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HALO'S PRODUCTION IN VITRO ON BRACHYTHERAPY EXPERIMENTS

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ABSTRACT

Since earlier of 1960, one of the most significant contributions of radiation biology has been the theory of cell killing as a function of increasing doses of a cytotoxic agent, as well as the demonstration of repair of sublethal or potentially lethal damage after irradiation. The impact of cellular and molecular radiobiology, by exploitation of cellular mechanisms related to apoptosis, may be the cell killing with irradiation by including changes other than unrepaired DNA damage. Based on the understanding of the tumor microenvironment and how growth factors and proteins produced by irradiated cells may alter cellular processes, improved combined-modality strategies may emerge. This effect was show since 1960's, but here we propose to demonstrate this phenomenon in Brachytherapy. The present goal is to verify the macroscopic response through the production and analysis of clonogenic control based on halos generation by radioactive seeds of Ho-165 and Sm-153, aiming to study the effect of this type of irradiation. Confluent cell culture flasks with HeLa cell line were subjected to radiation in a period up to five half-lives of radionuclide, respectively. Devices were introduced which set the polymer-ceramic Ho-165 and Sm-153 seeds in the vials. After a period of exposure, the flasks were stained with violet Gensiana. The results showed the formation of halos control of confluent cancer cells. This paper will describe these experiments in the current stage of the research and report the implications of this new way of therapy for cancer treatment.

Irradiation procedures: ...the value of R/h was measured (repeated in 2 paragraphs);

11/12/08 at 17h30min in shield device having been counted...sample #5 has six Ho-166 seeds placed (twice)...

Fixation and stained occurred at 08h00min in 17/12/2008 - the dates should be in a uniform way. Either /08 or /2008.; Figure 6 and 7.....first 12 hours of cells exposure;

1. INTRODUCTION

According to the National Cancer Institute - INCA, Brazil, the estimates of gross rates of cancer incidence both in men and women were 489,270 new cases in 2010. Still, according to a cost estimative based on data from private health plans in the period of 2008 to 2010, the treatment of advanced cancer is nearly eight times more expensive than if disease is early detected. For the same period, projections indicate that treatment costs will be seven times higher than the cost of preventive actions. In 2007, between hospital permanence, chemotherapy and radiotherapy, the public health system spent a value of R$ 1.2 billion a year on cancer treatment in Brazil. The authors believe that the cost of treatment from private plans, added to this value, may reach a total value of R$ 2-billion year. Thus, any advancement in the field of oncology that can lead to cost reduction and efficiency improvement of the treatment will bring incalculable benefits to the brazilian health and its economy. Also, the authors suggest that research financial Brazilian agencies increase its investments on cancer research giving opportunities to all research areas to reach them since cancer is a multidisciplinar research field.

The incidence of cervical cancer is estimated to be about 18,430 new cases. According to Weinberg (2008) cancer of the cervix (or cervical) is the one with the highest risk in Brazil. Indeed, as most current estimate of INCA on Cancer Incidence in Brazil, cervical cancer appears prominently in the third place, behind prostate cancer, cancer of the trachea, bronchus and lung and female breast cancer (INCA, 2010). Similar to cervical cancer, about one fifth of the worldwide incidence of cancer in humans is associated with infectious agents. The Hepatitis B and C (HBV and HCV) and human papillomavirus (HPV) play key roles in triggering cancer, as a common occurrence.

Many types of cancer cells are propagated in culture and seem able to proliferate indefinitely when kept under appropriate conditions in vitro culture. HeLa cells, for example, from the past half century, have been systematically cultivated in vitro, easy and rapid spread, so it is considered the human cell type most often used to study the molecular biology of human cells. The lineage is derived from a HeLa cervical adenocarcinoma, unusual and particularly aggressive, and remains in cultivation since 1951. Since then, these cells have proliferated in culture in hundreds of laboratories worldwide, which is transfer to other frask every two days. In turn, normal cells are known to spend up to 50 or 60 generations or cycles of growth, dividing in culture, and then become senescent and stop growing. Normal cells, extracted from a variety of tissues do not adapt readily to culture conditions in vitro. This fact is also observed in certain types of cancer cells, as these depend on several other cell types to support their viability and proliferation. The HeLa lineage has an active telomerase, which produces the shortening of their telomeres. This change in their DNA makes the cells lose its ability to limit proliferation or cellular aging. His lineage is already "immortalized." (Weinberg, 2008)

This article discusses the control of adenocarcinoma lineage of the cervix with the use of radioactive seeds Ho-166 and Sm-153. The research group NRI - Núcleo de Radiações Ionizantes - Ionizing Radiation Center has investigated such radioactive seeds since 1998. Earlier publications have shown the feasibility of contrl cancerous cells with those radioactive devices.

