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EFFECTS OF GAMMA RADIATION ON *SPOROTHRIX SCHENCKII* YEAST CELLS

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ABSTRACT

Sporotrichosis is a subacute or chronic infection caused by the fungus *Sporothrix schenckii*. Zoonotic transmission can occur after scratches or bites of animals, mainly cats, rodents, and armadillos. Up to the moment, no approved vaccine was reported for *S. schenckii* or to any important pathogenic fungi infection in humans, indicating the need to expand the research in this field and to explore new alternatives. The aim of this study was to evaluate the effects of gamma radiation in the viability, metabolic activity and reproductive ability of *S. schenckii* yeast cells for further studies on the development of a vaccine for immunization of cats and dogs. The culture of *S. schenckii*, in solid medium, was irradiated at doses ranging from 1.0 to 9.0 kGy. After each dose the reproductive capacity, viability and protein synthesis were estimated. The results showed that a reduction of 6 $\log_{10}$ cycles in the number of colonies was achieved at 6.0 kGy and after 8.0 kGy no colonies could be recovered. The viability analysis indicated that yeast cells remained viable up to 9.0 kGy. The results of protein synthesis analysis showed that the yeast cells, irradiated up to 9.0 kGy, were able to synthesize proteins. Our preliminary results indicated that for the yeast cells of *S. schenckii,* it is possible to find an absorbed dose in which the pathogen loses its reproductive ability, while retaining its viability, a necessary condition for the development of a radioattenuated yeast vaccine.
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1. INTRODUCTION

Sporotrichosis is the most common of the deep mycoses and is characterized by the subacute and chronic evolution of cutaneous or subcutaneous nodular lesions. It is caused by the fungus *Sporothrix schenckii*, a dimorphic fungus that exist in the hyphal form at temperatures below 26ºC and as yeast above 37ºC [1]. The major known areas of current endemicity are in Japan and in North and South America, especially Mexico and Brazil [2]. Sporotrichosis is the most common subcutaneous mycosis in Latin America. This mycosis develops mainly through penetration 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 [3]. Zoonotic transmission can occur after scratches or bittes of animals,

mainly cats, rodents, and armadillos. Since the 1980s, the zoonotic transmission of sporotrichosis has been mentioned in different reports. The largest epidemic of sporotrichosis due to zoonotic transmission was described in Rio de Janeiro. Between 1998 and 2004, at the Instituto de Pesquisa Clínica Evandro Chagas, 1503 cats, 64 dogs and 759 humans were diagnosed. As a rule, feline disease preceded human disease [4]. Different drugs are used for the treatment of sporotrichosis, including potassium iodide, itraconazole, terbinafine, fluconazole and amphotericin B. Most patients require prolonged treatment. The duration of treatment until clinical cure is 6 to 8 weeks, on an average, in immunocompetent patients.

Up to the moment, no approved vaccine was reported for S. schenckii or to any important pathogenic fungi infection of medical relevance [5] indicating the need to expand the research in this field and to explore new alternatives. The potential of irradiation as a tool for creating highly effective attenuated vaccines has been recognized since the 1950s [6]. Irradiated pathogens frequently lose their virulence, but retain the metabolic activity and morphology, and consequently are able to induce a high level of immunity [7]. This approach was successfully used in studies of an experimental radioatenuated vaccine for the fungus of *Paracoccidioides brasiliensis* [8]. The aim of this study was to evaluate the effects of gamma radiation in the viability, metabolism and reproductive ability of *S. schenckii* yeast cells for further studies on the development of a vaccine for immunization of cats and dogs.

2. MATERIAL AND METHODS

2.1. Culture

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

2.1. 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 1500 Gy/h.

2.3. Yeast growth analysis

The reproductive capacity was measured by the ability of the yeast cells to form colonies. Control and irradiated cells were transferred to PBS solution, vortexed and counted. The suspensions, containing 10^2 to 10^7 yeast cells, were spread on Petri dishes with a high plating efficiency medium. The plates were incubated at 37º C for 7 days and colonies counted under magnifying glass. The mean colony counting was obtained from duplicate determinations.

2.4) Yeast viability analysis

The viability of irradiated cells was measured using the FungaLight™ CFDA, AM/Propidium Iodide Yeast Vitality Kit (Invitrogen) that combines a cell permeable esterase substrate with a membrane integrity indicator to evaluate the vitality of yeast cells by microscopy. The acetoxymethyl ester (AM) of the esterase substrate 5-carboxyfluorescein diacetate (CFDA) allows this reagent to permeate cell membranes. Once inside the cell, the

lipophilic blocking and diacetate groups are cleaved by nonspecific esterases, resulting in a fluorescent charged form that leaks out of cells very slowly. In contrast, the membrane integrity indicator, propidium iodide, penetrates yeast with damaged membranes. Esterase active yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red (Figure 1). The excitation/emission maxima for these dyes are 492/517 nm for CFDA, AM and 490/635 nm for propidium iodide.

