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Gamma Radiation Effects on *Sporothrix schenckii* Yeast Cells

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Abstract Sporotrichosis is a subcutaneous mycosis caused by *Sporothrix schenckii*. Zoonotic transmission to man can occur after scratches or bites of animals, mainly cats. In this study, the gamma radiation effects on yeast of *S. schenckii* were analyzed with a view of developing a radioattenuated vaccine for veterinary use. The cultures were irradiated at doses ranging from 1.0 to 9.0 kGy. The reproductive capacity was measured by the ability of cells to form colonies. No colonies could be recovered above 8.0 kGy, using inocula up to 10^7 cells. Nevertheless, yeast cells irradiated with 7.0 kGy already were unable to produce infection in immunosuppressed mice. Evaluation by the FungaLight™ Kit (Invitrogen) indicated that yeast cells remained viable up to 9.0 kGy. At 7.0 kGy, protein synthesis, estimated by the incorporation of [L-^{35}S] methionine, continues at levels slightly lower than the controls, but a significant decrease was observed at 9.0 kGy. The DNA of 7.0 kGy irradiated cells, analyzed by electrophoresis in agarose gel, was degraded. Cytoplasmic vacuolation was the main change verified in these cells by transmission electron microscopy. The dose of 7.0 kGy was considered satisfactory for yeast attenuation since irradiated cells were unable to produce infection but retained viability, metabolic activity, and morphology.

Keywords *Sporothrix schenckii* · Sporotrichosis · Gamma radiation · Attenuation · Vaccine

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Introduction

Sporotrichosis is the most common of the subcutaneous mycosis in South America and is characterized by the subacute and chronic evolution of cutaneous or subcutaneous nodular lesions [1]. It is caused by the fungus *Sporothrix schenckii*, a thermally dimorphic fungus that exists in the hyphal form at temperatures below 37°C and as yeast above 37°C. This mycosis develops mainly through traumatic inoculation of the fungus into the dermis after wounds or abrasion of the skin produced by infected materials or by the inhalation of spores through the respiratory tract. Zoonotic transmission can occur after scratches or bites of animals, mainly cats, rodents, and armadillos. Since the 1980s, zoonotic transmission of sporotrichosis has been mentioned in different reports and domestic cats have gained importance in the

transmission to man [2–4]. The largest epidemic of sporotrichosis due to zoonotic transmission was described in Rio de Janeiro (Brazil). Between 1998 and 2004, at the Evandro Chagas Clinical Research Institute, 1,503 cats, 64 dogs, and 759 humans were diagnosed. The feline disease preceded human and canine diseases [5].

Up to this moment, no vaccine has been reported for *S. schenckii* [6]. A veterinary vaccine for sporotrichosis would be useful for avoiding mycosis transmission from pet to man and also to protect animals.

Ionizing radiation has been successfully used to attenuate parasites and microorganisms for vaccine development and research. Irradiated pathogens frequently lost their virulence, but retained metabolic activity and morphology, and consequently are able to induce a high level of immunity. Radioattenuated live vaccines can expose their antigens sequentially to the host, as in a natural infection allowing the immune system to recognize them as viable agents without the risk of a progressive infection. Effective immunization of laboratory animals has been achieved with various irradiated protozoan parasites, helminths, and nematodes [7]. Recently, gamma radiation was used for attenuation of yeast cells of the pathogenic fungus *Paracoccidioides brasiliensis* [8, 9]. Immunization of Balb/c mice with radioattenuated yeast cells confers protection against challenge with highly infective yeasts forms of *P. brasiliensis* [10, 11], demonstrating the potential of this methodology for the development of live attenuated vaccines against fungal infections.

In the present study, the effects of gamma radiation on the reproductive ability, metabolic activity, morphology, and infectivity of yeast form of *S. schenckii* were analyzed. The main purpose was to evaluate whether yeast cells of *S. schenckii* could be attenuated by gamma radiation for subsequent studies on the development of a radioattenuated vaccine for veterinary use.

Materials and Methods

Culture Conditions

S. schenckii yeast cells, strain ATCC 201679, was grown in BHI agar, supplemented with thiamine, at 37°C. The yeast cells were subcultured every 7 days.

Gamma Irradiation

The cultures, in solid medium, were irradiated at doses ranging from 1.0 to 9.0 kGy, in the presence of oxygen and at room temperature. The irradiation was performed in a uniform source of ^{60}Co gamma rays at dose rate of 1,500 Gy/h. Adequate controls were maintained outside the source. After each dose the growth, viability and metabolic activity were estimated.

