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EFFECTS OF 60 COBALT IONIZING RADIATION IN MORPHOLOGY AND METABOLISM OF YEASTS AND CHLAMYDOSPORE OF *CANDIDA ALBICANS*

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ABSTRACT

Candida albicans is a fungus responsible for 80-90% of fungal infections, as the symptoms are similar to those of systemic bacterial infections there is a difficulty for immediate diagnosis. These difficulties can lead to delays of antifungal therapy, which contributes to the high mortality rates associated with this infection. Resistance structures referred to as chlamydospores are very common in the pathogen, representing different cell types that form in response to certain genetic or environmental conditions. Recently, various antifungal agents and new therapeutic strategies have come into use, allowing the fungus to acquire a resistance to the drugs. The use of ionizing radiation has been widely employed for the production of immunogens against various parasites. In this work, we evaluate the effects of gamma radiation (^{60}Co) in yeast and chlamydospore of *C. albicans* with doses ranging from 320 to 10.240 Gy with Cobalt 60. Subsequently the samples were plated and after seven days, the colony forming units (CFU) told. The viability of irradiated cells were evaluated using the Janus green dye. A dose of 6000 Gy was considered ideal for the mitigation of chlamydospore and yeast. The dimorphic change mechanisms of both fungal structures were not harmed. The viability of chlamydospores remained above 70% while the yeast viability remained above 85%. By transmission electron microscopy and fluorescence microscopy may be noted cytoplasmic changes, defects in the cell wall, mitochondria, and the presence of partially preserved vesicles of both morphological forms of *C. albicans*. Irradiation both chlamydospore as *C. albicans* yeast allows the suppression of their reproduction, opening the possibility of their use in future candidate immunogens.

1. INTRODUCTION

The genre Candida yeasts are unicellular microorganisms, including about 150 species, of which about 20 were described as agents of candidiasis. Epidemiological studies have

demonstrated that the genre *Candida ssp*. It is the fourth most common group of nosocomial pathogens isolated from patient [3].
This high frame candidemia is not clear and may be related to the resources available to physicians, difficulty in implementing infection control programs in developing country hospitals and the limited number of health professionals to care for patients in care therapy.
An untimely and accurate diagnosis of systemic fungal infections is difficult due to enespecificidade of clinical symptoms and lack of patterning. A prophylactic vaccine and / or therapeutic would be safer and more effective to meet this need for health [9].
Khares and collaborators (1982) showed a decrease in the influx of various amino acids after the yeast *C. albicans* were irradiated at doses 2.5 to 10 Gy of gamma radiation. Although these low doses did not provoke death of yeast, it was noticed that the plasma membrane can be one of the prime targets that precedes nuclear injury.
Gamma radiation is a type of electromagnetic radiation with power of ionization and great capacity to penetration, acting directly and indirectly throughout the cell body.
The use of ionizing radiation can be an important tool for maintaining the initial characteristics of the preserved parasite, mimicking the body all naturally caused to the host response and ensuring the absence of disease development [12].

2. MATERIALS AND METHODS

2.1. Fungi:

The strain of *C. albicans* ATCC 6458645, was kindly donated by Dr. Gilda Maria B. Del Negro (LIM-53 / IMTSP / LIMHCFMUSP). The fungi were maintained routinely in Protozoology Laboratory of Tropical Medicine Institute of São Paulo (USP - Universidade de São Paulo) in Agar Sabouraud and Agar cornmeal [3].

2.2. Obtaining chlamydospore:

After induction chlamydospores for five days at 27°C was added 4 mL buffer solution of phosphate - 0.15M NaCl / 0.01M sodium phosphate buffer pH 7.2 (PBS) and homogenized. With the aid of vortex apparatus (Phoenix Luferco AP 56) the suspension was stirred for 1 min. It centrifuged at 1500 rpm at 4°C for 10 min. The supernatant was collected and the pellet discarded and zimoliase reagent added (10 mg/mL) and kept at 27°C for 2 hours. Samples were shaken and centrifuged for 1500 rpm at 4°C for 3 min. The supernatant is collected and the pellet discarded. The suspension is centrifuged at 10000 rpm for 10 min. The formed pellet was resuspended in PBS, v/v pH 7.2.

