Compton Scattering by Internal Shields Based on Melanin-Containing Mushrooms Provides Protection of Gastrointestinal Tract from Ionizing Radiation

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Abstract

There is a need for radioprotectors that protect normal tissues from ionizing radiation in patients receiving high doses of radiation and during nuclear emergencies. We investigated the possibility of creating an efficient oral radioprotector based on the natural pigment melanin that would act as an internal shield and protect the tissues via Compton scattering followed by free radical scavenging. CD-1 mice were fed melanin-containing black edible mushrooms *Auricularia auricula-judae* before 9 Gy total body irradiation. The location of the mushrooms in the body before irradiation was determined by *in vivo* fluorescent imaging. Black mushrooms protected 80% of mice from the lethal dose, while control mice or those given melanin-devoid mushrooms died from gastrointestinal syndrome. The crypts of mice given black mushrooms showed less apoptosis and more cell division than those in control mice, and their white blood cell and platelet counts were restored at 45 days to preradiation levels. The role of melanin in radioprotection was proven by the fact that mice given white mushrooms supplemented with melanin survived at the same rate as mice given black mushrooms. The ability of melanin-containing mushrooms to provide remarkable protection against radiation suggests that they could be developed into oral radioprotectors.

Key words: apoptosis, black mushrooms, GI syndrome, melanin, radioprotection

Introduction

There is a need for radioprotectors to protect normal tissues, in particular the gastrointestinal (GI) tract, from the deleterious effects of ionizing radiation in patients receiving high doses of diagnostic or therapeutic radiation. In addition, the recent nuclear accident at Fukushima-Daiichi nuclear power plants and its aftermath has highlighted the need to protect emergency personnel from high doses of ionizing radiation in a fast, efficient, and cost-effective manner. Ionizing radiation can be shielded with lead or titanium, which attenuate radiation via Compton scattering. Obviously, it is not possible to achieve this *in vivo* with toxic heavy metals.

However, nature has devised effective defenses against ionizing radiation. For example, some microorganisms survive high radiation fluxes by virtue of efficient DNA repair mechanisms, while others are aided by pigments that absorb radiation or quench oxidants. One radioprotective pigment is melanin, which is a high-molecular-weight polymer that is ubiquitous in nature, and its structure is highly conserved from fungi to mammals.¹ Many fungi, such as human pathogen *Aspergillus niger*, are black due to melanin presence, but the pigment is widespread among the edible fungi (mushrooms) as well. There are two major classes of melanin—melanins that do not contain sulfur in their structure are called eumelanins, while melanins which incorporate divalent

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FIG. 1. Chemical structures of melanin oligomers: (A) eumelanin; (B) pheomelanin.

sulfur are called pheomelanins² (Fig. 1A, B). This pigment has been consistently associated with the extreme tolerance of dark fungi to high levels of ionizing radiation. Dramatic examples of such radiation protection are provided by the reports that melanized microorganisms are colonizing the highly radioactive environment inside the damaged nuclear reactor in Chernobyl, cooling pools in nuclear reactors, and space stations.²

The mechanism of melanin interaction with ionizing radiation has remained largely unexplored, which prompted us to investigate this subject several years ago. Initially, we demonstrated that microbial melanin could function in transducing the energy of γ radiation in fungal cells³ and that the efficacy of the radioprotection of melanized cells by melanin was dependent on its chemical composition and spatial arrangement.⁴ The observation that γ irradiation of melanin caused changes in melanin chemical structure, paramagnetism,⁵ reversible oxidation, and electrical current production⁶ provided direct evidence for the ability of γ radiation and melanin to interact. In addition to free reactive radical scavenging, radioprotection by melanin involved gradual energy dissipation of Compton recoil electrons through their interaction with the π -electron-rich melanin structural units until the kinetic energy of recoil electrons becomes low enough to be trapped by stable free radicals present in the pigment.⁷ Given such radioprotective properties of melanin, we hypothesized that melanin could be harnessed as a mammalian radioprotector and that the physical presence of melanin in the GI tract during irradiation would provide internal shielding and protect the vulnerable dividing cells of the intestine through both Compton scattering and free radical scavenging. Such protection should translate into reduced damage to the gut mucosa, which, in turn, should reduce the likelihood of gut leakage and mortality due to sepsis. Here, we report that orally administered edible melanin-containing mushrooms protected mice against lethal doses of radiation, suggesting their potential for development into low-cost oral radioprotectors.