Yeast Growth Analysis

Reproductive capacity was measured by the ability of yeast cells to form colonies. After irradiation, control and irradiated cells were transferred to PBS solution, vortexed and counted in a Neubauer chamber. The suspensions, containing 10^3–10^7 yeast cells depending upon the dose, were spread on Petri dishes with BHI agar supplemented with thiamine. The following numbers of cell were used for each dose: 10^3 (control and 1.0 kGy), 10^4 (2.0 kGy), 10^5 (4.0 and 5.0 kGy), 10^6 (6.0 kGy), and 10^7 (7.0, 8.0, and 9.0 kGy). The plates were incubated at 37°C for 7 days, and colonies were counted using a magnifying glass. The mean colony counting was obtained from triplicate determinations.

Yeast Viability Analysis

The viability of irradiated cells harvested 2 and 24 h after irradiation was measured using the FungaLight™ CFDA, AM/Propidium Iodide Yeast Vitality Kit (Invitrogen) according to the manufacturer's protocol. The kit combines a cell permeable esterase substrate (5-carboxyfluorescein diacetate) with a membrane integrity indicator (propidium iodide) to evaluate the viability of yeast cells by fluorescence microscopy. Esterase-active yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red. The excitation/emission maxima of dyes are 492/517 nm for CFDA, AM and 490/635 nm for propidium iodide. The kit was validated comparing the results with those obtained using the fluorescein diacetate–ethidium bromide stain method [12].

Protein Synthesis Assay

Controls and irradiated yeast cells (4, 7 and 9 kGy) harvested 2 and 24 h after irradiation were transferred

to 10 ml of liquid medium (BHI), at an initial inoculum density of 1×10^7 cells/ml. To each sample were added 10 μCi of [L-^{35}S] methionine, and cells were incubated for 12 h at 37°C. The cells were harvested, washed in PBS, and disrupted using glass beads. After the centrifugation at 10, 000 × g, the protein concentration in the supernatant was estimated by the Lowry method. Then, a volume containing 0.3 mg of protein was placed in scintillation vials, and 4.5 ml of scintillation fluid was added. Counts were determined in a liquid scintillation analyzer and expressed in counts per minute (cpm). All samples were submitted to triplicate determinations.

Infectivity Assay

The assay was performed using BALB/c mice immunosuppressed by gamma radiation. The animals received a dose of 2.5 Gy, at dose rate of 75 Gy/h, in a uniform source of ^{60}Co gamma rays. Immunosuppression was evaluated by counting leukocytes. An aliquot of peripheral blood was incubated with Türk's solution for microscopy, and the number of leukocytes per ml of blood was determined with the aid of a Neubauer chamber. On average, 24 h after irradiation, the leukocyte population was reduced around ten times. Twenty-four hours after irradiation, mice were inoculated intratracheally with 60 μl of PBS containing 5×10^6 yeast cells irradiated with 7.0 or 9.0 kGy. Controls were inoculated with the same number of viable virulent yeast cells. Organ colony-forming units (CFUs) were determined 15 days after infection in the lung, spleen, and liver. The organs were removed, weighed, homogenized, and washed three times in PBS by centrifugation. The final suspensions in PBS were plated in the medium described earlier in the yeast growth analysis section. The plates were incubated at 37°C and read after 20 days. The results were expressed as the number of CFUs per gram of tissue per mouse in each experimental group ($n = 5$).

DNA Fragmentation Evaluation

DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the supplier's instructions. DNA from control and irradiated yeast cells (7.0 kGy) were extracted 2 h after irradiation. Purity and concentration of DNA were determined by UV spectroscopy (260–280 nm). DNA (0.25 μg) from control and irradiated yeast was run on 1% (w/v) agarose gel at 100 V for 40 min. Following staining with ethidium bromide, DNA was visualized using a UV transilluminator.

Transmission Electron Microscopy

Non-irradiated yeast and those harvested 2 or 24 h after irradiation (7.0 kGy) were washed three times in PBS. The pellet was suspended in Karnovsky solution (3.5% (v/v) glutaraldehyde and 4% paraformaldehyde) in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.4, and fixed overnight. After fixation, the cells were rinsed three times in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.4, and then embedded in 4% molten agar (Merck, Darmstad, Germany). The resulting agar pellets were fixed in 1% osmium tetroxide and 0.1 mol l^{-1} sodium cacodylate, pH 7.4, for 1 h at 4°C and dehydrated with increasing concentrations of ethanol. After the 100% ethanol washes, cells were washed with 100% acetone and infiltrated with acetone/Epon (1:2). The sections were examined with a transmission electron microscope (EM10 A/B Zeiss)(Carl Zeiss, Oberkochen, Germany).

