Original article

Ultrastructural changes in *Paracoccidioides brasiliensis* yeast cells attenuated by gamma irradiation

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Summary

Paracoccidioides brasiliensis is a thermally dimorphic fungus agent of paracoccidioidomycosis, a deep-seated systemic infection of humans with high prevalence in Latin America. Until now no vaccine has been reported. Ionizing radiation can be used to attenuate pathogens for vaccine development and we have successfully attenuated yeast cells of *P. brasiliensis* by gamma irradiation. The aim of the present study was to examine at ultrastructural level the effects of gamma irradiation attenuation on the morphology of *P. brasiliensis* yeast cells. *Paracoccidioides brasiliensis* (strain Pb-18) cultures were irradiated with a dose of 6.5 kGy. The irradiated cells were examined by scanning and also transmission electron microscopy. When examined 2 h after the irradiation by scanning electron microscopy, the 6.5 kGy irradiated cells presented deep folds or were collapsed. These lesions were reversible since when examined 48 h after irradiation the yeast had recovered the usual morphology. The transmission electron microscopy showed that the irradiated cells plasma membrane and cell wall were intact and preserved. Remarkable changes were found in the nucleus that was frequently in a very electrondense form. An extensive DNA fragmentation was produced by the gamma irradiation treatment.

Key words: *Paracoccidioides brasiliensis*, gamma irradiation, ultrastructure, electron microscopy.

Introduction

Paracoccidioides brasiliensis is a thermally dimorphic fungus agent of paracoccidioidomycosis (PCM), a deep-seated systemic infection of humans with high prevalence in Latin America.[1] Until now no vaccine has been reported and there are only few reports describing the role of *P. brasiliensis* antigens on induction of protective immune response in experimental PCM.[2–3] The potential of irradiation as a tool for creating highly effective attenuated vaccines has been recognized since the 1950s.[4] Gamma and X irradiation have been consistently proved to be successful as attenuating agents for a remarkably wide range of pathogens.[5] In every case, the irradiated pathogen induces a high level of immunity during its abbreviated lifespan and dies before reaching the stage associated with pathogenicity. In a previous work we successfully attenuated yeast cells of *P. brasiliensis* by gamma irradiation. We found an absorbed dose (6.5 kGy) in which the pathogen loses its reproductive ability and virulence, while retaining its viability, metabolic activity and antigenic profile.[6] This behaviour allows the immune system to recognize the irradiated yeast as a viable agent without risk of progressive infection. The radioattenuated yeast provides a novel tool for immunological studies in experimental paracoccidioidomycosis and vaccine research, as it can expose their antigens sequentially to the host, as in a natural infection, avoiding problems associated with single immunization schedules and can synthesize antigens induced only during the infection. The aim of the present work was to examine at ultrastructural level the effects of gamma irradiation attenuation on the morphology of *P. brasiliensis* yeast cells.

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Material and methods

Culture conditions

Paracoccidioides brasiliensis, strain Pb-18, was maintained in the yeast-form, at 35 °C, in brain infusion agar medium (BHIA) (Biobrás, Montes Claros, MG, Brazil) supplemented with 1% glucose. The yeast cells were subcultured every 10 days.

Gamma irradiation

Cultures of *P. brasiliensis*, in solid medium, were irradiated in the presence of oxygen and at room temperature. The irradiation was performed in a uniform source of ^{60}Co gamma rays, at a dose rate of 950 Gy h^{-1}. Adequate controls were maintained outside the source. To achieve the attenuation a dose of 6.5 kGy was used.

Yeast viability analysis

The viability was determined by using the modified vital dye Janus green method.[7] In brief, 20 µl of 0.05% solution of the dye Janus green (Sigma, St Louis, MO, USA) was added to an equal volume of the fungal cell suspension, and the viability determined at intervals of 10 min. The counting was performed in a Neubauer chamber (BOECO, Hamburg, Germany). Viable cells remained unstained and dead cells stained blue. The viability was followed up for 21 days.

Extraction of DNA and gel electrophoresis

DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the supplier instructions. DNA from control and irradiated yeast cells (2.0, 4.0 and 6.5 kGy) was extracted 2 h after irradiation. DNA from 6.5-kGy irradiated yeast were also extracted 48 h after irradiation. Purity and concentration of DNA was determined by UV spectroscopy (260–280 nm). DNA from control and irradiated yeast cells were 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 cells and cells harvest 2 and 48 h after irradiation were washed three times in PBS. The pellet was suspended in Karnovsky nud (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 embedded in 4% molten agar (Merck, Darmstad, Germany). The resulting agar pellet containing the cell was 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 using a transmission electron microscope (EM10 A/B Zeiss; Carl Zeiss, Oberkochen, Germany).