2.3. Obtaining yeasts:

Superficial fungal material of the colony was scraped with the aid of a disposable handle and added to an *eppendorfs* with 1.5 mL PBS and stirred with the aid of vortex device (56 Phoenix Luferco AP) for 30 seconds. The sample is centrifuged at 10000 rpm for 15 min. The formed pellet was resuspended in PBS, v/v pH 7.2, sterile and stored for analysis.

2.4. Irradiation of samples:

The samples were irradiated at the Institute of Energy and Nuclear Research (IPEN) in partnership with Dr. Nanci do Nascimento. Yeasts and chlamydospore were subjected to radiation in their respective predetermined doses of 320 Gy, 640 Gy, 1280 Gy, 2560 Gy, 5120 Gy, 6000 Gy, 7000 Gy, 8000 Gy, 9000 Gy and 10240 Gy, with exposure to rays of γ a source of 60Co (Gammacell, Atomic Energy of Canada, Ltd.) evenly in the presence of O_2

2.5. Evaluation of the replication ability of yeast and chlamydospore:

The multiplication of capacity of yeast and chlamydospore it was monitored by plating. An aliquot removed (40 uL) in concentrations of 10^7 cells/mL, and added to their respective plates. Yeasts and chlamydospore irradiated at doses of 320 Gy, 640 Gy, 1280 Gy, 2560 Gy 5120 Gy, 6000 Gy, 7000 Gy, 8000 Gy, 9000 Gy and 10240 Gy were plated in their respective culture media and their respective temperatures for 7 days.

2.6. Analysis of the ability to reversal of yeast and chlamydospore:

After the irradiation process removed an aliquot (40 uL) cells with concentrations of 10^7 and added to plates. The irradiated yeast in doses of 320 Gy, 640 Gy, 1280 Gy, 2560 Gy, 5120 Gy 6000 Gy, 7000 Gy, 8000 Gy, 9000 Gy and 10240 Gy were plated onto cornmeal agar and maintained at 27 ° C for 7 days, chlamydospores and irradiated at doses of 320 Gy, 640 Gy, 1280 Gy, 2560 Gy, 5120 Gy, 6000 Gy, 7000 Gy, 8000 Gy, 9000 Gy and 10240 Gy were plated on agar Sabouraud at 37ºC for 7 days.

2.7. Cell viability after irradiation:

The controls yeasts, the controls chlamydospores and the respective doses where there it was no growth observed colonies had its concentration adjusted to 10^6 cells/ml in 1x PBS and monitored for a period of 2 hours, 24 hours and 48 hours after the irradiation process. A percentage (10 uL) of the cell solution was removed and homogenized with Janus Green-10 uL 0.05% after 10 min incubation at room temperature. The proportion of viable cells was determined by counting in a Neubauer chamber [11]

2.8. Eletron microscopy:

The irradiated samples and controls were washed with PBS and then resuspended and fixed in 3% glutaraldehyde / 3% formaldehyde in 0.1 M cacodylate buffer with sucrose at 2%; post-fixation in osmium tetroxide 2% and 0.1M s-colidine buffer, pH 7.4. Thereafter the samples were washed in 0.1 M cacodylate buffer with sucrose at 2%; and staining with ethyl uralina 5% to 50% ethanol; then washed with 70% ethanol; Dehydrated 2,2-dimetroxipropeno in acidified with 1N hydrochloric acid and with 100% acetone. The materials were infiltrated with 100% acetone and Epon Araldite Polybed 812 and 502. Polymerization 100%, ultrathin sections, staining with further saturated uranyl acetate and lead citrate. The samples were viewed in electron microscope JEOL JEM-1010-Department of FMUSP pathology. All will electron microscopy submitted material was processed in the laboratory of pathology of Infectious Diseases, Department of Pathology FMUSP

2.9. Analyze the distribution of chitin

To evaluate the effects of radiation in the cellular structure of *C. albicans* fungi were labeled with 1 ul of 0.05% Calcofluor-white (Sigma, St. Louis, MO, USA) suspension in 10 uL of cells (10^6 cells/mL) and viewed with a fluorescence microscope with UV filter.