Methods and Materials

Melanin sources and physicochemical analyses

Commercial synthetic eumelanin made from tyrosine was obtained from Sigma-Aldrich. We selected the black

edible mushroom *Auricularia auricula-judae* (common name Jelly Ear) as a source of edible melanin and the white mushroom *Boletus edulis* (common name porcino) as a melanin-devoid control. Both mushrooms are basidiomycetes that are used in Western and Asian cuisines and are commercially available. Dried *Auricularia auricula-judae* and *Boletus edulis* were purchased from Trader Joe's (Monrovia, CA). Melanin from black mushrooms was purified as previously described.⁸ An elemental analysis of melanin was carried out by QTI (Whitehouse, NJ). Electron paramagnetic resonance (EPR) of mushrooms and high-performance liquid chromatography (HPLC) of melanin using permanganate oxidation were performed as in.⁷ The antioxidant capacity of methanol extracts from black and white mushrooms in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was measured as in.⁹

In vivo fluorescent imaging

All animal studies were carried out in accordance with the guidelines of the Animal Care and Use Committee. Six- to eight-week-old CD-1 female mice (Charles River Breeding Laboratories, Portage, MI) were used in all experiments. The *in vivo* imaging was performed with an IVIS Spectrum Imaging System (Caliper Life Sciences, Hopton, MA) in the epifluorescence mode equipped with 675/30 nm and 840/20 nm filters for excitation and emission, respectively. Mice were fed with nonfluorescent chow for 5 days and then fasted overnight before the imaging experiment to exclude the interference from the remnant autofluorescence of the chow. They were given 1 g/kg body weight white mushroom suspension in water via a gavage needle and imaged in the supine position under Isoflurane anesthesia at 15, 30, and 60 minutes postfeeding.

In vivo radioprotection with melanin-containing mushrooms

CD-1 mice were divided into groups of 5–6 and fed with a gavage needle 1 g/kg body weight black mushroom suspension in PBS, or PBS alone, or 1 g/kg white mushroom suspension, or 1 g/kg white mushroom suspension supplemented with 100 mg/kg synthetic melanin. One hour after mushroom administration, the mice were irradiated with 9 Gy dose of Cs-137 radiation at a dose rate of

FIG. 2. Physicochemical characterization of black and white mushrooms: (A, B) Electron paramagnetic resonance of dried mushrooms: (A) black mushrooms; (B) white mushrooms (inserts show the appearance of dried mushrooms); (C–E) oxidative high-performance liquid chromatography of melanin purified from black mushrooms: (C) background solution; (D) Pyrrole-2,3-dicarboxylic acid (PDCA) standard eluting at 8 minutes; (E) melanin from black mushrooms showing PDCA peak; (F, G) results of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay for antioxidant presence: (F) BHA positive control; (G) methanol extracts from black and white mushrooms.

2.5 Gy/min. The mice were evaluated daily for body weight and physical condition for 45 days. The experiment was performed twice. At the conclusion of the experiment, the surviving mice were humanely sacrificed; their blood chemistry was analyzed for white blood cells and platelet count; gross pathology was performed; and the stomach, small and large intestines, spleen, and bone marrow were

subjected to histological evaluation. In addition, the blood from irradiated mice was taken on days 1–5 postirradiation and analyzed for WBS and platelet count. The survival of the mice was analyzed using log-rank test; the WBC and platelet counts were analyzed by one-tail Student's test. The differences in results were considered statistically significant when p was <0.05 .

