Chapter- IV

EFFECT OF PLEUROTUS OSTREATUS ON GENE EXPRESSION OF CATALASE DURING AGEING

1. Introduction

Ageing 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 process of ageing (Ashok and Ali, 1999). Inflammatory or ageing processes are associated with disruption of the oxidant/antioxidant (redox) balance, resulting in cellular and tissue oxidative stress and leading to cell death by apoptosis (Chandra et al., 2000; Wickens, 2001; Lang et al., 2002). Ageing, or the determination of functions of various organs, is believed to occur after adulthood due to alterations in the expression of genes that maintain the functions of adulthood. This hypothesis has found proof in the expression of the ovalbumin gene in birds during the process of egg formation (Upadhyay et al., 1999). Gene profiling studies from several laboratories using DNA microarray methods to screen thousands of genes in various tissues, mostly from mice, have identified mRNA products during ageing and with caloric restriction (Park and Prolla, 2005). However, the regulation of gene expression is sensitive to subtle changes in the redox condition, as observed during ageing; these changes would account for many altered gene products in aged organisms (Park et al., 2003). Recent molecular studies on oxidative stress have also shown the occurrence of altered gene regulation during ageing (Yoon et al., 2002; Kim et al., 2002a, 2002b), which provides 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 (Weindruch et al., 2002).

Mammalian cells are generally shown to have more tolerance to oxidative stress than bacteria because of their better developed defenses (Suzuki et al., 2002). These natural defenses are essentially composed of specialized enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathioneperoxidase (Gpx), and also of non-enzymatic antioxidant molecules such as vitamins, thiols and β-carotene.

Nutritional antioxidant supplementation can have health-promoting effects if it is able to control the endogenous redox system (Fang et al., 2002; Kritharides and Stocker, 2002). Consequently, there is an increasing interest in natural antioxidants, such as the proanthocyanidine in grape seeds and red wine (Machiex et al., 1990), ferulic acid in orange juice (Peleg et al., 1992), catechins in green tea (Penman and Gordon, 1997), carotenoids in mango (Mercadante et al., 1997) and quercetin in tomato, onion and lettuce (Crozier et al., 1997). Administration of rose-flower extract, a potent antioxidant, has been reported to alter the gene expression of the antioxidant enzymes, catalase and glutathione peroxidase, in the liver of aged mice (Ng et al., 2005). The present chapter describes an attempt to investigate whether an ethanolic extract of *P. ostreatus* could enhance the gene expression of the antioxidant enzyme, catalase, in the liver and kidneys of aged rats.
2. Materials and Methods

2.1. Chemicals

Total RNA isolating reagent (Trizol), agarose, primers for CAT and β-actin, 2, 4-dinitrophenylhydrazine (DNPH) and ethidium bromide were all purchased from Sigma Chemical Co. (St Louis, MO, USA). The Qiagen one-step RT-PCR kit was purchased from Qiagen (Germany). All other chemicals and reagents used were of analytical grade.

2.2. Animal experiments

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

2.3. Total RNA isolation

Total RNA was extracted from the freshly isolated liver and kidneys of the rats using 1 ml of the Trizol reagent as described by the suppliers (Sigma Aldrich, USA). Briefly, the fresh samples of liver and kidney (100 mg /ml) were homogenized in Trizol reagent. The homogenates were then incubated at 4°C for 5 min. To this, 0.2 ml of chloroform was added and thoroughly mixed by vortexing. The mixture was again
incubated at 4°C for 5 min. After incubation, the mixture was centrifuged at 12000×g for 15 min to obtain an aqueous layer (600 μl). To this, an equal volume of isopropanol was added followed by thorough vortexing; the solution was then again incubated at 4°C for 10 min. The incubated mixture was then centrifuged at 12000 xg for 10 min; the supernatant thus obtained was washed with 75% ethanol by centrifugation at 7500 xg for 5 min. After centrifugation, the ethanol was removed carefully and the pellet obtained (RNA) was allowed to dry for 10 min. The isolated RNA was resuspended in 50 μl of RNase free water; the quantity of the total RNA was determined by spectrophotometry and the purity checked by agarose gel electrophoresis (Sambrook et al., 1999).

2.4. Primers

The primers for CAT and β-actin used in the present study have previously been described in the literature (Table 8); these were procured from Sigma Aldrich, USA. The Tm values of the forward and reverse primers of catalase (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%.

2.5. Reverse transcription –polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with the Qiagen one-step RT-PCR kit (Qiagen, Germany). The RNA was reverse-transcribed to make a DNA copy for use in PCR. Briefly, 2 μg of each of the RNA templates was added to a tube containing, 0.6 μM of each of the forward and reverse primers of the gene of interest (CAT) along with an housekeeping gene (β-actin), 10 mM Tris Buffer pH 8.3, 1mM each of dNTP (ATGC) and 50 units of MuLV reverse transcriptase. The
mixture was then placed in a Techne (UK) thermal cycler. The PCR reaction was run under the following conditions:

(1) reverse transcription for 30 min at 50°C
(2) initial PCR activation for 15 min at 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.