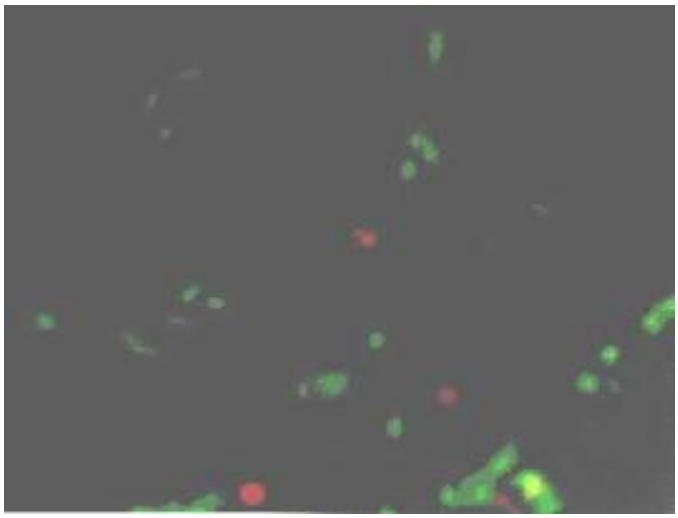

Figure 1 – Viability analysis using the Kit FungaLight CFDA, AM/ Propidium Iodide. Esterase active yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red.

2.5) Protein synthesis assay

Controls and irradiated yeast cells (4, 7 and 9 kGy) were transferred to 10 ml of liquid medium (BHI), at an initial inoculum density of 1 x 10^7 cells/ml. To each sample were added 10 µCi of [L-^{35}S] methionine and cells were incubated for 12 hours at 37^{o}C. The cells were harvested, washed in PBS and disrupted using glass beads. After the centrifugation at 10 000 x 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 were added. Counts were determined in a liquid scintillation analysis and expressed in counts per minute (cpm).

3. RESULTS AND DISCUSSION

The reproductive ability of irradiated cells was monitored by their capacity to form colonies. The survival curve was presented in figure –2. The number of surviving cells falls off with increasing doses of radiation. A reduction of 6 $\log_{10}$ cycles in the number of colonies was achieved at 6.0 kGy and at 8.0 kGy no colonies could be recovered, even a if large inoculum was used. This curve represents an average of two independent experiments.

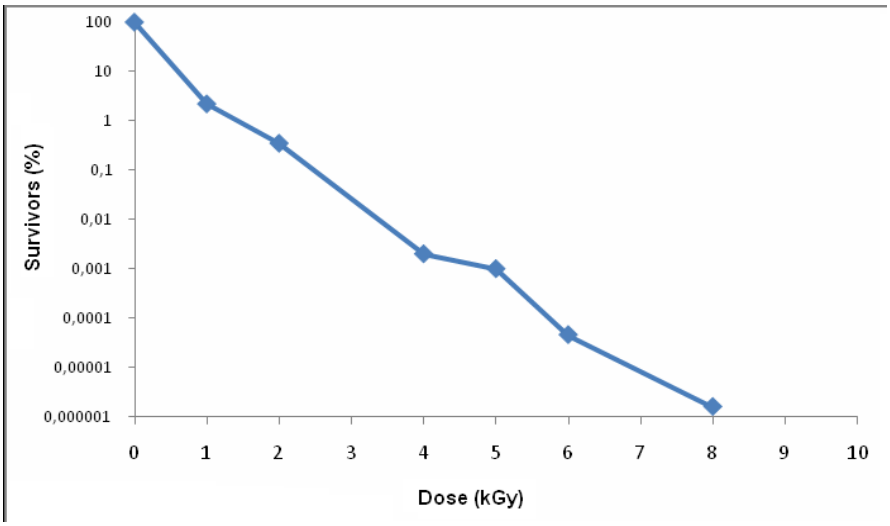

Figure 2- Effect of gamma irradiation on survival of yeast cells of *S. schenckii*. Yeast cells were irradiated with increasing doses of external gamma radiation and the fraction of surviving cells determined. At 8.0 kGy no more colonies could be recovered.