2. METHODOLOGY

The choice of the HeLa cell line for this experiment was based on the facts that the cells are rapidly dividing and its relatively simple culture medium. The strain was kindly provided by Dr. Luciana Maria Silva, FUNDED / MG. The cells were kept stocked in appropriate tubes suitable for cryogenics in gallon, stored at -196 °C in liquid nitrogen in the laboratory of the research group.

2.1 *In vitro* culture

HeLa cells were thawed following specific protocol and seeded in 25 mL bottles, suitable for cell culture. The culture medium used was MEM CULTILAB laboratory, plus 10% Fetal Bovine Serum (FBS). In 40 to 60 hours in CO_2 incubator at a constant temperature of 37°C, cells were already attached to the bottom of the bottle and due to the clonogenic active process, it covered the whole area under cultivation. At this confluent stage, the flasks were taken for the experiment.

2.2 Irradiation and activation of radioactive seeds

The ceramic seeds incorporating natural samarium and holmium were synthetised in NRI research group. Irradiation was taken on the reactor core of CDTN in two positions: on a side table and on the center tube (center of the reactor core), type TRIGA IPR-R1, 100 kW, at thermal neutron flux of $2.8x10^{12}$ n/cm²s and epithermal of $2.6x10^{11}$ n/cm²s.

2.3 Radiation protection

For handling the seeds, the following apparatus was used: an apron and thyroid shield of 2 mm lead, and glass tank containing water 15 cm thick, which served as a transparent shield, placed in front of a shield box.

The seeds were manipulated by the principal researcher using tweezers behind the transparent tank. All utensils and manipulated objects were contained in an isolated environment on a sterile tray. The seeds were removed from the shield and inserted into the T25 flask, scattered or placed in fixed positions (fixer). The T-25 flasks with the seeds were put in shield boxes of 3 mm lead thick, and inserted in CO_2 incubator at 37^{o}C. After finishing the procedure, an area monitor and surface contamination scanner was made to ensure the absence of surface contamination and the acceptable values of the environment. The room was kept closed by the time of seed exposure. Measures of personal dose in the hands and chest were made in a standard experiment, showing no measurable exposure. Pen dosimeter was used and accumulative personal dose were documented. All the experiments were limited to a maximum of 200MBq of Ho-166 and 100MBq of Sm-153; however, the manipulation of individual seed with maxiumum activity of 120MBq (~ 3mCi).

2.4 Control experiment

Control flask was produced in the same period as the irradiation experiments, kept in CO_2 incubator at 37°C away from the radioactive seeds.

2.5 Bottles stained with gentian violet

At the end of exposure time, the bottles are removed from their lead box. The support apparatus of seeds is also removed, and the bottles are subject to staining. The contents of these bottles are drained and subjected to the action of PBS and methanol repeatedly, following cell staining protocol. After this procedure, 02 mL of gentian violet was added for 02 min and then drained. Thus, cell layers had acquired staining.

2.6 Irradiation procedures

2.6.1 Experiment of low activity and 05 days exposition

Irradiation samples #1 and #2 seeds with 03 Sm-152 and 03 Ho-165 seeds, respectively, were irradiated in the lateral of the reactor for a period of 08h IPR1/CDTN reaching activity measured in gauge Capintec dose of 3.7 MBq (0.1 mCi). The samples were received on the 31/10/08 - 17h30min in shield with 12μR/h, having count on the surface

after receipt by MRA7027 detector. These samples were handled in an isolated area with adequate radiation protection devices. The maximum surface radiation background contamination reached 90 to 100 cpm. After manipulation, the surface counts ranged from 98 to 110 cpm, indicating non radiological contamination. The 10% increasing in counters are related to source proximity in the same area. After loading the seed in the culture medium, the monitoring the area in the surface of the door of the greenhouse at time zero was done by MRA7027 detector. After setup the experiments, the room was kept closed for 5 days. Samples #1 and #2 were prepared in 18h00min at 31/10/08. In summary, sample #1 had 03 Ho-166 seeds placed in five 03 T25 culture flasks, one seed per bottle. Sample #2 had 03 Sm-153 seeds placed in four (04) T25 culture flasks on 31/10/2008, and one seed per bottle, however, received the two (02) seeds.