3. RESULTS

3.1 Evaluation of the replication ability of yeast and chlamydospore irradiations:

By plating method found that yeast and chlamydospore lose their ability to reproduce at a dose of 6000 Gy (Tab. 1).

Table 1 - Growth of the colonies of *C. albicans* in Sabouraud agar

Doses (Gy)	Yeast			Chlamydospore		
	Plates 1	Plates 2	Plates 3	Plates 1	Plates 2	Plates 3
0	+++	+++	+++	+++	+++	+++
320	+++	+++	+++	+++	+++	+++
640	+++	+++	+++	+++	+++	+++
1280	+++	+++	+++	++	++	++
2560	++	++	++	+	+	+
5120	+	+	+	-	-	+
6000	-	-	-	-	-	-
7000	-	-	-	-	-	-
8000	-	-	-	-	-	-
9000	-	-	-	-	-	-
10240	-	-	-	-	-	-

Presence of high (+++) Medium (++), down (+) number of colonies of *C. albicans*

3.2 Reversal process:

To verify that the irradiation process interferes with the dimorphic fungus change mechanism, we analyze its reversal capability as described in item 2.6 in materials and methods.
By analyzing the ability of reversal of the samples, there were no growth in chlamydospore at a dose of 5120 Gy and yeasts at a dose of 6000 Gy (Tab. 2).

Table 2 - Growth of the colonies of *C. albicans* yeast in cornmeal agar and growth of chlamydospores colonies on agar Sabouraud.

Doses (Gy)	Yeast to Chlamydospores (27ºC)			Chlamidospores to Yeast (37ºC)		
	Plates 1	Plates 2	Plates 3	Plates 1	Plates 2	Plates 3
0	+	+	+	+	+	+
320	+	+	+	+	+	+
640	+	+	+	+	+	+
1280	+	+	+	+	+	+
2560	+	+	+	+	+	+
5120	+	+	+	-	-	-
6000	-	-	-	-	-	-
7000	-	-	-	-	-	-
8000	-	-	-	-	-	-
9000	-	-	-	-	-	-
10240	-	-	-	-	-	-

Presence of colonies of *C. albicans* (+) colonies

3.3 Viability:

Established the lowest dose at which no growth (6000Gy), analyze the viability of chlamydospores and yeasts. In the control group the yeasts remained in its most viable, corresponding to 98% after 2 hours, 98% after 24 hours and 96% after 48 hours. The irradiated yeast showed 87.6% after 2 hours, 84% after 24 hours, and 80.2% after 48 hours (Fig. 1). The non-irradiated samples of chlamydospores corresponded to 98% after 2 hours, 96% after 24 hours and 95% after 48 hours. The chlamydospores irradiated there was a percentage of 74.5% after 2 hours, 73.1% after 24 hours and 70.6% after 48 hours (Fig. 2).

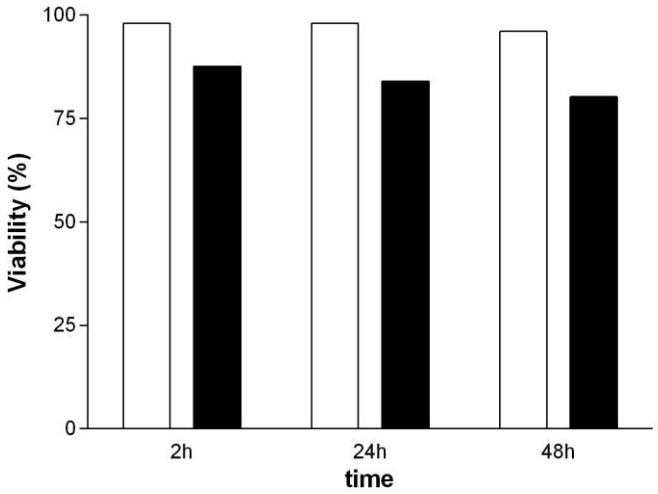

Figure 1 - Viability of *C. albicans* yeast irradiated (black) and not irradiated (white). The viability was evaluated 2 hours, 24 hours and 48 hours after irradiation.

