Inhibition of lipid peroxidation induced by \( \gamma \)-radiation and AAPH in rat liver and brain mitochondria by mushrooms

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Exposure to radiation or 2,2’-Azobis(2-amidopropane) dihydrochloride (AAPH) induces generation of reactive oxygen species (ROS) especially hydroxyl radical (’OH) and peroxyl radical (ROO\(^{\bullet} \)) which are capable of inducing lipid peroxidation. Our earlier studies have demonstrated that extracts of the medicinal and edible mushrooms \textit{Ganoderma lucidum}, \textit{Pleurotus flori\textordmasculine{a}}, \textit{Pleurotus sajor-caju} and \textit{Phellinus rimosus} possessed significant antioxidant activity, measured as radical scavenging. In the present study, we examined the protective effect of these mushroom extracts against radiation- and AAPH-induced lipid peroxidation using rat liver and brain mitochondria as model systems. The results obtained showed that the investigated mushroom extracts significantly inhibited the formation of lipid hydroperoxide and thiobarbituric acid reactive substances, indicating membrane protective effects. The finding suggests the profound protective effect of the extracts of the fruiting bodies of \textit{G. lucidum}, \textit{P. flori\textordmasculine{a}}, \textit{P. sajor-caju} and \textit{P. rimosus} against lipid peroxidation by two major forms of ROS capable of inducing this type of damage in a major organelle, the mitochondria from both rat liver and brain. This observation can possibly explain the health benefits of these mushrooms.

The study of lipid peroxidation (LP) is attracting much attention in recent years due to its role in disease processes. Membrane lipids are particularly susceptible to LP due to the presence of polyunsaturated fatty acids. Since membranes form the basis of many cellular organelles like mitochondria, plasma membranes, endoplasmic reticulum, lysosomes, peroxisomes, etc. the damage caused by LP is highly detrimental to the functioning of the cell and its survival. It has been implicated in the pathogenesis of a number of diseases and clinical conditions. These include atherosclerosis, cancer, adult respiratory distress syndrome, Alzheimer’s disease, Parkinson’s disease, ischaemia-reperfusion injury of various organs, chemical and radiation-induced injury, diabetes, etc. Experimental and clinical evidence suggests that aldehyde products of LP can also act as bioactive molecules in physiological and pathological conditions. It is now generally accepted that LP and its products play an important role in liver, kidney and brain toxicity.

Exogenous chemicals and radiation produce peroxidation of lipids leading to structural and functional damage to cellular membranes. Ionizing radiation damages cellular molecules directly by transferring energy or indirectly by generation of oxygen-derived free radicals. Excited states and other reactive species are collectively known as reactive oxygen species (ROS). Polyunsaturated fatty acids present in cellular membranes are especially prone to damage by ROS and the resulting LP can have serious consequences. LP plays a major role in mediating oxidative-damage in biological systems. There are also several toxic by-products of peroxidation which can damage other biomolecules away from the site of generation. Among the subcellular organelles mitochondria are one of the key components of the cell killed by radiation-induced oxidative stress.

Endogenous antioxidants constitute important defence systems in cells and elicit their action by suppressing the formation of ROS, their scavenging or by repairing the damage caused. Besides this, a number of natural antioxidants are found in plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. Some of them exhibit significant antioxidant activity and are commonly utilized for pharmaceutical purposes and in health foods. Recent evidence indicates that mushrooms contain a large number of biologically active components that offer protection against degenerative diseases. A number of medicinal mushrooms have recently been reported to possess significant antioxidant activity.