2.6.2 Experiment of low activity and 03 days exposition

Irradiation samples with 05 Ho-166 seeds and 05 Sm-152 seeds were placed in the reactor IPR1/CDTN on lateral of reactor core for a period of 08h and had their activity measured in dose calibrator Capintec. The samples were received from the reactor and put into counting device with the surface transport and exposure, measured in MRA7027 detector. These samples were handled in the area prepared with adequate radiation protection. The radiation background surface contamination was kept below 100cpm. After manipulation, no radiological contamination had occurred. After loading the seed in the culture medium, monitoring the area in the door surface of the greenhouse at time zero was measured by MRA7027 detector. Samples #3 and #4 were prepared in 18h00min at 31/10/08. In summary, sample #3 had 05 Ho seeds placed in five (5) T25 culture flasks, one seed per bottle. Sample #4 had 05 Sm seeds placed in four (4) T25 culture flasks on 31/10/2008, one seed per bottle, the latter received two seeds.

2.6.3 Experiment of high activity

02 irradiation samples were prepared with high activity. Each sample had six seeds and Ho-165 was irradiated in the central tube for eight hours. The samples were received on the 11/12/08 at 17h30min in shield device having count on the surface after receiving 30.1 kcpm measured in MRA 7027. In this case there was a cooling time of 24 h previous manipulation. The activities were measured in the seed dose calibrator Capintec. Arrangements were developed with six seeds, with three seeds placed into a polymeric tube, separated 8 mm from the center of each other. The two tubes were separated 9 mm, fixed in a holder. The area dosimetry, afterload in the greenhouse, in front of the CO_2 incubator, presented 440 μR/h. In summary, sample #5 and #6 had six Ho-166 seeds placed in 01 arrangement on 11/12/2008 in T25 culture flask with six seeds per vial, arranged in spaced array 8x9mm preset. Fixation and stain occurred 08h00min at 17/12/08.

3. RESULTS

3.1 Control experiment

HeLa cells were growing and had produced viable clonogenic cells confluent over the whole bottle. Fig. 1 illustrates the HeLa cells in control flask having a polygonal shape

characteristic of normal cells with microvilli extensions, which are protrusions in the cell membrane which increases the surface area of the cell, as well as connections to the plasma membrane, possibly in the interface.

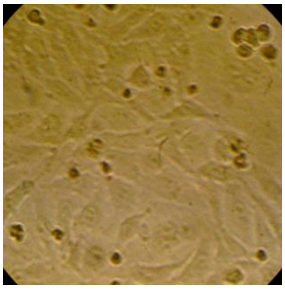

Figure 1 - Photograph of HeLa cells in the control flask.

3.2 Experiment of low activity and 05 days of exposition

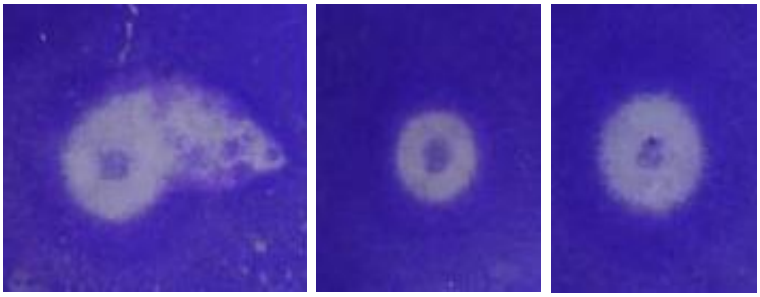

Figure 2 - Photos of halos control HeLa cells in culture generated by radioactive seeds Ho-166 activity of 0.3 mCi, exposed for 5 days.

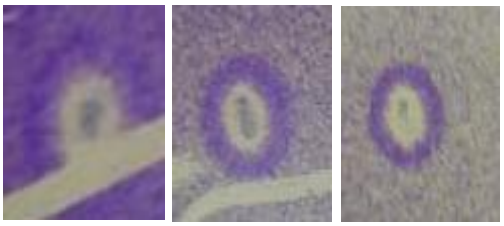

Figure 3 - Pictures of halos control HeLa cells in culture generated by radioactive seeds Sm-153 activity of 0.1 mCi, exposed for 5 days.