After the completion of the PCR reaction, a 10µl 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 the RT-PCR reaction. To quantitate the transcript level, the ratio of the corresponding gene product to the β-actin gene product was calculated. Experiments were performed in duplicate.

3. Results

The level of the catalase gene transcript was found to be significantly (p<0.05) lower in the liver and kidney of aged (Group II) rats, when compared to levels in young (Group I) rats (Figs. 35 & 36). In aged rats that had been treated with the extract of *Pleurotus ostreatus* (Group III rats), the level of the transcript of CAT gene was found to be higher than that in the aged untreated rats (Group II).
Table 8. Primer sequences and expected product sizes for the genes amplified.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>Forward</td>
<td>ACAACTCCCAAGCCTAAGAATG</td>
<td>76</td>
<td>Depreter et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTTTGCCCTTGCCAGCTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>ATCGCTGACAGGATGAGAGAAG</td>
<td>108</td>
<td>Noda et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAGCCACCAATCCACACAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CAT- catalase; bp – base pair
Fig. 35. Agarose gel electrophoresis of RT-PCR products from total RNA isolated using Trizol.

M- 100bp DNA ladder

L1- Liver of young rats
L2- Liver of aged rats
L3- Liver of aged rats treated with mushroom extract
L4- Kidney of young rats
L5- Kidney of aged rats
L6- Kidney of aged rats treated with mushroom extract.
Fig. 36. Catalase gene transcripts in the liver and kidneys of different groups of rats.
4. Discussion

Determination of alterations in enzyme activity during the process of ageing has been an important approach in gerontological research (Jayachandran et al., 1996). While SOD, Gpx and CAT are regarded as the first line antioxidant defense system enzymes against ROS which are generated \textit{in-vivo} during oxidative stress, catalase has the advantage of catalysing the dismutation of H$_2$O$_2$ into H$_2$O and O$_2$ without requiring an additional source of reducing power, as all peroxidases do. In fact, CAT has been found to act $10^4$ times faster than peroxidase (Pryor, 1986). Various studies have reported the reduction in the activity of the catalase enzyme in the liver (Augustyniak et al., 2005; Valls et al., 2005), liver and kidney (Arivazhagan et al., 2000), liver, kidney, heart and brain (Jayakumar et al., 2007) and in various region of the brain (Balu et al., 2005) of aged rats.

While it is now widely accepted that oxidative damage plays an important role in the ageing process (Lombard et al., 2005), 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 (Rao et al., 1990; Thomas et al., 2002). Chen et al. (2006) reported a decreased expression of the catalase gene and no significant change in Cu/Zn- SOD and Gpx in the liver of aged male Fischer 344 rats. Significant decreases in mRNA expression of catalase in the liver of aged mice (Ng et al., 2005) and aged rats (Chen et al., 2006) have also been documented. Furthermore, Hatao et al. (2006) have reported a decreased level of gene expression of CAT enzyme in the lungs of aged rats. Limaye et al. (2003) reported an elevated expression of antioxidant enzymes, such as CAT, SOD and Gpx, in streptozotocin-induced diabetic rats. In addition, a decrease in the expression of the
CAT gene was also noted in the kidneys of rats exposed to endotoxin (Ghosh et al., 1996). So also, in the present study, a decline in the level of gene expression was recorded for the catalase enzyme in the liver and kidneys of aged rats, when compared to young rats.

Supplementation of the diet with fruits and vegetables is reported to be beneficial in reversing the deleterious effects of ageing on neuronal communication and behavior (Joseph et al., 2005). Balu et al. (2005) reported that supplementation of the diet with grape seed extract improved the antioxidant status of the central nervous system of aged rats. Augustyniak et al. (2005) reported that the administration of green tea extract increased the activity of CAT in the liver of aged rats. A recent study has shown that administration of an extract of the mushroom *Pleurotus ostreatus* increased the activity of catalase in the liver, kidneys, heart and brain of aged rats (Jayakumar et al., 2007). Such supplementation altering the gene expression of antioxidant enzymes has been well documented with reference to melatonin (Becker-Andre et al., 1994; Wiesenberg et al., 1995; Steinhiller et al., 1995), and hence supplementation with melatonin during ageing has been proposed as a means of increasing the gene action responsible for elevated activity of the antioxidant defense system (ADS). Ng et al. (2005) reported that the administration of rose-flower extract increased the CAT gene expression in the liver of aged mice. One study suggests that dietary polyphenols can stimulate antioxidant transcription and detoxification defense systems through antioxidant responsive elements (ARE) (Masella et al., 2005). So also in the present investigation, supplementation with an extract of the mushroom *P. ostreatus* resulted in an increased expression of the CAT gene in the liver and kidney tissues of aged rats, when compared to aged untreated rats.
5. Conclusion

This study demonstrated that there was a decrease in the expression of the catalase gene in the liver and kidneys of aged rats, when compared to young rats. On the other hand, treatment with the *P. ostreatus* extract caused a remarkable increase in the expression of the CAT gene in the liver and kidney tissues of aged rats, when compared to aged untreated rats. This result suggests that administration of the mushroom extract to aged rats can upregulate the gene expression of the antioxidant enzyme catalase, which possibly contributes to the anti-ageing action of the extract of *P. ostreatus*.