FIG. 3. Fluorescent imaging of CD-1 mice fed with white mushrooms at different times postfeeding: (A) 15 minutes, (B) 30 and (C) 60 minutes. Mice were given 1 g/kg body weight white mushrooms suspension in water via a gavage needle and imaged in a supine position under anesthesia.

Histological analysis of GI tissue for dividing cells (Ki67 marker) and apoptotic cells (TUNEL)

Mice were divided into the same groups and irradiated as described earlier; this was followed by sacrifice 24 hours later. Their duodenum was harvested, fixed in 10% neutral-buffered formalin, and paraffin embedded. For immunohistochemistry of Ki67, primary antibody for Ki67 (Clone SP6; Thermo Scientific, Rockford, IL) was used. For TUNEL DNA strand-breaks analysis, the TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein; Roche Applied Science, Indianapolis, IN) was utilized according to the manufacturer's instructions.

Results

Analyses of melanin and antioxidant contents in black and white mushrooms

The presence of melanin in *Auricularia auricula-judae* (black mushrooms) and its absence in *Boletus edulis* (white mushrooms) was demonstrated by EPR with characteristic melanin "signature" signal in black mushrooms (Fig. 2A) and background only in white mushrooms (Fig. 2B). Since melanin is insoluble in all common solvents, EPR provides the only standardized qualitative and quantitative test for melanin since the 1990s,¹⁰ as it is a stable free radical with a strong and distinctive EPR signature.

Melanin purified from black mushrooms using the protocol developed in our laboratories⁸ constituted ~10% of their weight and was further characterized by elemental analysis and oxidative HPLC. Eumelanins are composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomer units with 6%–9% nitrogen.¹¹ In parallel, fungi also synthesize eumelanin from 1,8-dihydroxynaphthalene (DHN) via the pentaketide synthetic pathway, and such melanin does not contain nitrogen in its structure.¹² The elemental analysis determined 44% carbon, 5% hydrogen, and 2% nitrogen in black mushroom melanin. The low percentage of nitrogen suggested that the pigment was primarily DHN-melanin, while the HPLC of oxidized melanin detected the presence of pyrrole-2,3-dicarboxylic acid (PDCA) (Fig. 2C–E), which is an oxidation product of DHI-derived units. We concluded that melanin in black mushrooms was a mixture of DHN and DHI melanins.

In addition, we considered that mushroom-associated antioxidants could contribute to the radio-protective effect and compared the antioxidant contents of black and white mushrooms using the DPPH assay. The DPPH is a stable free radical having a deep violet color in solution. The radical scavenging activity of a sample can be measured as a decolorizing effect following the trapping of the unpaired electron of DPPH.⁹ There was no difference in soluble antioxidant content between black and white mushrooms (Fig. 2F, G).

Melanin-containing edible mushrooms offered efficient radioprotection in irradiated mice

We evaluated the radioprotective efficacy of melanin delivered as a natural food source. First, we needed to determine the time between feeding mice mushrooms and irradiation to ascertain the presence of mushrooms in the GI tract during irradiation. Imaging of mushroom-fed mice on a IVIS Spectrum Imaging System at 15, 30, and 60 minutes

FIG. 4. Survival of irradiated CD-1 mice fed with black mushrooms and irradiated with 9 Gy Cs-137 radiation at a dose rate of 2.5 Gy/min, blood counts in the surviving mice, and histology of the gastrointestinal tract and bone marrow. (A) Kaplan-Meier survival curves. The experiment was performed twice and was terminated at day 45; (B) white blood cell counts at day 45; (C) platelet counts; (D–H) H&E stained slides with tissues from control and irradiated mice. Left, nonirradiated controls; middle, black mushroom group; right, white mushroom supplemented with melanin. (D) stomach; (E) large intestine; (F) small intestine (SI); (G) bone marrow; (H) spleen. Original magnification: D,E,G—400, SI-200, spleen-100.

postfeeding revealed that the mushrooms were in the stomach at 15 and 30 minutes (Fig. 3A, B) and moved into the intestines to the large extent at 60 minutes (Fig. 3C). Since intestines are more sensitive to ionizing radiation than the stomach, we selected 60 minutes as the time to administer radiation in order to ensure maximum protection for the most sensitive part of the GI tract.

