An extract of the oyster mushroom, *Pleurotus ostreatus*, increases catalase gene expression and reduces protein oxidation during aging in rats

Thanasekaran Jayakumar¹, Philip Aloysius Thomas², Mathivanan Isai¹, Pitchairaj Geraldine¹

1. Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
2. Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirappalli 620 001, Tamil Nadu, India

**Objective:** The objective of the present study was to address the effect of mushroom *Pleurotus ostreatus* on the catalase (CAT) gene expression and the protein carbonyls in liver and kidney of aged (24 months old) rats.

**Methods:** Eighteen acclimated rats were divided into 3 groups of 6 each; group I, normal young (4 months old) rats; group II, normal aged (24 months old) untreated rats; group III, normal aged rats treated with mushroom *P. ostreatus* extract (200 mg/kg body weight administered intraperitoneally for 30 days). On the 31st day, rats were sacrificed by decapitation, the livers and kidneys were removed, washed free of blood, blotted dry and processed immediately. Reverse transcriptase-polymerase chain reaction (RT-PCR) and spectrophotometry were utilized for the analyses of CAT gene expression and protein carbonyl content in the tissues of livers and kidneys.

**Results:** In aged rats that had been treated with the extract of *P. ostreatus* (group III), the level of the transcript of CAT gene was found to be higher than that in liver (*P*<0.01) and kidney (*P*<0.05) of aged untreated (group II) rats, respectively. Treatment of aged rats with *P. ostreatus* extract (group III) resulted in protein carbonyl levels being significantly lower in liver (*P*<0.05) and kidney (*P*<0.01) than those observed in aged untreated (group II) rats.

**Conclusion:** These results suggest that an extract of *P. ostreatus* can enhance the antioxidant enzyme (CAT) gene expression and could decrease the incidence of free radical-induced protein oxidation in aged rats, thereby protecting the occurrence of age-associated disorders that involve free radicals.

**Keywords:** *Pleurotus ostreatus*, catalase, reactive oxygen species, reverse transcriptase-polymerase chain reaction, protein carbonyl, rats

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蚝菇提取物对老年大鼠肝肾组织内过氧化物酶表达及蛋白质氧化的影响

Thanasekaran Jayakumar¹, Philip Aloysius Thomas², Mathivanan Isai¹, Pitchairaj Geraldine¹

1. Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
2. Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirappalli 620 001, Tamil Nadu, India

**目的:** 讨论蚝菇提取物对老年大鼠（24 月龄）肝肾组织内过氧化物酶基因表达及蛋白质羰基含量的影响。

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**Correspondence:** Pitchairaj Geraldine, PhD, Professor; Tel: +91-431-2407040; E-mail: gerryarchup@yahoo.co.in
方法：18 只大鼠被分为 3 组，每组 6 只。组 1 为健康年轻大鼠（4 月龄）对照组；组 2 为健康老年大鼠（24 月龄）对照组；组 3 为健康老年大鼠（24 月龄）蚯蚓提取物干预组。干预措施为每天腹腔注射蚯蚓提取物 200 mg/kg 体重，连续注射 30 d。第 31 天处死全部大鼠并取出肝组织。使用逆转录聚合酶链反应及分光光度法定法分别测定大鼠肝组织内过氧化物酶基因表达情况及蛋白质羰基的含量。

结果：组 3 老年大鼠注射蚯蚓提取物后，其肝组织内过氧化物酶基因表达显著高于组 2 老年大鼠，且组 3 老年大鼠肝组织内蛋白质羰基含量显著低于组 2 老年大鼠。

结论：蚯蚓提取物能够增加老年大鼠肝组织内抗氧化酶基因的表达并减少自由基引起的蛋白质氧化，对于自由基引起的年龄相关性疾病可能有保护作用。

关键词：蚯蚓；过氧化氢酶；活性氧；逆转录聚合酶链反应；蛋白质羰基；大鼠

Aging is a universal biological phenomenon associated with histological, biochemical, and functional alterations. It is generally accepted that free radicals play an important role in the progress of aging[1]. Inflammatory or aging processes[2] are associated with the disruption of the oxidant/antioxidant (redox) balance resulting in cellular and tissue oxidative stress leading to cell death by apoptosis[3, 4]. Mammalian cells are generally shown to be more tolerant of oxidative stress than bacteria because of their better developed natural defenses[5]. These defenses are essentially composed of specialized antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) and also non-enzymatic antioxidant molecules such as vitamins, thiols and β-carotene.