\textit{Phellinus rimosus} is a wood-inhabiting polypore-macro-fungus often found growing on jackfruit tree trunks in Kerala. The basidiocarp of this fungus has been used by some local tribes for the treatment of ailments like mumps. Our earlier investigations showed that ethyl acetate extracts of \textit{P. rimosus} possessed antioxidant, antitumor, anti-inflammatory, hepatoprotective and nephroprotective activities. Oyster mushrooms (\textit{Pleurotus spp.}) are widespread throughout the hard wood forests of the world; they are edible, nutritious and rank second among the cultivated mushrooms in the world. They are known as efficient blood pressure-lowering agents, diuretics, cholesterol reducers, adjuvant and aphrodisiac. They have also been found to modulate the immune system. \textit{Ganoderma lucidum}, commonly known as Reishi, is considered as a panacea in Chinese medicine because of its effectiveness in the treatment of a large number of diseases. It is known to possess DNA protective properties. Recent investigations in our laboratory have shown that fruit bodies of \textit{G. lucidum} occurring in South India possessed significant antioxidant, anti-inflammatory and anti-nociceptive properties. Since the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of mushrooms is of significant importance in exploiting their therapeutic potential. The proof of their antioxidant activity can also explain their...
mechanism of action and hence, it was considered desirable to evaluate the effect of these mushroom extracts against lipid peroxidation induced by γ-radiation and 2,2’Azobis(2-amidopropane)dihydrochloride (AAPH), which generates two potent ROS capable of inducing LP, namely hydroxyl radical (‘OH) and peroxyl radical (ROO’). AAPH generates model peroxyl radicals. These radicals are similar to such peroxyl conditions that are physiologically active. γ-Radiation also generates physiologically relevant ROS such as hydroxyl radical, superoxide, hydrogen peroxide, single oxygen, etc. that are capable of damaging many crucial cellular molecules, including membrane lipids. Hence, inhibition of lipid peroxidation induced by these two agents is physiologically relevant.

Hydrogen peroxide, ethylene diamine tetraacetic acid (EDTA), 2-thiobarbituric acid, triphenylphosphine (TPP), trichloroacetic acid, xylenol orange and butylated hydroxy toluene (BHT) were purchased from Sigma Chemical Co, USA. AAPH was from Aldrich Chemical Co, USA. Other chemicals used in our study were of the highest quality commercially available from local suppliers.

Fruiting bodies of Pleurotus florida and Pleurotus sajor-caju were obtained from the small-scale cultivation unit of Integrated Rural Technology Centre, Palakkad, Kerala. G. lucidum and P. rimosus were collected locally from the outskirts of Thrissur. The specimens were identified and the identifications were authenticated by K. M. Leelavathy (Department of Botany, Calicut University Calicut). Voucher specimens were deposited in the herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai (G. lucidum: HERB.MUBL-3175, P. rimosus: HERB. MUBL-3171).

Fruiting bodies of P. florida, P. sajor-caju, G. lucidum and P. rimosus were dried at 45 to 50°C for 48 h and powdered. The powdered material was defatted with petroleum ether in Soxhlet apparatus for 8 to 10 h and the defatted material was then extracted with ethyl acetate or 70% methanol. The ethyl acetate extract of P. rimosus and methanol extracts of P. florida, P. sajor-caju and G. lucidum corresponding to the active fractions from these mushrooms were used for the experiments.

One percent extracts of P. florinda (Pr), P. sajor-caju (Ps) and G. lucidum (Gl) were prepared in dissolving 0.1 g of the extract in 10 ml of distilled water and stirring for 1 h. In the case of P. rimosus (Pr), 0.01 g of the extract was dissolved in 10 ml of methanol and stirred for 30 min. All the above extracts were centrifuged for 15 min and supernatants were stored at -20°C. The supernatants were used to examine the antioxidant properties. The final concentration of Ps, Pr and Gl extracts used in our study was 0.1%, which gives significant protection against LP in rat liver mitochondria. In the case of Pr, the final effective concentration of the extract was 0.01%. This was chosen from our earlier study on radical scavenging. In our earlier experiments on radical scavenging activities, such as FRAP, DPPH and ferrylmyoglobin/ABTS assays, we had used three different concentrations, i.e. 0.1, 0.5 and 1% for Ps, Pr and Gl extracts, while in the case of Pr, 0.005, 0.01 and 0.1% concentrations were used. We have chosen the effective concentration based on earlier studies.

Three-month-old female Wistar rats (weighing about 250 g) were used for the preparation of mitochondria. In brief, rat liver and brain tissues were excised, homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3000 g for 10 min to remove cell debris and nuclear fraction. The resultant supernatant was centrifuged at 10,000 g for 10 min to sediment mitochondria. This pellet was washed thrice with 50 mM phosphate buffer, pH 7.4 to remove sucrose. The protein was estimated and pellets were suspended in the same buffer.