Results

The reproductive ability of irradiated cells was monitored by their capacity to form colonies. The effect of gamma irradiation on this parameter was somewhat variable. A representative curve was presented in Fig. 1. The number of growth competent cells falls off with increasing doses of radiation. A reduction of 5 $\log_{10}$ cycles in the number of colonies was achieved at 4.0 kGy and at 9.0 kGy no colonies could be recovered, even when large inocula containing up to 10^7 cells were used. However, when massive inocula (large pellets) with uncountable cells were used, eventually, some colonies were found following irradiation with up to 12.0 kGy, demonstrating the presence of cells with high radioresistance in the yeast population (data not shown).

The viability of irradiated cells evaluated with the FungaLight™ CFDA, AM/Propidium Iodide Yeast Vitality Kit (Invitrogen), 2 and 24 h postirradiation, indicated that yeast cells remained viable (esterase

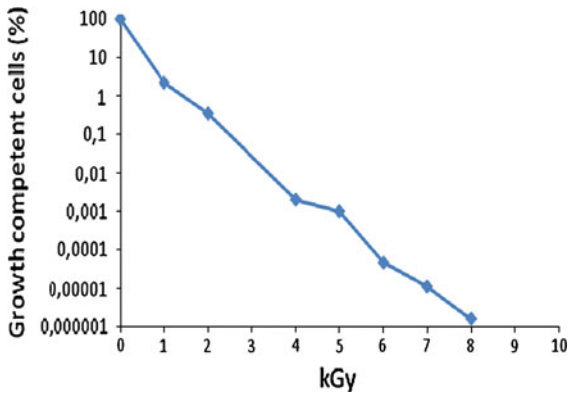

Fig. 1 Effect of gamma radiation on the reproductive ability of *Sporothrix schenckii* yeast cells. Yeast cells were irradiated with increasing doses of external gamma radiation and the fraction of cells with reproductive ability determined by the capacity to form colonies. The mean colony counting was obtained from triplicate determinations. These results were representative from three independent experiments

active and with intact cell membranes) up to 9.0 kGy, the highest dose tested (Fig. 2).

Synthetic protein metabolism was analyzed by incorporation of [L-^{35}S] methionine. The effects of gamma irradiation on synthetic protein metabolism are shown in Fig. 3. A remarkable increase in protein synthesis was verified at 4.0 kGy, 24 h after irradiation, indicating an intense process of protein turnover. At 7.0 kGy, the protein synthesis continued at levels slightly lower than the controls. A significant decrease in protein synthesis was observed 24 h after irradiation at 9.0 kGy.

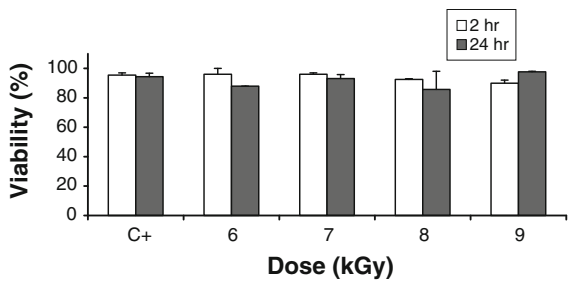

Fig. 2 Effect of gamma radiation on the viability of *Sporothrix schenckii* yeast cells. Yeast cells were irradiated with increasing doses of gamma radiation, and viability was measured 2 and 24 h later using the Kit FungaLight CFDA, AM/Propidium Iodide (Invitrogen). Non-irradiated control (*C* +). The *values* represent the median and standard deviations of three independent experiments