The halos produced by Ho-166 seeds at 0.3mCi can be seen in Figure 2. It is observed that the halos are well defined and well characterized, demonstrating that the circular region received a high dose of radiation, enough to control the radial growth toward the central direction. Close to the central region one can observe a deeper coloration halo, however this color refers to cell fragments together with viable cells that, due to the region of maximum dose, is joined by biochemical substances produced by irradiated cells, in the bottle.

The halos produced with 0.1mCi Sm-153 seeds can be seen in Figure 3. It is observed that there are well defined halos with characteristic sizes smaller than those produced by the Ho-166.

3.3 Experiment of low activity and 03 days exposition

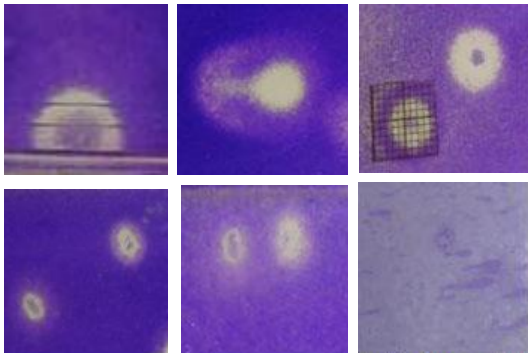

Figure 4 - Photographs obtained directly from the culture flasks exposed to seeds of Ho-166 (Sample #1), 0.3 mCi, exposed for 3 d.

Figure 5 - Photographs obtained directly from the culture flasks exposed to seed after Sm-153 (Sample #2), 0.1 mCi.

3.4 Experiment of high activity

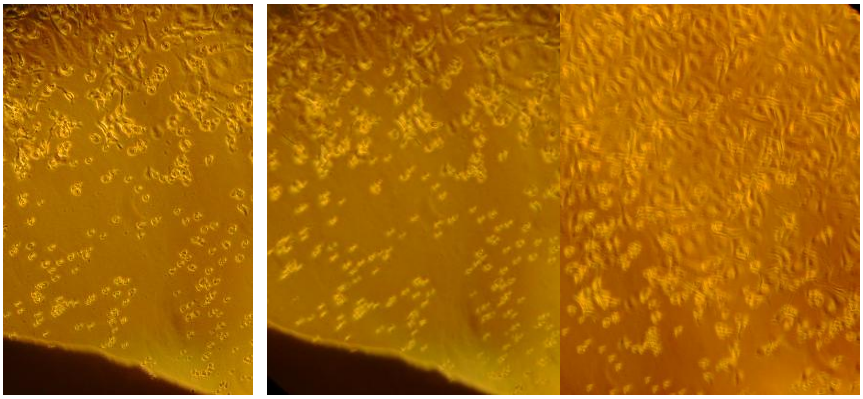

Figure 6 - Sequence of microscopic images obtained in the first 6 and 12 h in the radial direction away from a radioactive seed Ho-166 demonstrating the effectiveness of control HeLa cell *in vitro* exposure time arbitrarily.

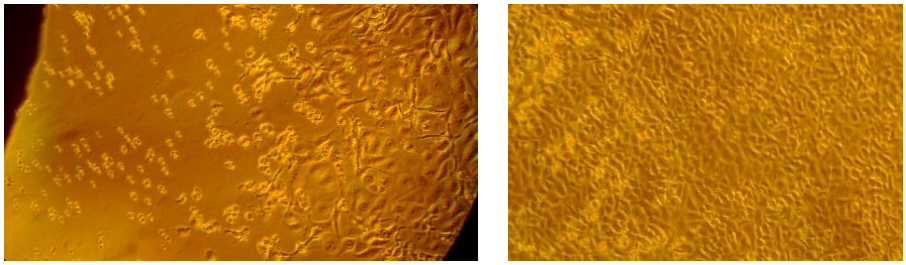

Figure 7 - Characteristics that define distinct morphological deleterious stages in HeLa cancer cells as a function of radial distance from the seed of Ho-166, investigated in the first six hours of exposure. In function of radial distance, it is possible identify fragments in cell detachment from the bottle, cells in spherical shape, cells attached but with sharp shapes, evolving cancer cells equivalent to the control. (Left) Photography of HeLa cells far from seed surface showing a high density of cell culture.

Figure 6 and 7 shows the dynamics of formation of halos in the first 12 hours of exposure of cells to radioactive seed. The dark spot in the images represents the shadow of the surface of the seed. This process can be observed along the time showing the deleterious effects on cancerous cells in processing death (necrosis or apoptosis), leaving fragments that slowly give off the bottle. It produces a characteristic well-defined halo provided by the beta emitters of Ho-166 or Sm-153.

