX, have significantly lost their enzymatic activities under OXS attack (Figure 3). Such a loss in their activities clearly suggests that the cellular antioxidant (defense) system might have been severely weakened or collapsed. This is the devastating situation because cells without a defense system would be further vulnerable to even relatively weak OXS, resulting in total cell destruction (cell death). Nevertheless, to better understand how OXS would lead to the cell viability reduction or cell death, we examined the possible effect of OXS on glycolysis, which was an essential metabolic process required for cell growth and survival [18,19]. Such study showed that PE specifically targeted HK, the key glycolytic enzyme to trigger and carry out the glycolytic pathway [18]. As a result, the rest of the pathway was shut down and cellular ATP synthesis also declined (as the reduced cellular ATP level), indicating the inhibition of glycolysis. Cells would subsequently stop growing and result in cell death. In fact, we found that such cell death could be more likely associated with apoptosis. This may further account for a significant reduction in cell viability (due to apoptosis) by OXS (through PE). However, further studies are required for fully elucidating the intricate biochemical pathways leading to apoptosis.

Moreover, PE was found to be highly potentiated with VC, inducing a ~80% cell viability reduction. In other words, ineffective concentration (50 μg/ml) of PE can become highly cytotoxic to drastically reduce cell viability when combined with another ineffective concentration (100 μM) of VC. This finding is rather interesting since VC is a well-known "antioxidant" but could have acted as a prooxidant (exerting OXS) with PE instead, further exerting severer OXS on cells. Nevertheless, it is not entirely new or surprised to find that VC could act as a prooxidant because its dual property has been documented [17]. It would be significant if this synergistic potentiation of PE and VC were indeed valid, but more studies are yet required for further confirmation.

## Conclusions

The present study demonstrates anticancer effect of *Poria* mushroom extract on renal cell carcinoma ACHN cells. Its anticancer mechanism is primarily associated with oxidative stress, which causes inactivation of antioxidant enzymes, glycolysis inhibition, and induction of apoptosis. In addition, *Poria* mushroom extract could be synergistically potentiated with vitamin C, becoming highly cytotoxic (feasibly due to enhanced oxidative stress). Therefore, *Poria* mushroom extract is a natural anticancer agent with prooxidant activity and may have clinical implications as oral supplement for more effective therapeutic control of renal cell carcinoma.

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