The viability analysis, performed 2 and 24 hours post irradiation, indicated that yeast cells remained viable up to 9.0 kGy, the higher dose tested. (Figure 3)

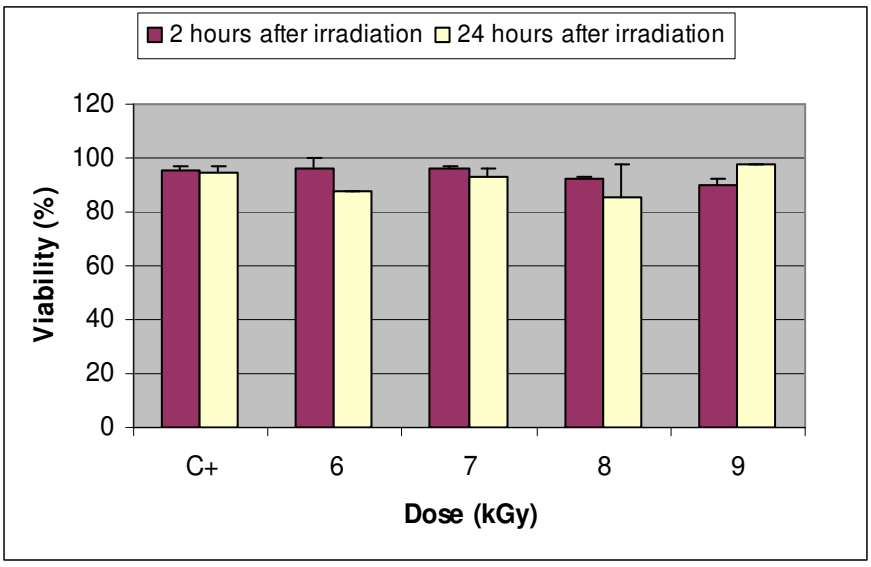

Figure 3 – Effect of gamma irradiation on the viability of yeast cells of *S. schenckii*. Viability was mesasured using the Kit FungaLight CFDA, AM/ Propidium Iodide Yeast cells were irradiated with increasing doses of external gamma radiation. C+ non-irradiated control. The values represent the median and standard deviations of two independent experiments.

The protein synthesis was analyzed by the incorporation of ^{35}S-methionine (Figure 4). The results showed that the yeast cells, irradiated up to 9.0 kGy, maintained the synthetic protein metabolism. A remarkable increase in the protein synthesis was verified at 4 kGy, 24

hours post irradiation, suggesting that an intense process of cellular repair was in course. At 7.0 kGy the protein synthesis continued but less intense than in the controls. A significant decrease in the protein synthesis was observed 24 hours post irradiation at the dose of 9.0 kGy, indicating that this parameter was affected by the irradiation.

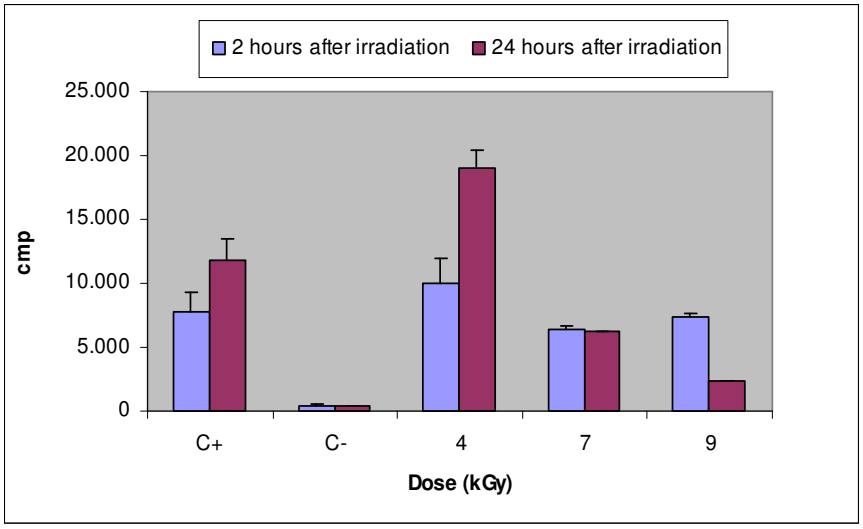

Figure 4 - ^{35}S-Methionine incorporation in cells of *S. schenckii* according to the dose of radiation.
Yeast cells were irradiated with increasing doses of external gamma radiation and the metabolism of protein synthesis monitored by the incorporation of [L-^{35}S]-methionine. C+ non-irradiated control, C- cells killed by heating. The values represent the median and standard deviations of three independent experiments

4. CONCLUSIONS

Our preliminary results indicated that for yeast cells of *S. schenckii* was possible find absorbed doses (between 7.0 and 9.0 kGy) in which the reproductive ability of the yeast cells was greatly reduced, while the viability and the metabolic activity still were present. This is a requirement for the development of a radioattenuated yeast vaccine. Now, we are evaluating the capacity of the yeast cells attenuated with these doses to stimulate a protective immunity against *S. schenckii.*

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