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Edible mushroom *Morchella esculenta* (L.) Pers. mycelium protects DNA and mitochondria from radiation induced damages

B Nitha[1]*, Strayo De[2], TPA Devasagayam[2] & KK Janardhanan[3]

[1]Department of Microbiology, Sree Ayyappa College, Eramalliakkra, Chengannur, Alappuzha, Kerala, India

[2]Bhaba Atomic Research Centre, Trombay, Mumbai, Maharashtra, India

[3]Department of Microbiology, Amala Cancer Research Centre, Thrissur, Kerala, India

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Mushrooms have gained great attraction not only for their nutritional and medicinal values but also for diversity of their bioactive components. *Morchella esculenta*, commonly called Morel, is an edible and nutritious mushroom. In the present study, we evaluated the radioprotective effect of cultured mycelium of *M. esculenta* against radiation induced damages in mouse spleen lymphocyte DNA and rat liver mitochondria. The mitochondria were exposed to 450 Gy γ-radiation and lipid peroxidation caused by radiation was studied by LOOH assay and TBARS assay. *M. esculenta* extract at a concentration of 200 and 50 μg/mL significantly inhibited the formation of LOOH and TBARS formation in mitochondria. The protective effect of *M. esculenta* against radiation-induced damage to DNA in the spleen lymphocyte was measured using comet assay. Spleen lymphocytes were exposed to γ-radiation at a dose of 6 Gy in the presence and absence of the extract and the strand break were analyzed. The extract inhibited the strand break significantly. The study indicate that *M. esculenta* mycelia protect mitochondria form oxidative stress and DNA from deleterious effects induced by radiation and has potential for the development of safe and non-toxic radio protector with significant nutritional properties.

Keywords: Morel Mushroom mycelium, Nutraceuticals, Radioprotection

Radiation has been considered an enigma to the general public and the use of radiation for therapeutic and other uses has always been associated with some skepticism. The level of radiation is increasing day by day due to rapid technological advancement; therefore there is a need to protect human, animals, and even plants against such harmful effects of ionizing radiation[1]. Presently, ionizing radiation is being used in a large number of therapeutic, industrial and other applications. Radiation therapy is among the most effective treatment modalities for patients with cancer. About 60% of all patients with cancer receive ionizing radiation (IR) as part of their therapeutic regimen[2]. While IR is a powerful tool for destroying cancer cells, it is also toxic to normal cells and causes cellular damage and unwanted side effects. IR affects biological molecules both directly and indirectly. Direct effects are mediated by direct interaction of IR with individual DNA moieties, and indirect effects occur via reactive oxygen species (ROS) produced from the molecules surrounding DNA[3]. Mainly two types of changes are observed in DNA at the molecular level, namely altered bases and strand breaks. Both types of changes, if not repaired, affect the cell structure and function[4,5]. The deleterious effects of ionizing radiation, especially those having low LET, in biological systems are mainly mediated through the generation of oxygen-derived intermediates in the formation of free radicals and excited states[5]. It generates a number of reactive oxygen species (ROS) and excited triplets capable of damaging different crucial biomolecules[6,7]. Polyunsaturated fatty acids, present in cellular membranes, are especially prone to damage by the ROS, generated during radiation[8]. Among the various species generated, peroxyl radical ($ROO^{\cdot}$), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($O_2^{\cdot}$) are capable of inducing peroxidation which has been implicated directly or indirectly in several cytotoxic and genotoxic reactions leading to various diseases[6-9].

Due to increased use of ionizing radiation in various aspects of human life, there is a need to develop an effective and non-toxic radioprotector. Radioprotectors are also required to reduce injury to normal tissues during radiotherapy of cancer. Several novel approaches are on to locate a potent and non-toxic radioprotector. Use of natural products as

*Correspondence:
E-Mail: nitha.balan@gmail.com

possible radioprotectors has gaining considerable momentum in last decade due to less toxicity, reduced cost and other associated advantages[10,11].