Scanning electron microscopy

The yeast samples, the same used for transmission electron microscopy, were washed three times in PBS and left for 18 h in 2.5% glutaraldehyde. After that, the samples were washed in PBS and cut. The fragments were washed in PBS again, and fixed in 2.5% osmium-tetroxide, 1.6% potassium ferricyanide for 2 h. The samples were dehydrated with increasing concentrations of ethanol. After the 100% ethanol washes, cells were washed with 100% acetone, dried in a critical-point drier, gold sputter-coated and observed under the scanning electron microscope JSM-5600 (Jeol B.V., Zaventem, Belgium).

Results

Radioattenuated yeast cells of *P. brasiliensis* were first examined by scanning electron microscopy (Fig. 1). The non-irradiated yeast cells presented spherical or oval shapes and often appeared surrounded by multiple buds (Fig. 1a). When observed 2 h after irradiation, the 6.5 kGy irradiated cells showed signals of cellular damage: the majority of the cells presented deep folds or were collapsed (Fig. 1b). However, these alterations were reversible since when examined 48 h after irradiation the yeast cells had recovered the usual morphology (Fig. 1c), presenting the same appearance of the non-irradiated controls.

Figure 2 represents the comparison of the viability between the radioattenuated yeast and the non-irradiated controls. The method used to determine the cell viability was the vital dye exclusion test by Janus green, a very popular method for determination of cell viability of fungi and reliable for *P. brasiliensis*.[7] The viability curve showed that the irradiated yeast cells retained the viability up to 21 days after irradiation, at the same

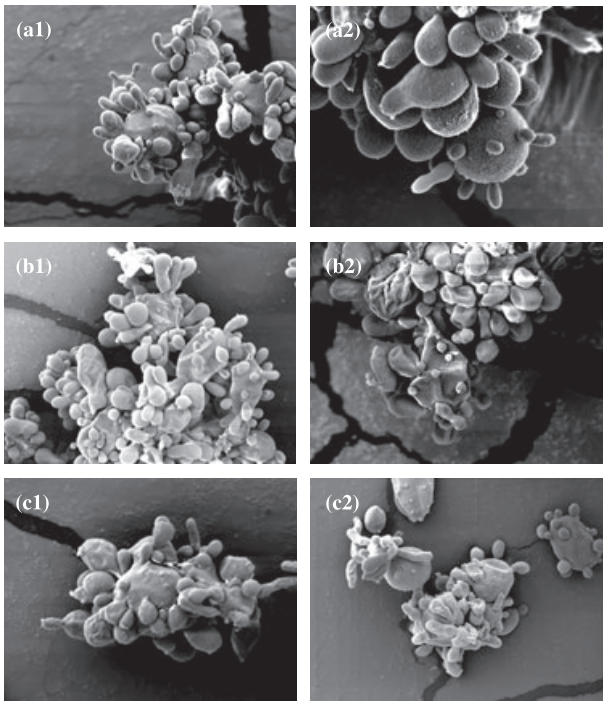

Figure 1 Scanning electron microscopy analysis of 6.5 kGy irradiated *Paracoccidioides brasiliensis* yeast cells. Control (A1 and A2), yeast cells 2 h after irradiation (B1 and B2) and yeast cells 48 h after irradiation (C1 and C2). Original magnification: A1, B1, C1 and C2 × 200, A2 × 400, B2 × 300.

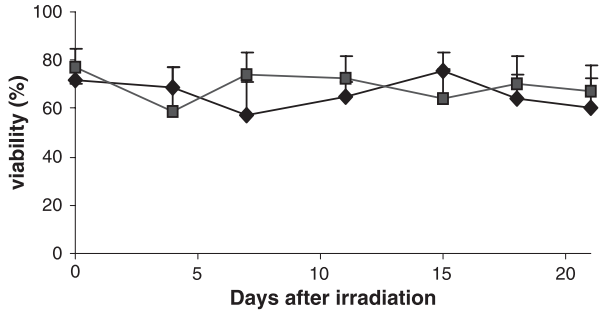

Figure 2 Cell viability analysis of 6.5 kGy irradiated yeast with the vital stain Janus green. Cells viability was analysed by counting the viable and nonviable cells in a Neubauer chamber. The values were plotted as a percentage of viable cells. ◆ – cells irradiated with 6.5 kGy, ■ – non-irradiated cells (control). The values represent the mean and standard deviations of three experiments.

level of the non-irradiated controls, indicating the cell survival after the gamma irradiation treatment.