For radioprotection experiments, groups of 5–6 CD-1 mice were fed with 1 g/kg body weight black mushrooms. The control groups included mice given only sterile PBS, or 1 g/kg white mushrooms. To establish that the melanin pigment was indeed the radioprotective substance in black mushrooms, an additional group of mice was given 1 g/kg white mushrooms dose supplemented with 100 mg/kg synthetic melanin to match 10% melanin contents in black mushrooms as per elemental analysis. All mice in the PBS and 80% in the white mushrooms groups died by day 14 postirradiation (Fig. 4A). The remaining 20% of mice in the white mushroom-treated group died by day 25. This trend toward prolongation in survival in comparison with the PBS-alone group that was not statistically significant ($p=0.07$) might be explained by the presence of antioxidants in white mushrooms which could have produced transient protective effects in the gut. Strikingly, in black mushrooms and in white mushrooms supplemented with synthetic melanin groups, 60 and 75% of mice survived ($p=0.002$ and 0.001), respectively, up to day 45 when the experiment was terminated (Fig. 4A). Simultaneously, the white blood cell counts in black mushroom and in melanin-supplemented groups were not different from the nonirradiated controls ($p=0.06$) (Fig. 4B), while platelet counts were lower in both irradiated groups ($p=0.03$) (Fig. 4C), however, at the levels that ensure

recovery in mice receiving radiation treatment.¹³ In lethally irradiated mice, mortality results from damage to rapidly dividing tissues such as GI mucosa¹⁴ and bone marrow suppression.¹⁵ There were no signs of radiation damage in the stomachs, large and small intestines (LI and SI, respectively) in the surviving mice in black mushroom and melanin-supplemented groups (Fig. 4D–F). The bone marrow of irradiated mice had slight myeloid hyperplasia (Fig. 4G), and the spleen's architecture was normal with some extramedullary hematopoiesis (Fig. 4H).

To confirm that the survival of mice in black mushrooms and melanin-supplemented groups was due to the radioprotection of GI track, the GI tissues of irradiated mice were analyzed for the number of dividing cells (Ki67 index) and the presence of apoptotic cells (TUNEL) 24 hours after irradiation. We also measured their WBC and platelet on days 0–5 postirradiation. There were significantly fewer dividing cells in the small intestines of the mice receiving PBS or white mushrooms in comparison with nonirradiated controls ($p=0.03$ and 0.04 , respectively), while there was no statistically significant difference in Ki67-positive cells between nonirradiated controls and black mushrooms and melanin-supplemented groups ($p=0.08$ and 0.07 , respectively) (Fig. 5A, C–G [upper row]). Conversely, while very few apoptotic cells were detected by TUNEL in nonirradiated

FIG. 5. Histological analysis of SIs in irradiated CD-1 mice for dividing (Ki67) and apoptotic (TUNEL) cells and blood counts in these mice on days 0–5 after irradiation. (A) counts of Ki67-positive cells in the crypts. The insert on the left shows the villi, crypts, muscle, and cells that were counted as Ki67-positive (false-positive cells are shown with crosses); (B) counts of TUNEL-positive cells in the crypts. The insert on the left shows villi, crypts, muscle, and cells that were counted as TUNEL-positive (shown with black arrows, false-positive cells are shown with crosses); (C–G) Ki67 (upper row) and TUNEL (low row) stained slides with the tissues from the nonirradiated and irradiated mice: (C) nonirradiated group; (D) PBS; (E) white mushrooms; (F) black mushrooms; (G) white mushrooms supplemented with melanin. Original magnification 200; (H) WBC in irradiated mice on days 0–5 postirradiation; (I) platelet in irradiated mice on days 0–5 postirradiation.