Mushrooms are known for their nutritional and medicinal values, and the diversity of their bioactive components[12]. There is significant interest in the use of mushrooms and /or mushroom extracts as dietary supplements based on theories that they enhance immune function and promote health[13]. Mushroom metabolites are increasingly being utilized to treat a wide variety of diseases, particularly as they can be added to the diet and used orally, without the need to go through phase-I/II/III trials as a synthetic drug, and they are considered as safe and useful for disease treatment. Most mushroom derived preparations find their use not as pharmacy nutraceuticals but as a novel class of dietary supplements (DS) or nutraceuticals[14].

Morchella esculenta (L) Pers. is an edible and nutritious morel mushroom belonging to Ascomycotina division. Mycelium of mushrooms obtained from pure cultures is a preferable choice for developing consistent and safe health care products. In our previous study cultured mycelium of *M. esculenta* showed high antioxidant activity[15]. Here, we screened the extract of the mycelium of this mushroom which showed significant antioxidant property for possible radioprotective activity.

Materials and Methods

Production of mushroom mycelium

Culture of *Morchella esculenta* obtained from the Microbial Type Culture Collection (MTCC 1795), CSIR-Institute of Microbiology, Chandigarh, India, was employed for the study. The fungus was grown in submerged culture on potato-dextrose broth (PDB) for production of mycelia biomass. After ten days of growth in submerged culture on a shaker at 24-25°C, the fungal biomass was harvested, washed thoroughly and dried at 40-50°C[16].

Preparation of extracts

The dried mycelia was powered and 100 g of powder was extracted with hot aqueous-ethyl alcohol (ethyl alcohol: water 50/50 v/v) for 8-10 h. The extract was concentrated at low temperature and solvent completely evaporated under vacuum. The residue thus obtained was used for the experiments.

Animals

Four-six months old female Wistar rats and Female Swiss albino mice of 6 weeks old were employed for the studies. These animals were housed in polypropylene cages in the animal house at temperatures of 24±30°C, supplemented with food (Hindustan Lever Ltd. India) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethical Committee. All the experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India and by the approval of the Institutional Animal Ethical Committee (149/99/CPCSEA dated 23-10-2009).

Gamma-irradiation

A ^{60}Co Gamma Cell 220 (Atomic Energy of Canada Ltd) with a dose rate of 5 Gy/min was used for *in vitro* experiments.

In situ radioprotection assay

Isolation of mitochondrial fraction

Four-six months old female Wistar rats were used for the preparation of mitochondria. In brief, rat liver was 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 the nuclear fraction. The resultant supernatant was centrifuged at 10,000 ×g for 10 min to sediment mitochondria. This pellet was washed with 5 mM Phosphate buffer, pH 7.4, to remove sucrose. Protein was estimated and pellets were suspended in the above buffer at the concentration of 10 mg protein/mL[17].

Exposure of mitochondria to γ-radiation

The mitochondria were suspended in buffer (50mM phosphate buffer, pH 7.4) and oxidative damage was induced in biological membranes by exposure to γ-rays from a ^{60}Co source (Atomic Energy of Canada Ltd). All *in vitro* gamma irradiations were carried out using a Gamma irradiator. Mitochondria (2.0 mg protein/ml) from rat liver treated with or without extract *in vitro* were exposed to 450 Gy γ-radiation at a dose rate of 7 Gy/min. The lipid peroxidation caused by radiation was studied after exposure of mitochondria to γ-radiation[17,18].

