Ciências Exatas e da Terra e a Dimensão Adquirida através da Evolução Tecnológica 4

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Alan Mario Zuffo
(Organizadores)

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APRESENTAÇÃO

A obra "*Ciências Exatas e da Terra e a Dimensão Adquirida através da Evolução Tecnológica vol. 4*" aborda uma publicação da Atena Editora, apresenta, em seus 22 capítulos, conhecimentos tecnológicos e aplicados as Ciências Exatas e da Terra.

Este volume dedicado à Ciência Exatas e da Terra traz uma variedade de artigos que mostram a evolução tecnológica que vem acontecendo nestas duas ciências, e como isso tem impactado a vários setores produtivos e de pesquisas. São abordados temas relacionados com a produção de conhecimento na área da matemática, química do solo, computação, geoprocessamento de dados, biodigestores, educação ambiental, manejo da água, entre outros temas. Estas aplicações visam contribuir no aumento do conhecimento gerado por instituições públicas e privadas no país.

Aos autores dos diversos capítulos, pela dedicação e esforços sem limites, que viabilizaram esta obra que retrata os recentes avanços científicos e tecnológicos nas Ciências Exatas e da Terra, os agradecimentos dos Organizadores e da Atena Editora.

Por fim, esperamos que este livro possa colaborar e instigar mais estudantes e pesquisadores na constante busca de novas tecnologias para a área da Física, Matemática, e na Agronomia e, assim, contribuir na procura de novas pesquisas e tecnologias que possam solucionar os problemas que enfrentamos no dia a dia.

Jorge González Aguilera
Alan Mario Zuffo

SUMÁRIO

CAPÍTULO 1

ANALYSIS OF CANINE OSTEOSARCOMA CELLS SURVIVAL AFTER IRRADIATION WITH COBALT EQUIPMENT

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ABSTRACT: Radiation therapy is widely used in the treatment of cancer in human patients and it is equally applicable in Veterinary Medicine. This study aims to evaluate the cell survival of primary cell culture of canine osteosarcoma after irradiation of different intensities of ionizing radiation. Cell survival was analyzed by MTT assay and the dual acridine orange/ethidium bromide (AO/EB) staining 24 and 72 hours after the treatment with different intensities of radiation. By the MTT assay, the cell unviability was significant for dose of 30 Gy (9.33% of cells were viable) and the AO/EB staining was excellent to analysis of slides, which enabled evaluate qualitatively cells at different stages of apoptosis, being verified increase of cellular unviability from 24 to 72 hours results from late effects of ionizing radiation and also by the duration time of the cell cycle. The results were proven the resistance of osteosarcoma to treatment with ionizing radiation. We expect to contribute to researching comparative and multidisciplinary oncology to serve as search incentive for new therapeutic approaches.

KEYWORDS: Osteosarcoma, *In vitro* models, Ionizing radiation, MTT assay, Fluorescence microscopy.

ANÁLISE DA SOBREVIDA DE CÉLULAS DE OSTEOSSARCOMA CANINO APÓS IRRADIAÇÃO COM EQUIPAMENTO DE COBALTOTERAPIA

RESUMO: A radioterapia é amplamente utilizada no tratamento do câncer em pacientes humanos e é igualmente aplicável na Medicina Veterinária. Este estudo teve como objetivo avaliar a sobrevivência celular de uma cultura primária de osteossarcoma canino após esta ser irradiada com diferentes intensidades de radiação ionizante. A sobrevivência celular foi analisada pelo ensaio MTT e pela dupla coloração com laranja de acridina e brometo de etídio (AO/EB) 24 e 72 horas após o tratamento. Pelo ensaio MTT a inviabilidade celular foi significativa para a dose de 30 Gy (9.33% das células estavam viáveis) e a coloração AO/EB demonstrou ser excelente para a análise das lâminas, o que permitiu avaliar qualitativamente células em diferentes estágios de apoptose, sendo verificado aumento da inviabilidade celular de 24 para 72 horas como resultado dos efeitos tardios da radiação ionizante e também pelo tempo de duração do ciclo celular. Os resultados comprovaram a resistência do osteossarcoma ao tratamento com radiação ionizante. Esperamos contribuir para a pesquisa em oncologia comparada e multidisciplinar a fim de servir de incentivo para a busca de novos enfoques terapêuticos.
PALAVRAS-CHAVE: Osteossarcoma, Modelos *in vitro*, Radiação ionizante, Ensaio MTT, Microscopia de fluorescência.