The radioattenuated yeasts were also analysed by transmission electron microscopy. The ultrastructural characteristics of *P. brasiliensis* yeast cells growing in

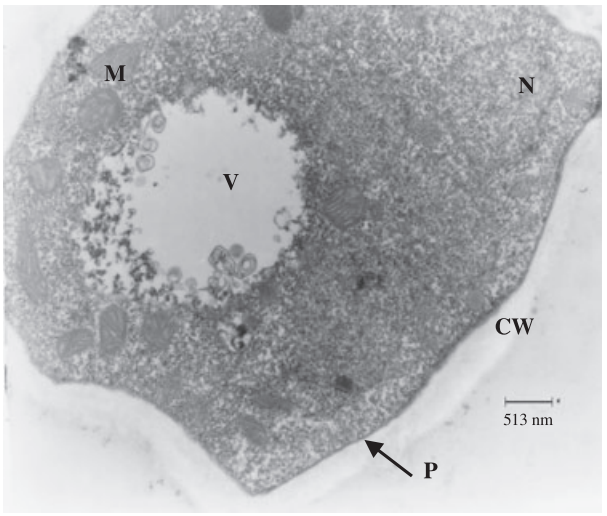

Figure 3 Transmission electron microscopy of a *Paracoccidioides brasiliensis* yeast cell. General ultrastructural features of a *Paracoccidioides brasiliensis* yeast cell. Cell wall (CW), plasma membrane (P), mitochondria (M), vacuole (V) and nucleus (N).

solid BHI medium agree with those reported by Queiroz-Telles [8] (Fig. 3). The cells presented rounded with thick walls of low electron density. The plasma membrane was easily visible. Vacuoles were observed in different numbers and shapes in the cytoplasm. The cytoplasm had a granulated appearance possibly because of the presence of large amounts of ribosomes. Mitochondria were numerous and tended to accumulate at the cellular periphery. The nuclear profiles enveloped by nuclear membrane were identified and the number of nuclei per cell varied.

The Fig. 4 shows the transmission electron microscopy analysis of the cell wall, plasma membrane and mitochondria of the radioattenuated yeast. The irradiated yeast plasma membrane was intact and preserved when examined after irradiation. No changes were also verified in the irradiated yeast cell wall. The mitochondria presented a less electrondense matrix but were not significantly different from controls. The most remarkable change was verified in the nucleus (Fig. 5). After being irradiated, the nucleus was frequently found in a very electrondense form (Fig. 5a) which, in many cells, was crescent-shaped (Fig. 5b). These nuclear changes, however, did not impair the cell viability, as shown in Fig. 2.

The ultrastructural changes in the nucleus seem to be related to DNA damage caused by gamma irradiation. The DNA degradation caused by the gamma irradiation attenuation can be visualized in Fig. 6, where the DNA integrity was analysed by