However, since regulation of gene expression is sensitive to subtle changes in the redox condition, as observed during aging, would account for many altered gene products in aged organisms[6]. Further, recent molecular studies on oxidative stress have shown altered gene regulation during aging[7,8], which provide additional evidence for the association of oxidative stress at the gene level. Therefore, gene profiling data permit documentation of alterations in gene expression with age and of changes in various functional parameters of aged organisms[9].

Oxidative damage to DNA, proteins and other macromolecules accumulates with age and has been postulated to constitute a major type of endogenous damage leading to aging[10]. Proteins are particularly susceptible to oxidative damage because of their inherent structural flexibility and reactive amino acid residues[11, 12]. Oxidative damage to protein is reflected in the level of protein carbonyl (PCO). The reaction of free radicals, such as hydroxyl (OH·) or superoxide (O2−) with the side chain of lysine, arginine, proline, threonine and glutamic acid residues of protein leads to the formation of carbonyl derivatives[13]. Carbonyl groups can also be introduced into proteins by glycation and glycoprotein reactions[14]. Therefore, modifications in protein carbonyl are good indicators of the presence of oxidized proteins that increase with advancing age[15, 16].

Nutritional antioxidant supplementation can have health-promoting effects if it could control the endogenous redox system[17]. Therefore, there is an increasing interest in the natural antioxidants that includes catechin in green tea, ferulic acid in orange juice, carotenoids in mango, quercetin in tomato, onion and lettuce and proanthocyanidine in grape seeds and red wine, which have been worked out as candidates for the prevention of oxidative damage. Its implication in elevating the antioxidant status in animal model has been extensively reported[18-20]. Further, administration of the rose-flower extract as a potent antioxidant has been reported to alter the gene expression on antioxidant enzymes CAT, and Gpx in liver of aged mice[21].

Mushrooms have long been considered as an essential part of the normal human diet; these are reported to contain comparatively large amounts of vitamins A, C and β-carotene, all of which have protective effects because of their antioxidant properties[22]. Mushrooms contain many phenols, which serve as very efficient scavengers of perox radicals[23]. Ethyl acetate and methanol extracts of Phleurotus floridus have been found to exhibit potent scavenging of hydroxyl radicals and inhibition of lipid peroxidation activities[24]. In addition, an extract of the mushroom Phleurotus ostreatus has been shown to prevent selenite-induced cataractogenesis[25] and to improve the antioxidant status of various organs during aging[26].

P. ostreatus is reported to contain higher concentrations of cystine, methionine and aspartic acid than the other edible mushrooms, such as Agaricus bisporus (brown), A. bisporus (white) and Lentinus edodes[27]. The aim of this study was to investigate whether an ethanolic extract of the P. ostreatus could enhance the gene expression of the antioxidant enzyme CAT and reducing effect on the protein carbonyls in the livers and kidneys of aged rats.

1 Materials and methods

1.1 Preparation of the mushroom extract The mushroom P. ostreatus was cultivated by adopting the “layer spawning” method. Freshly-harvested whole mushrooms were shade-dried and then finely
powdered. Five grams of the powder were extracted with 100 mL of 95% ethanol by using a Soxhlet apparatus. The material thus obtained was filtered, and the resulting filtrate was concentrated to a dry mass by vacuum distillation; this was used as mushroom extract.

1.2 Animal experiment Male albino Wistar rats weighing approximately 75 to 100 g (4 months old) and 350 to 375 g (24 months old) were used for the experiment. The animals were acclimatized for 20 days prior to dosing, during which time they had free access to food and water ad libitum. Eighteen such acclimatized rats were divided into 3 groups of 6 each: group I, normal young (4 months old); group II, normal aged (24 months old) untreated rats; group III, normal aged rats treated with mushroom extract (200 mg/kg body weight administered intraperitoneally for 30 days). On the 31st day, rats were sacrificed by decapitation; the livers and kidneys were removed, washed free of blood, blotted dry and processed immediately.

1.3 Total RNA isolation Total RNA was extracted from freshly isolated rat liver and kidney by using 1 mL of the TRIzol reagent as described by the suppliers (Sigma Aldrich, USA). RNA was resuspended in 50 µL of RNase-free water, and the quantity and purity of the total RNA were determined by spectrophotometry and agarose gel electrophoresis[21], respectively.