Lipid hydroperoxide (LOOH) assay

This is a simple and sensitive spectrophotometric procedure, which detects hydroperoxides present in lipid phase such as in lipoproteins, membranes and fats. Hydroperoxides oxidize Fe^{2+} to Fe^{3+} under acidic conditions. The resultant Fe^{3+} can be determined using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration. The dye xylenol orange, complexes with an equal molar concentration of Fe^{3+}

to produce a blue-purple colour complex with an extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} at 560 nm. Hence, the method is named FOX (Ferrous Oxidation in Xylenol Orange). Fox II reagent contains, solution A made up of 98 mg ammonium ferrous sulphate, 100 mL 250 mM H_2SO_4 and 79 mg xylenol orange, and solution B comprising of 969 mg BHT in 900 mL methanol, mixed in the ratio of 1:9. It can be stored in dark at 4°C for one month. About 875 µL of FOX II reagent was added to 125 µL of the reaction mixture and incubated at 37°C for 30 min. It was centrifuged at 10000 ×g for 15 min and absorbance was measured at 560 nm. For calculations, a standard graph of OD against concentration of H_2O_2 was plotted[19].

Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid (TBA) to give a pink coloured species that absorbs at 532 nm. The method involved heating of biological samples with TBA reagent (TBA-TCA-HCl-EDTA) for 20 min in a boiling water bath. TBA reagent contains 20% TCA, 0.5% TBA, 2.5 N HCl and 6 mM EDTA. After cooling, the solution was centrifuged at 2000 rpm 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 except the biological sample. Concentration of TBARS was then calculated with the help of standard graph using 1,1,3,3-tetraethoxypropane, as malondialdehyde equivalents[20].

Protection of spleenic lymphocyte DNA from γ-radiation induced damage

Isolation of spleenic lymphocytes

Spleen lymphocytewere obtained by gently squeezing the Swiss mouse spleen through a nylon mesh in a petriplate containing RPMI 1640 medium supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. The RBCs were lysed by brief hypotonic shock. Lymphocytes were suspended in RPMI 1640 medium. Cell viability was assessed by trypan blue dye exclusion[4,21].

Comet assay

Radiation-induced damage to DNA in the spleen lymphocytewas measured as strand breaks using alkaline single cell gel electrophoresis (popularly termed as ‘comet assay’). Spleen lymphocytes were exposed to γ-radiation at a dose of 6 Gy in the presence and absence of the extract, and the strand break were analyzed. In brief, frosted microscope slides (Gold Coin, Mumbai, India) were covered with 200 µL of 1% normal melting agarose (NMA) in phosphate buffered saline (PBS) at 45°C, immediately cover-slipped and kept at 4°C for 10 min to allow the agarose to solidify. Removal of cover slip from the agar layer was followed by addition of a second layer of 200 µL of 0.5% low melting agarose (LMA) containing approximately 10^5 cells at 37°C. Cover slips were placed immediately and the slides were kept at 4°C. After solidification of the LMA, the cover-slips were removed and slides were placed in the chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris–HCl, pH 10, and 1% DMSO, 1% Triton X100 and 1% sodium sarcosinate, for 1 h at 4 °C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na_2-EDTA and 0.2% DMSO, pH ≥13.0). The slides were equilibrated in the same buffer for 20 min and electrophoresis was carried out at 25 V for 20 min. After electrophoresis the slides were washed gently with 0.4 M Tris–HCl buffer, pH 7.4, to remove the alkali. The slides were stained with 50 µL of propidium iodide (PI, 20 µg/mL) and visualized using a Carl Zeiss Fluorescent microscope (Axioskop) with bright field phase-contrast and epi-fluorescence facility. The images (40-50 cells/slide) were captured with high-performance GANZ (model: ZC-Y11PH 4) colour video camera. The integral frame grabber used in this system (Cvfbo1p) is a PC based card and it accepts colour composite video output of the camera. The quantification of the DNA strand breaks of the stored images was done using the CASP software by which %DNA in tail, tail length, tail moment and Olive tail moment could be obtained directly[22,23].