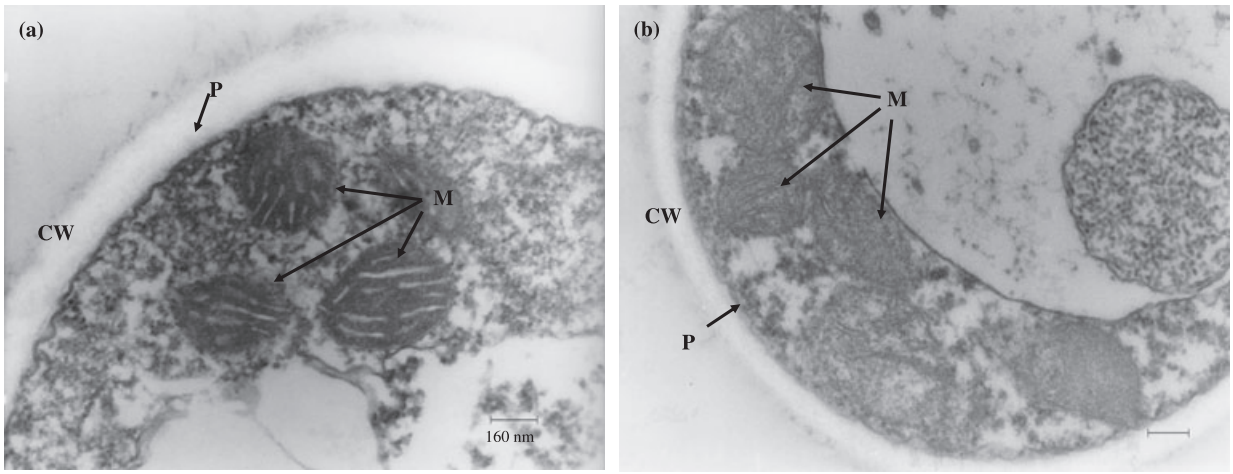

Figure 4 Transmission electron microscopy of a 6.5 kGy irradiated yeast cell. Control (a) and 6.5 radioattenuated yeast (b). Cell wall (CW), cytoplasmatic membrane (P) and mitochondria (M) were shown in details.

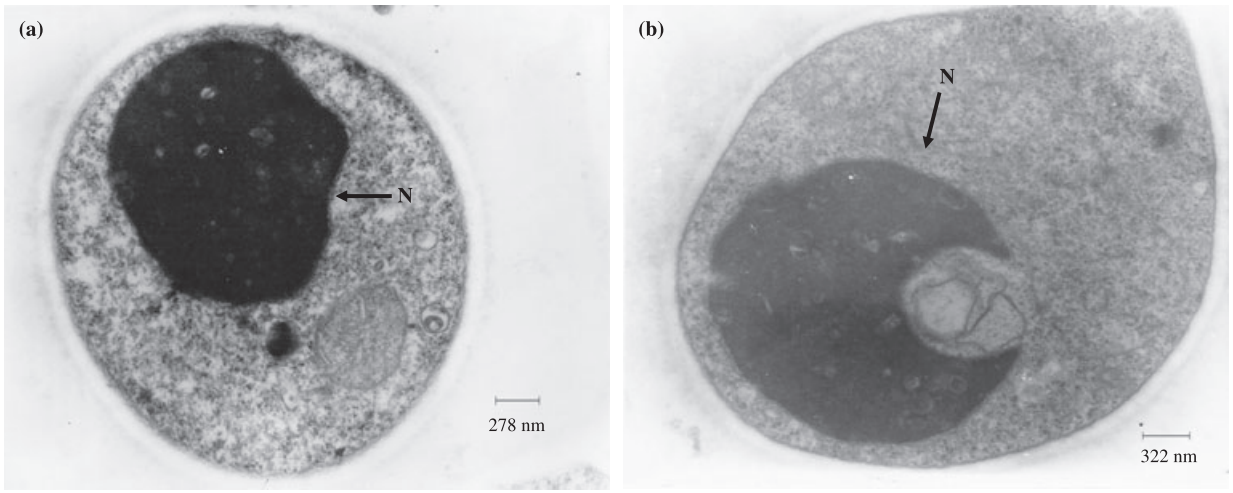

Figure 5 Vision of the nucleus of a 6.5 kGy irradiated yeast cell by transmission electron microscopy. After irradiation the nucleus was frequently found in a very electrondense form (5a) which, in many cells, was crescent-shaped (5b). Nucleus (N).

electrophoresis in agarose gel.[9] The analysis showed that yeast cells DNA was degraded by ionizing radiation in a dose relation way. At 6.5 kGy, the dose that disrupted the cell division capacity,[6] the DNA was completely fragmented (Fig. 6a). The degradation can be estimated by the disappearance of the band corresponding to the genomic DNA and the smearing, indicating small DNA fragments in the lower part of the gel. The extensive DNA fragmentation caused by the 6.5-kGy dose was not significantly repaired as the same picture was observed 48 h after irradiation (Fig. 6b).

Discussion

The DNA is considered the main target for ionizing radiation action on living cells. The gamma irradiation is able to induce multiple DNA lesions, including: base damage, base loss, DNA–DNA cross-links, DNA-protein cross-links, single-strand breaks and double-strand breaks. The double-strand breaks are the most dangerous lesions because they lead to the DNA fragmentation. Unrepaired double DNA breaks in the cell block the cell division leading to cell cycle arrest or unbalanced chromatin exchange in daughter cells and consequent