The mitochondria were suspended in buffer and exposed to γ-radiation from 60Co source (Atomic Energy of Canada Ltd) at a dose rate of 15 Gy/min. The effect of extract on the oxidative-damage caused by radiation was studied at a dose of 450 Gy. Mitochondria (2.0 mg protein/ml) were suspended in the buffer and exposed to radiation with or without the extracts. The effect of extracts on the oxidative-damage caused by AAPH was also studied. The mitochondria (2.0 mg protein/ml) were exposed to AAPH (10 mM) with or without extract for 30 min. The mitochondria after exposure to γ-radiation and AAPH were evaluated for LP.

Aliquots (90 μl) of brain/liver mitochondria, after exposure to radiation sample, were transferred to microcentrifuge tubes together with 10 μl of TPP in methanol/10 μl of methanol in blank and test samples respectively. The samples were then vortexed and subsequently incubated for 30 min at room temperature. Next 900 μl of Fox II reagent (xylenol orange (100 μM), butylated hydroxy toluene (4.4 μM), sulphuric acid (25 mM), ammonium ferrous sulphate (250 μM)) was added and samples were incubated for a further 30 min in dark. The samples were centrifuged at 12000 g for 10 min prior to reading absorbance of supernatant at 560 nm. The level of peroxide in the sample was then determined using the difference between mean absorbance of samples with and without TPP treatment and the final value was extrapolated to H2O2 concentrations in the standard graph. The effect of mushroom extracts on hydroperoxide induction by AAPH at varying time intervals was also determined.

Thiobarbituric acid reactive substances (TBARS) assay was performed by standard method using malonaldehyde equivalents derived from tetramethoxyxpropane. Malonaldehyde and other aldehydes have been identified as products of LP that react with thiobarbituric acid (TBA) to give a pink coloured species at 532 nm. The method involved heating of the samples after exposure to radiation and AAPH with TBA reagent for 20 min in a boiling water bath. TBA reagent contains 50 ml TCA (20%), 25 ml TBA (500 mg), 2.5 M HCl, 224 mg EDTA and the final volume is made up to 100 ml. After cooling, the solution was centrifuged at 2000 g for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at
532 nm against a blank that contained all the reagents minus the sample. The malonaldehyde equivalents of the sample were calculated using an extinction coefficient of 1.56 × 10^3 M⁻¹cm⁻¹. For collection of endogenous TBARS, fresh samples were boiled without radiation exposure, and values were subtracted. Vehicle controls were used for all the extracts. Methanolic extracts Ps, Pf and Gl were dissolved in distilled water, while ethyl acetate extract of Pr was dissolved in ethanol for LP experiments. These values were subtracted from the sample readings.

Data on the effect of mushroom extracts on lipid hydroperoxide (LOOH) induced by AAPH in rat liver mitochondria are presented in Figure 1. P. sajor-caju, with 100% inhibition, was the most effective in reducing AAPH-induced LOOH formation. P. rimosus showed 96% inhibition, P. florida 94.1% inhibition and G. lucidum inhibited 94.9% of LOOH induction. LOOH formation induced by AAPH at varying time intervals in brain mitochondria and its inhibition by P. rimosus extract was more effective than other extracts. LOOH formation was maximum at 60 min after exposure to AAPH. After 60 min, LOOH formation was inhibited in all treated groups (Figure 2).

Data on the effect of mushroom extracts on TBARS formation induced by AAPH in rat liver mitochondria are given in Figure 3. P. florida and P. rimosus were more effective in reducing LP induced by AAPH compared to other mushroom extracts.

Exposure to radiation, as a function of dose, ranging from a dose of 0 to 750 Gy, resulted in enhanced LP as evident by the formation of TBARS. The increase in TBARS formation was significant with the increasing doses examined. Exposure to 300–450 Gy showed steep increases, while higher doses were effective only in marginally enhancing peroxidation. Hence the optimum dose of 450 Gy was selected for the experiments, as this dose caused optimum damage in terms of LP in rat liver and brain mitochondria.