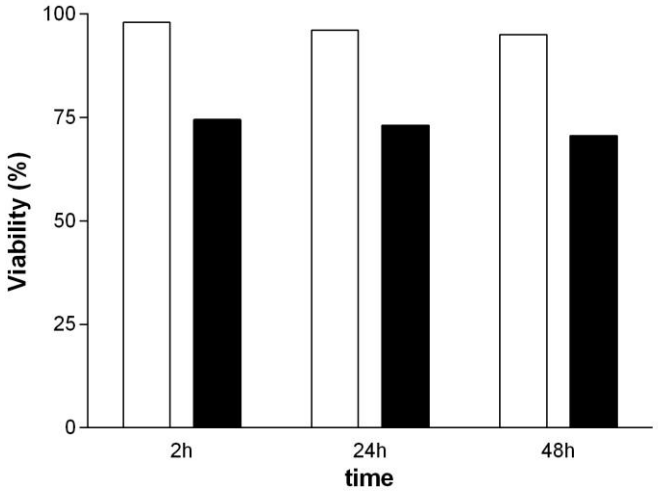

Figure 2 - Viability of *C. albicans* chamydospores irradiated (black) and not irradiated (white). The viability was evaluated 2 hours, 24 hours and 48 hours after irradiation.

3.4 Eletron microscopy:

The yeast *C. albicans* irradiated in comparison to non-irradiated sample, it is noted that less condensed cytoplasm, the cell wall indicating that remains preserved (Fig. 3), with irregularities of occurrences in plasma and membrane ultrastructure (Fig. 4).