Results

Protection of mitochondria from γ-radiation

Exposure of rat liver mitochondria to γ-radiation, as a function of dose, resulted in a significant increase in lipid peroxidation, as measured by the formation of LOOH and TBARS. Exposure to (300-600 Gy) caused a steep increase while higher doses were effective only marginally in increasing the extent of peroxidation further. Fig. 1 presents data on the inhibition of radiation-induced LOOH production by extract in rat liver mitochondria. The enhanced formation of LOOH obtained at the optimum dose of 450Gy was significantly reduced when irradiation

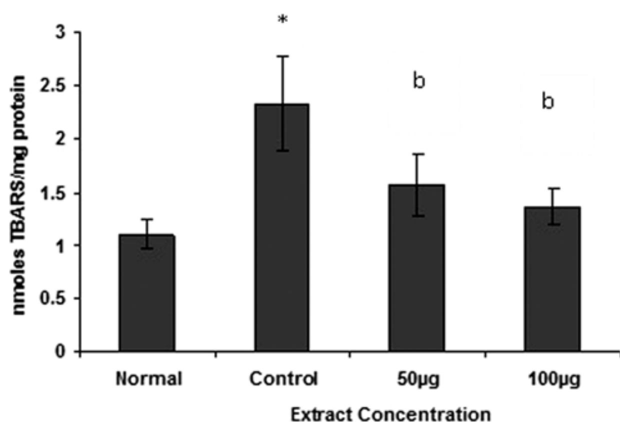

Fig. 1 — *In situ* effect of extract on γ radiation-induced LOOH production in rat liver mitochondria. [Values are mean ± SD, n=3. *P <0.001, significantly different from normal. [a]P <0.01, significantly different from control]

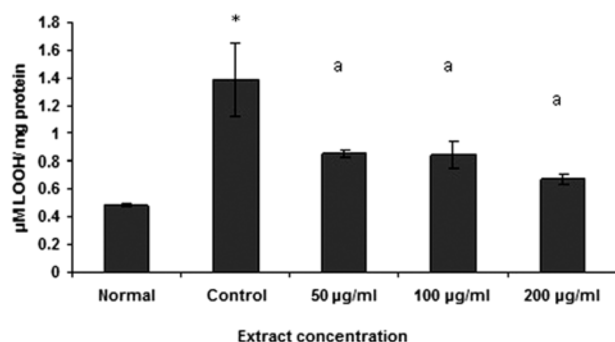

Fig. 2 — *In situ* effect of extract on γ radiation-induced TBARS formation in rat liver mitochondria. [All values are mean ± SD, n=3. *P <0.001, significantly different from normal, [a]P <0.01 and [b]P <0.05 significantly different from control]

was carried out in the presence of extract (P <0.01). Extract at a concentration of 200 µg/mL significantly inhibited the formation of LOOH, an intermediate of peroxidation, and restored the level to almost normal.

Upon exposure to γ-radiation a drastic increase in TBARS was found in rat liver mitochondria. Treatment with the extract at a concentration of 50 and 100 µg/mL along with the radiation reduced the TBARS formation is presented in Fig. 2 (P <0.05).

Protection of spleenic lymphocyte DNA fromγ-irradiation

Protection of spleenic lymphocyte DNA from γ-irradiation studied using comet assay showed significant protection of the DNA by the extract at a concentration of 200 µg/mL. In the extract treated groups the % tail DNA, tail length, tail moment and olive tail moment the indices of DNA damage were found to be decreased by the extract when compared

Table 1 — Protection against radiation induced damage in blood lymphocytes by *Morchella esculenta*

Parameter	Normal	Control	Extract (200 µg)	% inhibition by extract
Tail DNA (%)	2.28±0.61	18.43±1.45*	8.87±0.84[a]	51.87
Tail length (µ)	12.1±1.19	55.2±7.43*	16.7±3.62[a]	69.74
Tail moment	0.288±0.07	10.53±1.11*	1.35 ±0.30[a]	87.15

[All Values are Mean ± SD, (n=6), *P <0.001, significantly different from normal, [a] P <0.01, significantly different from control]