Mitochondria are crucial targets for radiation and free radical-mediated damage. Since mitochondria are devoid of cytosolic antioxidants, as in a whole cell, they are fairly resistant to γ-radiation. Hence a dose of 450 Gy is needed to achieve optimum concentration of free radicals capable of inducing significant damage measurable by simple spectrophotometric means. This dose is much higher than those used in radiotherapy (1–6 Gy) or for radioprotection pertinent to mammals (LD₅₀ in the range of 5–7 Gy). Such in vitro studies with easily measurable systems are usually animal models involving a large number of animals, and being subject to animal ethics committees. A case in point are such studies on the radioprotective effect of caffeine using in vitro systems and animal models. We plan to extend our investigations to animal studies in vivo in future.

Data on radiation-induced LP and its protection by mushroom extracts are given in Figure 4. P. florida extract showed significant ability to inhibit radiation-induced LP in rat liver mitochondria. P. florida at a concentration of 1% reduced TBARS formation significantly when it was present at the time of irradiation. The inhibition of LP by P. rimosus and P. sajor-caju was higher than G. lucidum extract.

The formation of LOOH, an intermediate of peroxidation, showed that LOOH formation induced by γ-radiation in rat liver mitochondria was inhibited more effectively by...
P. sajor-caju than other mushroom extracts, and the data are represented in Figure 5. However, G. lucidum was also as effective as P. sajor-caju.

Prevention of free-radical formation and maintenance of cellular structural integrity and of chemical environment are fundamental requirements of all cells. In biological systems, radiation-induced free radicals impair antioxidant defence leading to increased membrane lipid peroxidation. Generation of ROS by ionizing radiation (especially with low-LET radiation) and AAPH and its profound impact on cellular biomolecules are well established. The present investigation demonstrates that AAPH and radiation induced significant LP in mitochondria. Increase in peroxidation is observed as a function of radiation dose. Radiation generated ROS and is also capable of initiating LP. The initial products of peroxidation are conjugated dienes, to which is added oxygen to form LOOP that further breaks down to stable aldehydes and reacts with TBA to form thiobarbituric acid–malonaldehyde adduct.

Radiation therapy is one of the most important and popular tools for cancer treatment. Because human tissues contain 80% water, the major radiation damage is due to aqueous free radicals, generated by the action of radiation on water. The major free radicals resulting from aqueous radiolysis are ‘OH, ·H, eaqH2O2, HO2, etc. Among them ‘OH is the most potent, capable of inflicting severe molecular damage. This free radical reacts with cellular macromolecules such as DNA, proteins, lipids, etc. and causes dysfunction and mortality. These reactions take place in tumour as well as normal cells when exposed to radiation.

LP causes membrane damage as well as oxidative modification of critical targets. Agents that can interact with these secondary radicals formed during peroxidation and scavenging them, would be effective in inhibiting LP and in turn protect against radiation and AAPH-induced damage. Removal of excess reactive species, suppression of their generation or protection against peroxidation by repair of membrane damage may be an efficient way of preventing cancer and other diseases.

The effects of mushroom extracts on LP show significant inhibition of LOOH and TBARS formation. Our earlier studies have indicated that mushroom extracts are effective scavengers of both primary and secondary radicals. Protection of membranes at both primary and secondary levels explains the possible mechanism by which mushrooms inhibit LP by radiation and AAPH. Phenolics are a group of non-essential dietary components that have been associated with inhibition of atherosclerosis and cancer, by chelating metals, inhibiting lipooxygenases and scavenging of free radicals. Mushroom phenolic compounds are found to be excellent antioxidants and synergists that are not mutagenic. Our earlier results also have shown that mushroom extracts possess significant radical scavenging properties of both primary and secondary radicals, in a concentration-dependent manner. Hence the components present in mushroom may inhibit LP by scavenging of radicals that initiate or propagate LP.

Our earlier studies have revealed that all the mushrooms employed in this study possess antioxidant properties, mainly measured as radical scavenging. The present finding strongly suggests that the use of these mushroom extracts to prevent LP leading to membrane damage consequent to exposure to radiation and to certain chemicals
which generate potent ROS in the form of ‘OH or ROO’. This also explains the possible mechanisms behind the observed health benefits of these mushrooms.


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