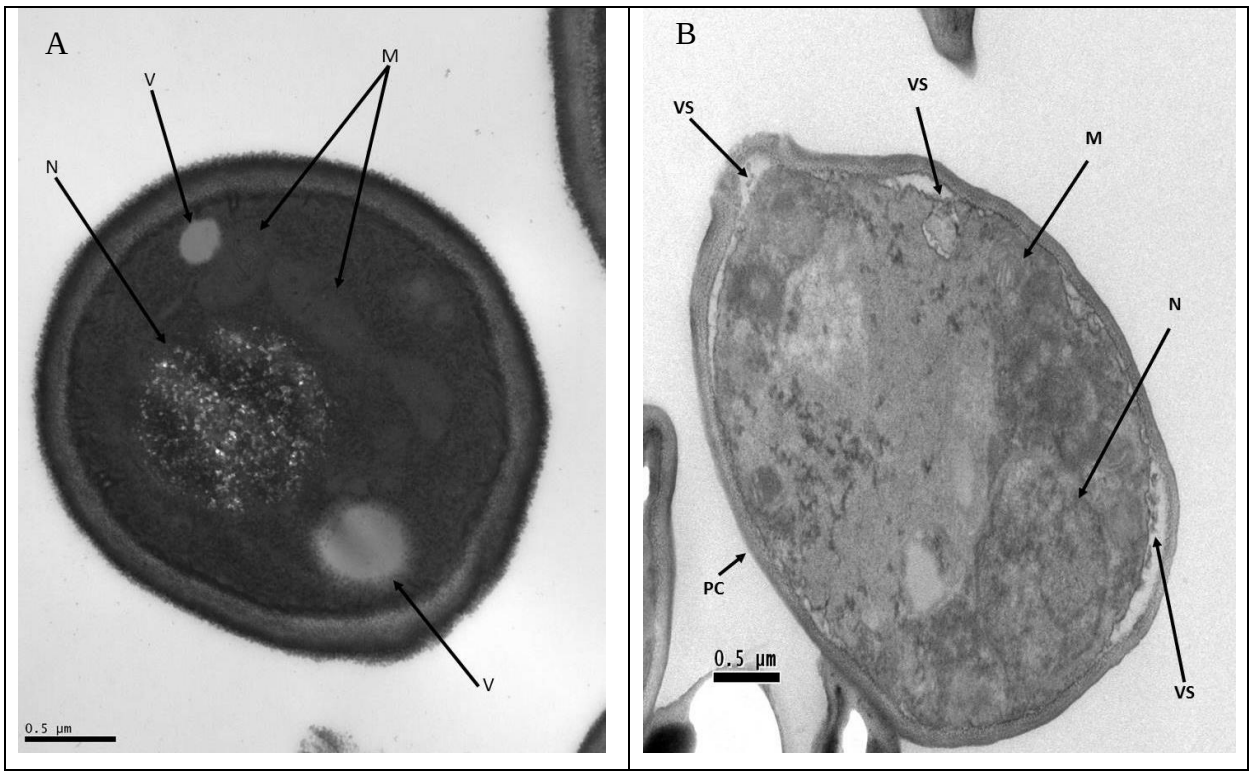

Figure 3 - Electron Microscopy non-irradiated yeast (A) with cell walls (PC) and set, mitochondria (M) shows core (N) and vacuole (V). Yeast irradiated (B) at a dose of 6000 Gy with cell wall (CW) preserved, mitochondria (M), nucleus (C) and secretory vesicles (SV) partially preserved.

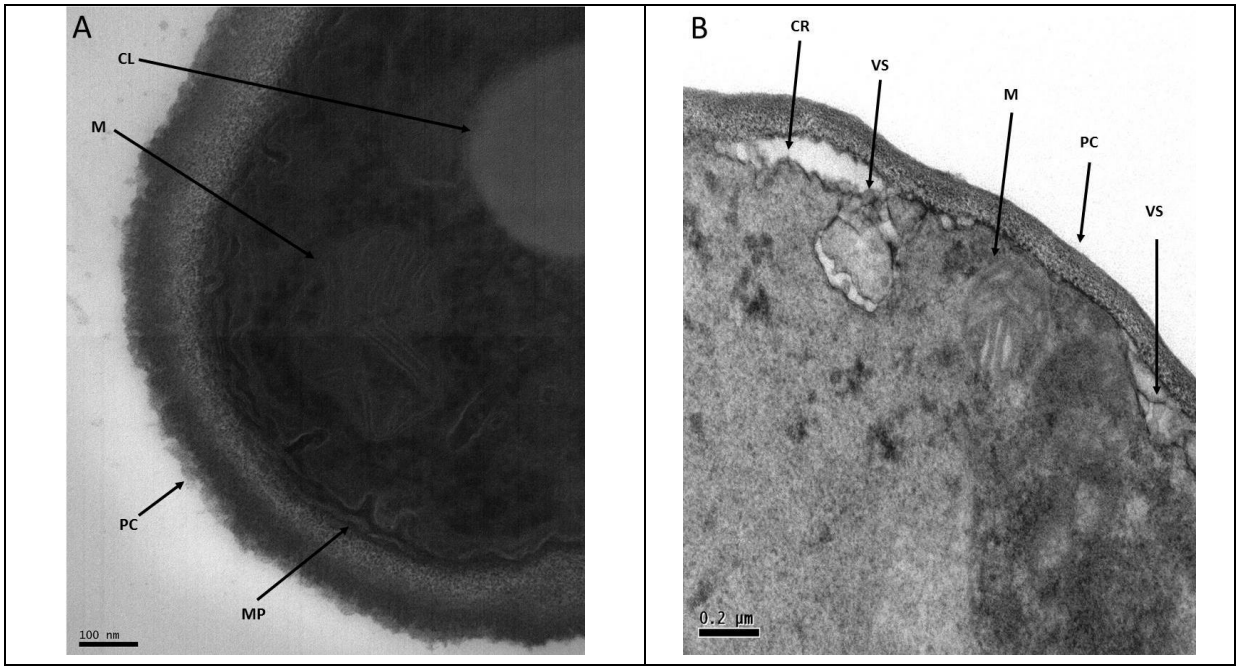

Figure 4 – The non-irradiated Yeast electron microscopy (A) and cell wall (CW) as well structured, mitochondria (M), plasma membrane (PM) and lipid corpuscle (CL). Yeast irradiated (B) at a dose of 6000 Gy retracted to the cytoplasm (CR), cell wall (CW) preserved, mitochondria (M) and secretory vesicles (SV) partially preserved.