Fig. 3 — Protection of spleenic lymphocyte DNA by the extract-Comet assay. (A) Normal lymphocyte DNA; (B) Control (Radiation alone treated); and (C) Radiation+ 200 µg extract

to the untreated control DNA (Table-1) (P <0.01). Treatment with the extract reduced the % tail DNA and tail length by 51.87 and 69.74%, respectively when compared to the spleenic DNA which received only irradiation. The tail moment and olive tail moment were also found to be decreased by 87.15 and 71.28%, respectively in the extract treated spleenic lymphocyte DNA as compared to untreated control DNA. The spleen lymphocyteafter exposure to γ-irradiation with and without drug, captured using Carl Zeis microscope with high-performance GANZ colour video camera, clearly indicated the protection of spleenic lymphocyte DNA from γ-irradiation by the extract (Fig. 3).

Discussion

Protecting living systems from the dangers of ionizing radiation is of paramount importance. However, radiation protection has also significant relevance in radiotherapy of cancer. Many natural and synthetic chemicals have been investigated in the recent past for their efficacy to protect against radiation-induced damage in biological systems[24-26]. Though a large number of compounds have been shown to be promising as radioprotectors in laboratory studies, only a few could pass the transition from bench to bedside. However, the inherent toxicity of some of the synthetic agents at the effective radioprotective concentration warranted further search for safer and more effective radioprotectors. In fact, no radioprotective agent is now available, either alone or in combination to meet all the requisites of an ideal radioprotector[27].

Radiation induced damages to cells and tissues involve generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), $^{\cdot}OH$ radicals and H_2O_2 which in turn cause alterations in DNA, membrane lipids and proteins eventually leading to cellular dysfunction or cell death[28]. Ionizing radiation-induced damages to cellular DNA are of prime biological significance. The types of damages suffered by DNA due to ionizing radiation include strand breaks of single and double-strand types, base damage, elimination of bases and sugar damage[29]. Protecting cellular DNA from radiation damage might result in prevention of the cancers/mutations induced by radiation. Apart from DNA, another major target of radiation inactivation is the membranes of cytoplasmic organelles and plasma membrane. Oxidative damage to membrane is generally mediated by the degradation of phospholipids, which are the major constituents of the membrane. Membrane lipids are easily peroxidised by ROS produced by ionizing radiation, causing structural and functional impairment of the membrane[29,30].

The results of our preliminary screening on the radio protective activity of the extract revealed that the extract efficiently protected both the mitochondria and DNA from deleterious effects of radiation by *ex vivo* conditions of radiation exposure. Exposure of mitochondria to γ-radiation along with the extract significantly reduces the lipid peroxidation, measured in terms of both LOOH and TBARS. The radio protective effect of the extract on mitochondria can be ascribed to its interaction with radiation induced free radical generation.

The single-cell gel electrophoresis (SCGE) or comet assay is a sensitive method for measuring DNA strand breaks in individual cells and is widely used in environmental toxicology, cancer research, and radiation biology to assess DNA damage. A small number of cells are embedded in agarose gel on a microscope slide, lysed, electrophoresed, and stained with fluorescent DNA binding dye. Damaged DNA migrates during electrophoresis from the nucleus towards the anode, forming a shape of a "comet" with a head (cell nucleus with intact DNA) and a tail (relaxed and broken DNA). The proportion of DNA in the tail is indicative of the frequency of breaks. The results of comet assay indicate that the extract could efficiently protect the spleenic lymphocyte DNA from radiation induced strand breaks and other damages. Treatment with the extract significantly reduced the % tail DNA, tail length, tail moment and olive tail moment considerably compared to the splenic DNA which received only the γ –radiation exposure.

Conclusion

The results indicate that morel *Mochella esculenta* mycelia extract is an effective antioxidant in rat liver mitochondria against oxidative stress induced by radiation and is capable to protect DNA from deleterious effects of radiation. This study suggests that the extract of cultured mycelia of *M. esculenta* has for the development of safe and non toxic radioprotector with significant nutritional properties.

Conflict of interest

The authors declare no conflict of interest

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