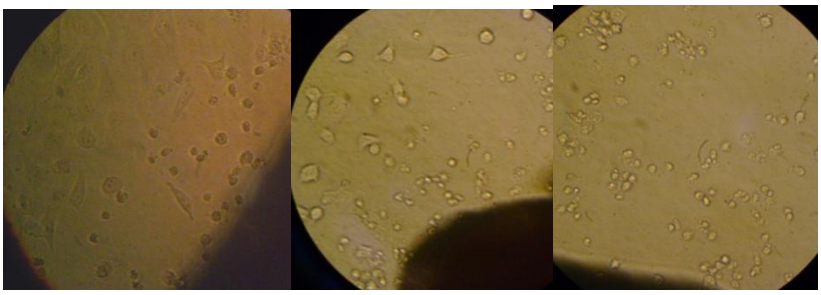

Figure 8 - Photos of harmful cells that have undergone death processes near the seed at the time of placement of seed; 03h, 06h and 12h after start exposure. The shadow represents the surface of the seed.

The morphology of HeLa cells that have suffered irreversible damage by radiation shows the presence of small bubbles and in some cases have a large diameter, which broke down after possibly releases the cytoplasmic contents into the middle. The following is for knowledge necrosis chromatin condensation with reduction of nuclear size. Some of these necrotic cells are connected to the substrate, generating a polygonal shape with nuclei altered. Such rectangular cells with polygonal morphology, for example, attach can be seen in the photographs on Figs. 6 to 8. One can see, at 20 mm position far from seed, normal cells with oval or slightly polygonal outer smooth surface, with possible features of normal mitotic cells, as shown in Fig.7 (left). Similar to Fig.7 (right), it is possible identify fragments in cell detachment from the bottle, cells in spherical shape, cells attached but with sharp shapes, evolving cancer cells equivalent to the control, which appers in function of radial distance.

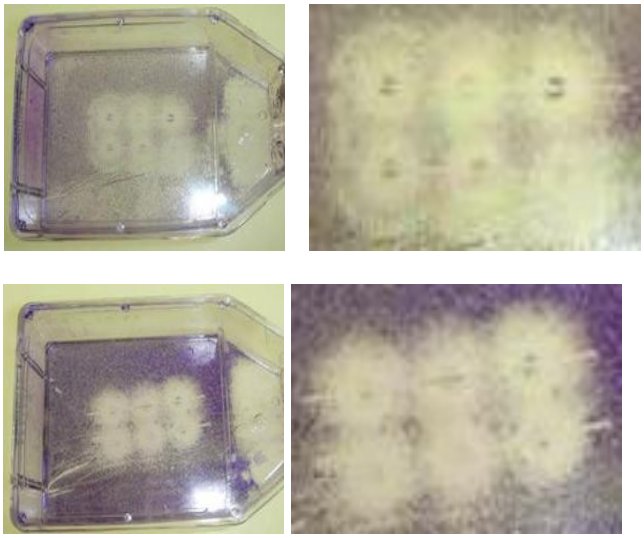

Figure 9 - Flask T25: #5 and 6, Lineage: HeLa, transfer number #152, incubated on 09/12/2008, subject to six Ho-166 seeds spaced 8x9mm in pre-set arrangement.

The activities obtained by the seed set of six Ho-166 were 2mCi each. Figure 9 demonstrates the clear halos around each seed. The dimensions of the halos shows that the seeds of Ho-166 with 2 mCi activity when grouped as described can control the clonogenic HeLa cells after 5 days of exposure.

4. CONCLUSION

Clear macroscopic halos control of HeLa cells in clonogenic *in vitro* assay are produced by ceramic radioactive Ho-166 seeds. The halos have diameters proportional to the activities of the seeds, from 0.1 to 2 mCi; however, this dimension may be limited to the maximum range of beta particles emitted by radionuclides. Besides, for visual analysis of bottles of high activity may speculate that the radius of biological response is larger than found by action range of the beta radiation emitted. Soluble chemical products expressed by high dose irradiation cells, or cell fragments, can induce apoptosis on close cells place on the same environment, extending the biological range of beta emitting. The NRI group has investigated the responses of other tumor lines and found the same radiation action. Experiments in cell viability as well as changes in cellular phenotype are in progress.

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