The primers for CAT and β-actin that were reported in literature were utilized for this study as shown in Table 1 and were procured from Sigma Aldrich, USA. The Tm values of the forward and reverse primers of CAT (67 °C) and β-actin (64 °C) were calculated. The length was chosen to be between 21—25 nucleotides and G/C content between 45%—50%.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>Forward: 5'-ACAACCTCCAGAGCCTAAGAGT-3'</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCTTTTGTCTGGACGCTATG-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5'-ACGCTGACAGGATGCGAGAAG-3'</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGAGCCACCAATTCCACGAGA-3'</td>
<td></td>
</tr>
</tbody>
</table>

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with one-step RT-PCR kit (Qiagen, Germany) as per the manufacturer’s instructions, using 2 µg each of the template RNA, 0.6 µmol/L of each of the forward and reverse gene specific primers CAT/β-actin in a Techne (UK) thermal cycler. The RT-PCR conditions were as follows: (1) reverse transcription, 30 min, 50 °C; (2) initial PCR activation step, 15 min, 95 °C; (3) 3-step cycling for 30 cycles, each cycle consisting of denaturation for 1.3 min at 94 °C followed by annealing for 1.3 min at 58 °C and extension for 3 min at 72 °C. Final extension was carried out at 72 °C for 10 min.

A 10 µL portion of the PCR product was electrophoresed in a 2% agarose gel. The ethidium bromide stained gel was photographed with a DS-34 type Polaroid camera and the band was scanned with a Biorad (Model GS-670) imaging densitometer. The β-actin gene was used as an internal standard for RT-PCR reaction. To quantify the transcript level, the ratio of the corresponding gene product to the β-actin gene product was calculated. Experiments were performed in duplicate.

1.4 Protein carbonyl content The protein carbonyl content was determined by the most common and reliable method based on the reaction of carbonyl groups with 2,4-dinitrophenyldrazine to form a 2,4-dinitrophenyldrazzone, described by Nakamura and Goto[22]. The protein in each liver or kidney tissue homogenate in two equal volumes (100 µL) was precipitated with 10% trichloroacetic acid (TCA). The precipitates were then treated with either 2 mol/L HCl alone (control) or 2 mol/L HCl containing 10 mmol/L dinitrophenyldrazine (DNPH) (test) at 15 °C for 1 h. After completion of the reaction, the mixture was centrifuged at 3 400×g for 10 min and the precipitates were then washed thrice with an ethanol: ethylacetate (1:1) mixture; the precipitates were finally dissolved in 8 mol/L urea. The absorbance was measured at 360 nm and the protein carbonyl content was determined as nanomoles per mg protein using molar extinction coefficient of 1/22 000.

1.5 Statistical analysis The values are expressed as x±s for six animals in each group. Differences between groups were assessed by a one-way analysis of variance (ANOVA) using SPSS 16.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Post hoc testing was performed for inter-group comparisons by using the least significant difference (LSD) test, and the chi-square test was applied wherever relevant. A value corresponding to P<0.05 was deemed to be statistically significant.

2 Results

2.1 CAT gene expression Gene profiling data permit documentation of alterations in gene expression with age[20]. Molecular studies on oxidative stress have shown the occurrence of altered gene regulation during aging[11]. In the present study, the level of the CAT gene transcript was found to be significantly lower in the liver (P<0.01) and kidney (P<0.05) of aged (group II) rats when compared with the levels in young (group I) rats.
The mean CAT gene transcript in the liver or kidney was lower in group II rats than that in group I rats. In aged rats that had been treated with the extract of *P. ostreatus* (group II), the level of the transcript of CAT gene was found to be higher than that in liver (*P*<0.01) and kidney (*P*<0.05) of aged untreated (group II) rats, respectively. So also, the expression of the CAT gene exhibits substantial variations during aging in a tissue-specific manner, which provides additional evidence for the association of oxidative stress at the gene level.

### 2.2 Level of protein carbonyl

An evaluation of the carbonyl level is possibly the most common method used for assessing the oxidative modification of proteins. Oxidative damage to protein is reflected in the protein carbonyl level. The increase in carbonyl derivatives, as a marker of protein oxidation with age was possibly due to the inability of the antioxidant defense system. In the present study, a significantly (*P*<0.01) higher level of protein carbonyl was observed in the liver and kidney tissues of aged rats, when compared with the levels in young rats (Table 2). The mean level of protein carbonyls in the liver and kidney was higher in group II rats than that in group I rats. However, treatment of aged rats with *P. ostreatus* extract (group III) resulted in protein carbonyl levels that were significantly lower in liver (*P*<0.05) and kidney (*P*<0.01) than those observed in aged untreated (group II) rats.