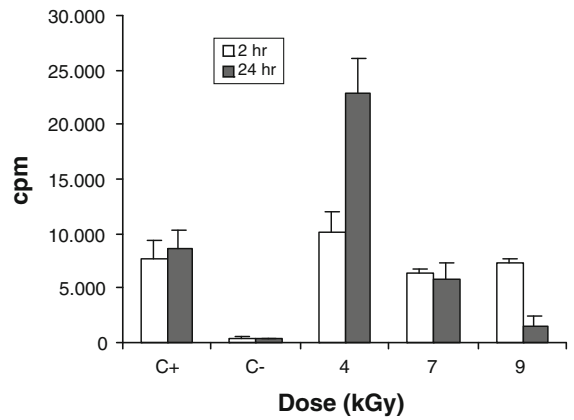

Fig. 3 Evaluation of protein synthesis after gamma irradiation. Yeast cells were irradiated with increasing doses of gamma radiation, and the metabolism of protein synthesis monitored 2 and 24 h later, by the incorporation of [L-^{35}S] methionine. The *positive control* (*C*+) was no irradiated cells. The *negative control* (*C*−) was cells killed by heating. The *values* represent the median and standard deviations of two independent experiments

The infectivity of 7.0 and 9.0 kGy gamma irradiated yeast cells was evaluated in immunosuppressed Balb/c mice. Table 1 shows the number of CFUs recovered by each organ from mice infected with the irradiated yeast cells in relation to controls. The 7.0 and 9.0 kGy gamma irradiated yeast cells failed to produce infection, given that no CFU could be recovered from mice infected with them. In view of these results, the dose of 7.0 kGy was selected for attenuation since at this dose, yeast protein synthesis was less damaged by radiation (Fig. 3), while the cells were unable to produce a progressive infection.

Yeast DNA integrity after irradiation with 7.0 kGy was analyzed by electrophoresis in agarose gels [9]. DNA degradation due to gamma irradiation can be

Table 1 CFUs recovery from immunosuppressed mice infected with radioattenuated yeast of *S. schenckii*

Organ	Control yeast cells (*)	Radioattenuated yeast cells (*)	
		7.0 kGy	9.0 kGy
Lung	210 (±45.6)	0	0
Spleen	714 (±425.7)	0	0
Liver	12 (±7.1)	0	0

Control yeast cells: non-irradiated. Values are the means and standard deviations

* CFUs per gram of tissue per mouse ($n = 5$)

Fig. 4 DNA banding pattern of 7.0 kGy irradiated yeast cells. DNA fragmentation of *S. schenckii* yeast cells treated with 7.0 kGy (*2*) was analyzed 2 h after irradiation by electrophoresis in an agarose gel. The positive controls (*1*) were no irradiated cells. This experiment was carried out three times and produced the same result

visualized in Fig. 4. Degradation can be estimated by the disappearance of the band corresponding to genomic DNA and the appearance of smearing, indicating small DNA fragments in the lower part of the gel.

Figure 5 shows transmission electron microscopy analysis of *S. schenckii* yeast submitted to 7.0 kGy, observed 2 h after irradiation. The irradiated yeast plasma membrane and cell wall were intact and preserved, but an extensive cytoplasmic vacuolation was verified in the irradiated cells. The same was verified 24 h after irradiation (data not shown).

Discussion

S. schenckii yeast, like many fungi, exhibited high radioresistance relative to other microorganisms and mammalian cells [13]. In the present study, no CFUs were recovered in vitro at doses above 8.0 kGy, using

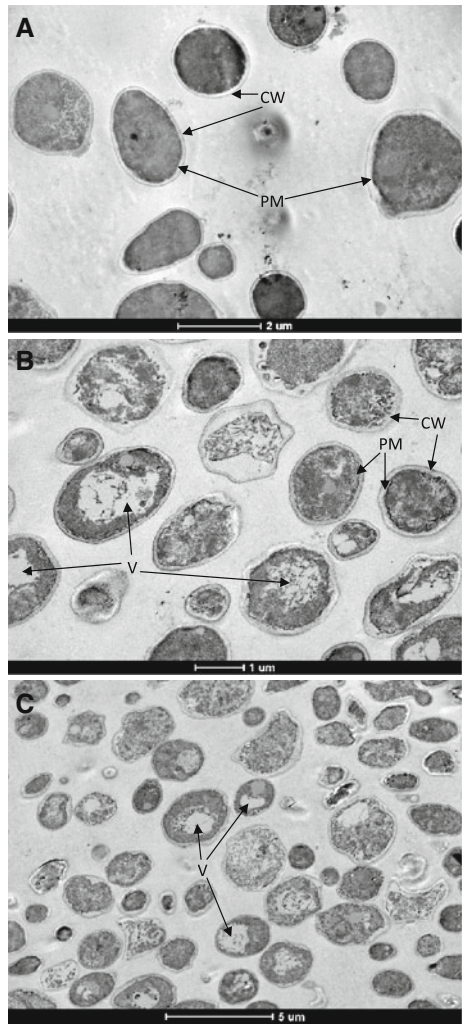

Fig. 5 Transmission electron microscopy of 7.0 kGy irradiated yeast cell. Control (**a**) and 7.0 kGy irradiated yeast (**b**, **c**) 2 h after irradiation. *CW* cell wall, *PM* plasma membrane, and *V* vacuoles