cells population—their number was 7–8 times higher post-irradiation in mice receiving PBS and white mushrooms ($p=0.001$ for both groups) (Fig. 5B, C–G [lower row]). In contrast, mice receiving black mushrooms exhibited significantly fewer apoptotic cells in comparison with PBS or white mushroom-fed animals ($p=0.015$ and 0.02 , respectively), while a similar trend was observed for the melanin-supplemented group ($p=0.06$ for both groups). WBC and platelet reached nadir in all groups at day 5 postirradiation (Fig. 5H, I), demonstrating that bone marrow was not protected by mushrooms during irradiation.

Discussion

We have demonstrated that it is possible to create an efficient oral radioprotector based on natural pigment melanin, which would act as an internal shield and protect the tissue by a mechanism based on Compton scattering followed by free radical scavenging. There is an ongoing need for oral radioprotectors that are inexpensive, and are suitable for both patients undergoing therapeutic and diagnostic radiation procedures and for the distribution to large numbers of people in the event of radiation emergencies. One potential radioprotector that has been studied extensively is amifostine.^{16,17} While this drug has some radioprotective efficacy, it also has several undesirable properties, including a relatively low radioprotective capacity, serious side effects such as anaphylaxis, and the need for intravenous administration. In a study conducted by Burdelya *et al.*,¹⁸ a different approach to radioprotection was taken by pharmacologically suppressing apoptosis in the irradiated cells. While this method showed promise, the drug also has to be given parenterally and might have carcinogenic side effects by virtue of interfering with the process of apoptosis.

We conducted *in vivo* studies to evaluate the protective effect of orally administered melanin on the GI tract of lethally irradiated CD-1 mice. The radioprotective effects of melanin are based on the controlled dissipation of Compton electron energy by melanin, which results in a decreased number of interactions between Compton electrons and cellular milieu and the scavenging of free reactive radicals by melanin.⁷ The equal survival of mice protected with either black mushrooms or white mushrooms supplemented with melanin established the causality between the presence of melanin in black mushrooms and their radioprotective properties. In addition, since both types of mushrooms contained the same amounts of antioxidants, the contribution of antioxidants to radioprotection in this study was ruled out. When compared with published data—black mushrooms were more protective than amifostine (60% survival after 9 Gy delivered at 1 Gy/min¹⁶), and equal to flagellin-derived polypeptide (80% survival after 9 Gy delivered at 2.3 Gy/min¹⁸). The increase in radiation dose rate is known to make the cellular repair mechanisms less efficient.¹⁹

It is known that endothelial apoptosis is the primary lesion which initiates intestinal radiation damage in mice.²⁰ The analysis of GI tissues for the presence of dividing and apoptotic cells at 24 hours postirradiation demonstrated that melanin presence prevented the apoptotic death of the cells. Given that a significant proportion of black mushroom or white mushroom supplemented with melanin-fed mice became long term survivors, it should follow that the presence

of melanin in the GI tract provided local protection that allowed these mice to recover. Since melanin is insoluble, it provided protection only in the gut area, but this protection must have extended to lymphoid cells that then repopulated tissues to produce a hematologic recovery through extramedullary hematopoiesis. The protection of GI mucosa would prevent death in the short term by a GI syndrome and sepsis, while gut-derived lymphoid cells could provide a hematologic reserve for long-term recovery.

In conclusion, local GI protection (shielding) with melanin-containing mushrooms translated into systemic protection, and this observation establishes a new concept in the approach for protecting against radiation syndrome. We anticipate that black edible mushrooms could be developed into low-cost oral radioprotectors for patients and affected populations exposed to ionizing radiation.

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Disclosure Statement

No competing financial interests exist.

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