Samples of irradiated chlamydospore showed the presence of vacuoles, cytoplasm less condensed regarding the non-irradiated samples. Note that the cell wall remains preserved there occur changes in the plasma membrane, mitochondria and existence of secretory vesicles can be observed (Fig. 5).

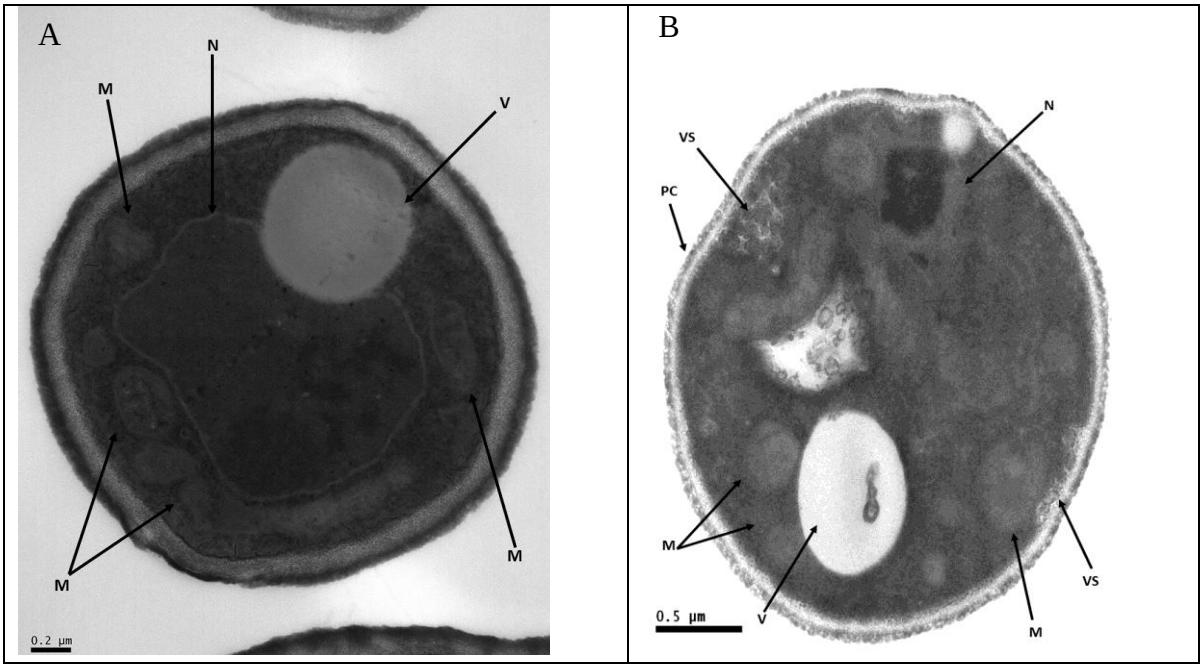

Figure 5 - Chlamidospores non-irradiated electron microscopy (A) and cell wall (CW) preserved vacuole (V) and mitochondria (M). Chlamidospore irradiated (B) at a dose of 6000 Gy with cell wall (CW) preserved vacuole (V), mitochondria (M), secretory vesicles (SV) and nucleus (N) partially preserved.

3.5 Analysis of the distribution of chitin samples of *C. albicans*:

It was observed in yeast samples and non-irradiated chlamydospore a chitin distribution uniform and without compromising the integrity of the cell wall, However the pattern shown in irradiated cells, as can be seen, there were anomalies in the cell wall of yeasts (Fig. 6) and chlamydospores (Fig. 7) at the dose of 6000 Gy.