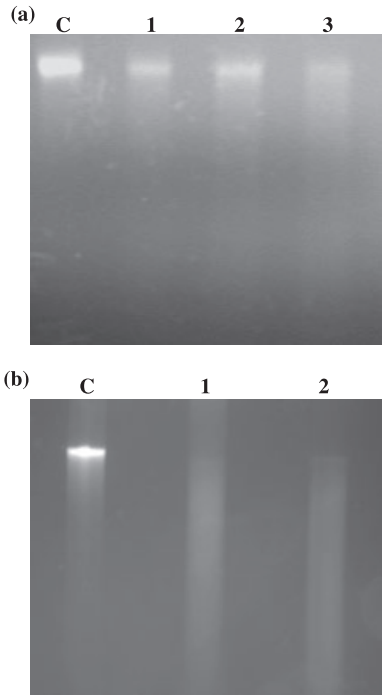

Figure 6 DNA banding pattern of yeast cells submitted to gamma irradiation. (a) Cells submitted to 2.0 kGy (1), 4.0 kGy (2) and 6.5 kGy (3). (b) Cell irradiated with 6.5 kGy and analysed two (1) and 48 h (2) after irradiation. Control (cells not irradiated) (C).

mitotic death.[10–11] The 6.5 kGy irradiated yeast loses the cell division capacity and virulence. However, the radioattenuated yeast retains its viability and the protein synthesis metabolism after irradiation[6] despite the extensive DNA fragmentation, likely because most of the breaks occur outside operons, usually a minor part of the genome, allowing adequate functions of the genes until cell division.

Various cytological changes have been reported in the few published descriptions of structural effects of radiation on yeastlike cells. The following cytoplasmic changes were reported: degeneration of mitochondria,[12] alterations in the internal membrane system,[13] cytoplasmatic vacuolation[12] and distortion of the vacuole in *Saccharomyces*.[14] The reported changes involving the nucleus included both fragmentation[13] and swelling[12] in *Saccharomyces*, and chromatin dispersion in *Rhodotorula*.[13] Disruption of the cell wall[13] and losing of capsule in *Cryptococcus neoformans*[15] were also reported. In this study with yeast cells of *P. brasiliensis*, using the dose of 6.5 kGy, remarkable changes were found in the nucleus that were parallel to the DNA fragmentation and the consequent disruption of the reproductive ability of the irradiated yeast cells.

Apoptosis in fungal cells follows many of the same steps evident in animal cells.[16] Nuclear fragmentation and chromatin condensation, sometimes like nuclear crescents, are characteristics of apoptotic cells.[17] The nucleus appearance in Fig. 5 as cell shrinkage aspect in Fig. 1b, verified in the yeast cells analysed 2 h after irradiation, resembles some morphological descriptions of apoptosis in yeast.[9,18] Our results, however, were not suggestive that a significant process of death by apoptosis was taking place, at least in the times analysed, as evidenced by the maintenance of the cells viability up to 21-days postirradiation (Fig. 2), and the recovery of the normal morphology when visualized 48 h after the gamma irradiation attenuation (Fig. 1c). Other markers of apoptosis, such as membrane blebbing and membrane fragmentation were not detected in the irradiated yeast. The hallmark of apoptosis is the DNA degradation but this parameter is useless as a marker of apoptosis for the radioattenuated yeast because gamma irradiation directly causes the same effect. DNA laddering is not observed in the yeast apoptosis, which could differentiate an apoptotic driven DNA degradation from the non-specific fragmentation caused by gamma radiation. Nevertheless apoptosis in yeast is an open question far from being well established and a comprehensive work will be necessary to evaluate the occurrence of gamma irradiation induced apoptosis in *P. brasiliensis* yeast cells.

The nuclear ultrastructural changes also could be an outcome from chromatin damage caused by gamma radiation leading to a similar overall aspect of an apoptotic nucleus. Chromatin condensation, formation of large dense chromatin clumps and nuclear bodies (ring-like chromatin aggregates) are known nuclear morphological aspects of cells surviving irradiation.[19] The prolonged viability retention by the radioattenuated yeast cells has allowed their utilization as a live vaccine in experimental paracoccidioidomycosis (our unpublished data).

In a previous work, we demonstrated that the *P. brasiliensis* yeast attenuated by gamma irradiation loses its reproductive ability and virulence but retains the viability, the metabolic activity and the antigenic profile.[6] In the present work, we showed that the fundamental ultrastructural aspects were preserved after irradiation, although extensive DNA fragmentation, along with nuclear changes, were verified. At the moment, the capacity of the radioattenuated yeast to elicit a protective immunity against a *P. brasilienis* infection is under study.

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