![Figure 1](image.png)

**Figure 1** Catalase gene transcripts in the liver and kidney of different groups of rats

![Figure 2](image.png)

**Figure 2** Agarose gel electrophoresis of RT-PCR products from total RNA isolated by using TRIzol

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Liver (μmol/mg)</th>
<th>Kidney (μmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>6</td>
<td>5.11±0.02</td>
<td>2.23±0.04</td>
</tr>
<tr>
<td>Group II</td>
<td>6</td>
<td>6.66±0.31**</td>
<td>4.20±0.27**</td>
</tr>
<tr>
<td>Group III</td>
<td>6</td>
<td>6.13±0.54ΔΔ</td>
<td>3.39±0.62ΔΔ</td>
</tr>
</tbody>
</table>

**P*<0.01 vs group I; ΔΔ*P*<0.01 vs group II.

### 3 Discussion

Determination of alteration in enzyme activity during the process of aging has been an important approach in gerontological research. While, SOD, Gpx and CAT are regarded as the first line of the antioxidant defense system enzymes against reactive oxygen species (ROS) which are generated *in vivo* during oxidative stress, CAT has the advantage to catalyse the dismutation of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and O<sub>2</sub> without requiring any additional source of reducing power as all peroxidases do. Various studies have reported the reduction in the activity of CAT enzyme in liver, liver and kidney and various regions of brain of aged rats.

While it is now widely accepted that oxidative damage plays an important role in the aging process, the influence of age on the gene expression of antioxidant enzymes has not been widely studied; moreover, the few studies that have been performed have yielded conflicting results. Limaye *et al.* reported the elevated expression of antioxidant enzymes such as CAT, SOD and Gpx in a streptozotocin-induced diabetic rats, while a significant decrease in the mRNA expression of CAT in the liver of aged rats has been documented. A decrease in the level of gene expression of CAT has already been reported in liver and lung of aged rats. A significant decrease in the expression of CAT gene was also noted in the kidney of endotoxin-induced rats. So also in the present study, a decline in the level of gene expression of CAT was recorded in liver and kidney of aged rats when compared with young rats.
Supplementation of the diet with fruits and vegetables is reported to be beneficial in reversing the deleterious effects of aging on neuronal communication and behavior. Augustyniak et al. reported that the administration of green tea extract increased the activities of CAT in the liver of aged rats. Balu et al. reported that the supplementation of the diet with grape seed extract improved the antioxidant status in the central nervous system of aged rats.

Such supplementation altering the gene expression of antioxidant enzymes, with reference to melatonin has been well documented and hence supplementation with melatonin during aging has been proposed as a means of increasing the gene action responsible for elevated activity of antioxidant defense system (ADS). A previous study has reported that the administration of rose-flower extract increase the CAT gene expression in the liver of aged mice. So also in the present investigation, supplementation with mushroom extract to aged rats possibly would occurred, resulting in the increased expression of CAT gene in liver and kidney tissues.

Protein oxidation, an exothermic event where peptides react with free radicals results in the modification of several amino acids, protein aggregation, and protein fragmentation. Evaluation of carbonyl level is possible the most common method used for assessing the oxidative modification of proteins. Increased level of this protein derivative has been documented in the lung, spinal cord and brain region and the hippocampus of patients with Alzheimer’s disease. So also, in the present investigation, the levels of protein carbonyl were significantly increased in the liver and kidney of aged rats when compared with young rats. The increase in carbonyl derivatives, as marker of protein oxidation with age, may suggest the inability of the antioxidant defense system to cope with increases in H₂O₂. However, aged rats when treated with mushroom extract recorded a significant decrease in the concentration of protein carbonyls in the liver and kidney than that of aged untreated rats. Balu et al. reported that the administration of the grape seed extract could reduce the protein carbonyls in the spinal cord and brain region of aged rats. The possible reason could be the antioxidant principles present in mushroom extract (data was not shown) that functions as in vivo antioxidants by virtue of their ability to directly scavenge ROS.

In conclusion, the present study clearly demonstrates that there was a decrease in the expression of CAT gene and the increased concentration of protein carbonyl in liver and kidney of aged rats when compared with young rats. Treatment with the mushroom P. ostreatus extract caused a remarkable increase in the level of CAT gene-expression in liver and kidney tissues. On the other hand, mushroom extract supplementation to aged rats decreased the oxidative damage which was confirmed by the protein carbonyl evaluation. It remains to be elucidated whether the mushroom extract increases the gene expression levels of SOD and Gpx during aging.

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