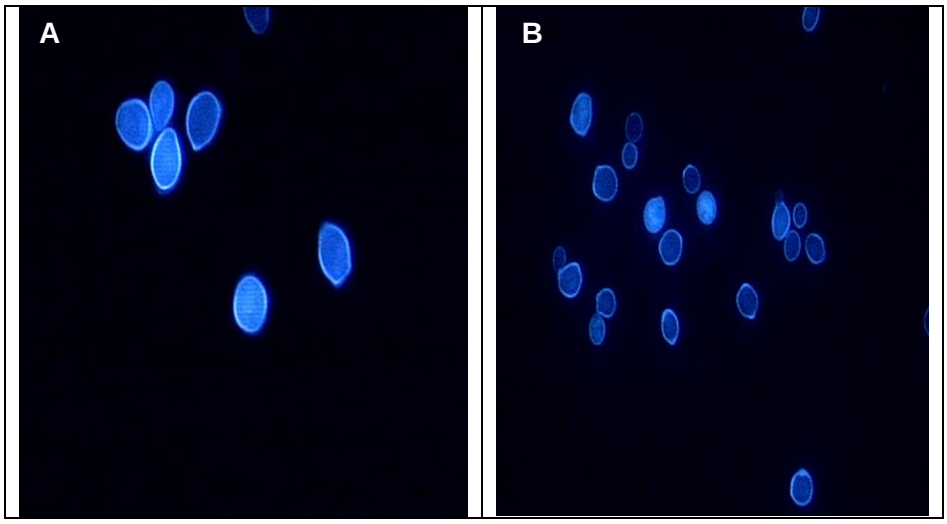

Figure 6 - Yeast *C. albicans* did non-irradiated (A). Yeast *C. albicans* irradiated with 6000 Gy dose of (B), marked with calcofluor-white.

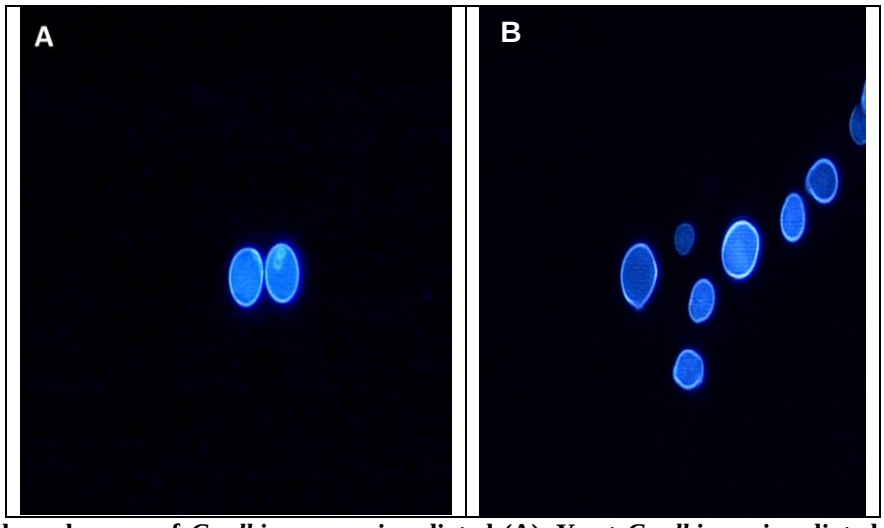

Figure 7 - Chlamydospore of *C. albicans* non-irradiated (A). Yeast *C. albicans* irradiated with 6000 Gy dose of (B), marked with calcofluor white

3. DISCUSSION AND CONCLUSIONS

By ionizing radiation can be eliminated microorganisms pathogenic to man and animals, especially in sterilizing medical and pharmaceutical products, food irradiation and control of insect pests on agricultural network [13]. Gamma radiation has been convincingly useful in mitigating or sterilization of biological agents of all kinds. In all cell models studied, radiation essentially promotes the loss of reproductive capacity, maintaining a certain level suitable immunogenic [14].