inocula of up to 10^7 cells. Nevertheless, yeast irradiated with 7.0 kGy already were unable to produce infection in immunosuppressed mice. A very small fraction of cells (0.00001%) were able to multiply at the dose of 7.0 kGy, as verified in the plating experiment in vitro. However, these growth competent cells, probably because they were very scarce or damaged, were unable to produce infection in vivo. Immunosuppressed mice were used to allow study of their ability to eliminate irradiated yeast in the absence of normal immunity.

The 7.0 kGy irradiated yeast cells retained viability, and to some degree, the capacity to synthesize

proteins, while for 9.0 kGy irradiated cells synthetic protein metabolism was strongly affected. The effects of gamma irradiation on protein synthesis could be demonstrated only 24 h after irradiation, since 2 h after irradiation, no significant difference in this parameter was seen among the different doses tested. The dose of 7.0 kGy was considered satisfactory for yeast attenuation since irradiated cells were unable to produce infection but retained metabolic activity. The use of higher radiation doses increases unnecessarily the cellular damage, while lower doses enhance the number of growth competent cells.

The increase in protein synthesis verified at 4.0 kGy was probably due to protein turnover. Gamma irradiation causes intense oxidation of proteins, which then are replaced by newly synthesized molecules. A fraction of the [L-^{35}S] methionine incorporation was presumably related to the synthesis of proteins and enzymes associated with repair mechanisms. The high [L-^{35}S] methionine incorporation at this dose indicated that yeast cells were metabolically able to react to damage caused by ionizing radiation.

The inability of irradiated cells to divide results from DNA fragmentation, which leads to cell cycle arrest or unbalanced chromatin exchange in daughter cells and consequent mitotic death [14, 15]. The DNA of 7.0 kGy irradiated cells was widely fragmented. Nevertheless, these lesions did not eliminate protein synthesis, likely because most of the breaks occur outside operons, which usually are a minor part of the genome, allowing genes to work until cell division.

Few studies about structural effects of ionizing radiation on yeast cells have been published. The reported changes include disruption of the cell wall, alterations in the internal membrane system, chromatin dispersion, and nucleus fragmentation in *Rhodotorula* [16], loss of capsule in *Cryptococcus neoformans* [17], mitochondria degeneration, cytoplasmic vacuolation, swelling of the nucleus [18] and vacuole distortion in *Saccharomyces* [19], and chromatin condensation in *P. brasiliensis* [9]. Cytoplasmic vacuolation was the main change verified for *S. schenckii* in the present study.

Successful resolution of diseases caused by pathogenic fungi is dependent on the coordinated interaction of many constituents of the host immune response [20]. Attenuated whole cell vaccines can be an alternative tool to achieve this goal since it induces strong, broad responses involving multiple arms of the immune system that recapitulates natural immunity to disease, differently from immunization with protein or killed agents [21]. In addition, radioattenuated pathogens frequently are more immunogenic than the normal counterparts [7].

A worry associated with attenuated whole cell vaccines is the potential for reversion to virulence. This problem was minimized in this work by the use of gamma radiation for yeast attenuation. Adequate doses of ionizing radiation cause an extensive DNA fragmentation beyond the capacity of cell repair mechanisms [9] leading to an irreversible loss of yeast reproductive ability and virulence [10]. Preparation instability and toxicity are other potential problems that demand careful quality control procedures. Nevertheless, there are several effective examples of live attenuated vaccines in the field of vaccinology, as the Bacille Calmette–Guérin (BCG) vaccine for tuberculosis [22] and the vaccine for prevention of ringworm caused by *Trychophyton verrucosum* in cattle [23].

We conclude that it is possible to use gamma radiation to eliminate *S. schenckii* infectivity, with the pathogen retaining its viability, metabolic activity, and morphology. Now, we are evaluating the capacity of the radioattenuated yeast cells to elicit protective immunity against *S. schenckii*.

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