Saleh and collaborators (1988) showed a high resistance to gamma radiation in fungi such as Aspergillus, Cladosporium and others regarding to the cells of different microorganisms. These cells with low doses, had their abilities to reproduce interrupted while the yeast cells, lacked very high doses to preclude their reproductive capacity. The various fungi, have strength parameters doses of between 1.7 and 2.5 KGy, but stood out species with higher inactivation radioresistance to display, which varied between 17 and 20 KGy. According to the author this high resilience can be related to concentration of melanin, present in the cell wall.

Analyzing the radioresistência yeast and chlamydospore of *C. albicans*, we found that high doses had to be used to incapacitate the growth of both. Doses of 6000 Gy were required to be no observable colonies of yeast and chlamydospore. Melanization processes in *C. albicans* are common in times of stress. Episode that can be related to the high radioresistance of both forms of the fungus, also involve other factors, such as the fact that cells possess enzymes able to inactivate and antioxidants toxic and/or mutagenic induced by radiolysis of water, moreover, there are systems enzyme complexes able to repair the damage in proteins and nucleic acids, promoting greater integrity of their essential cellular components for life [8].

Generally the simplest organisms are more resistant to the effects of ionizing radiation. The difference in resistance of these microorganisms is not restricted only to gender but also among strains of the same species [6].

Culture can influence the sensitivity to irradiation. Cultures 3 weeks of age or *Aspergillus flavus, Penicillium viridicatum* require a dose of 2 kGy to prevent their growth, while 1KGy doses are sufficient to inhibit the growth of such cultures at 6 weeks [2].

The concentration of lipid content also influences the damage caused by irradiation. Fungi more lipid content have a greater radioresistance to compare the effects of radiation other minor lipid concentration [1].

To analyze the dimorphism loss of cell process, the *C. albicans* yeast, it can be noted that at doses lower than 6000 Gy irradiation procedures the fully impaired not change its state. Later the chlamydospore showed a loss of its dimorphic capacity at doses lower than 5120 Gy. Showing that depending on the mobile phase radiation has a greater effect on the morphological change mechanism of the cell. Samples irradiated *Alternaria alternata*, *Aspergillus flavus*, *Trichoderma viride* and *Curvularia geniculata* capacida indicated that the germination capacity of both fungi were delayed and reduced growth rates between 2.5 and 3.0 KGy. According to the author, this variation may be related to the high capacity of DNA repair of fungi [7].

When assessing the viability of yeast and chlamydospores after irradiation, there was a small decrease in cell number over the time period examined, but the yeasts remained irradiated with viability greater than 85% and chlamydospores maintained their viability in the upper 70%. Note that if the selective permeability of the plasma membrane of the irradiated material was preserved, as evidenced by the lack of incorporation, Janus Green dye, another aspect taken into consideration, are the mitochondria, and secretory vesicles partially preserved, encountered when viewed in transmission microscopy, showing that the metabolic activity of both morphological states of *C. albicans*, are partially preserved. Factors that have been observed in Sporothrix schenkii irradiated with doses equivalent to 7.0 kGy. This being satisfactory dose to mitigate these yeasts, which were unable to produce infection, but maintained their viability, metabolic activity and morphology preserved [4].

The *C. albicans* yeasts and chlamydospores lose their reproductive capacity at a dose of 6000 Gy, however doses of 5120 Gy is sufficient to inhibit the activity of dimorphic chlamydospores. Demonstrating that there is an increased radiosensitivity in chlamydospore that the yeast *C. albicans*. The transmission electron microscopy showed significant changes

in the cytoplasmic contents of both morphological forms, resulting in the loss of its virulence, keeping their morphology. Significant changes to the cell nucleus are apparent and that probably is related to loss of reproductive capacity.
All these data show that the irradiation both chlamydospore or *C. albicans* yeast allows the suppression of their reproduction, opening the possibility of its future use as vaccine candidate.

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