1 I INTRODUCTION

The incidence of bone tumors in dogs is 7.9/100000, of which 98% are malignant. The classification includes primary, metastatic and invasive tumors. Among the primary ones, osteosarcoma (OSA) is the most diagnosed, from 80 to 85% of cases and 3 to 4% of all tumors diagnosed in these animals (DALECK, et al. 2002; GOMES, et al. 2006; OLIVEIRA; SILVEIRA, 2008; ANDRADE, 2009; ANGSTADT, et al. 2011; PIMENTA, et al. 2013; WYCISLO; FAN, 2015; WESTROM, et al. 2016; DE SANCTIS, et al. 2017). Despite of its low frequency in comparison to neoplasms from other organs, the study about osteosarcoma is relevant due to difficulties in its diagnostic and therapeutic conduction. Besides, it represents an excellent *in vivo* model for the study of human osteosarcoma and for presenting advantages in relation to studies performed on rats, since dogs live in the same environment as humans, for example (BERSANO, 2006; MUELLER, et al. 2007; PIMENTA, et al. 2013; WYCISLO; FAN, 2015; SHAHI, et al. 2015; DE SANCTIS, et al. 2017). OSA is characterized by chromosomal alterations, aggressive local infiltration of subjacent tissues with rapid hematogenic dissemination and the presence of pleomorphic osteoblasts (DALECK, et al. 2002; BERSANO, 2006; HOSKINS, 2008; ANDRADE, 2009; ANGSTADT, et al. 2011; FRACASSO, 2012; WYCISLO; FAN, 2015; WESTROM, et al. 2016). Both, the axial and appendicular skeleton can be affected, being more frequent in areas of metaphysis of long bones with

a greater prevalence in anterior limbs in relation to posterior ones, at a 2:1 proportion (DALECK, et al. 2002; OLIVEIRA; BERSANO, 2006; SILVEIRA, 2008; ANDRADE, 2009; NORTH; BANKS, 2009; FRACASSO, 2012; WYCISLO; FAN, 2015; ZHANG, et al. 2015). Surgery is considered among the most efficacious measures for the treatment of solid neoplasia by virtue of offering the best chances for providing a cure and pain relief. Another measure is alternative or co-adjuvant treatments such as chemotherapy and radiation therapy. The chemotherapeutic agents, however, has a low therapeutic index, since the dose that produces a therapeutic response is very close to that of a toxic response (HEIDNER, et al. 1991; DALECK, et al. 2002; OLIVEIRA; SILVEIRA, 2008; FRACASSO, 2012). Already radiation therapy, is widely utilized in the treatment of cancer in human patients and is equally applicable in Veterinary Medicine (HEIDNER, et al. 1991; OLIVEIRA; SILVEIRA, 2008; ANDRADE, 2009; BECKER, et al. 2013).

In radiation therapy, ionizing radiation (IR), when absorbed by live tissues, causes excitation and ionization of components of atoms or molecules in the path of the wave beam. With this, the subsequent chemical reactions result the breakage of molecular bridges and can cause the apoptotic death of cells, if the molecules critical for cellular viability are disrupted (MORRIS; DOBSON, 2007; BERTOLLO, 2010). The “critical target” more considered is deoxyribonucleic acid (DNA), but other molecules in other parts of the cells such as proteins and lipids, for example, also can be damaged and contribute to the cellular lesioning induced by IR (MORRIS; DOBSON, 2007). When irradiated, the majority of cells die due to mitotic catastrophe via standard clinical doses, in which the effect of IR is limited to cells undergoing division by the fact that the clinical effects depend on the growth fraction and duplication time of neoplastic cells (NORTH; BANKS, 2009).

Thus, according to the energy range utilized in the veterinary practice, the greatest effect of IR on cells is indirect due to the production of free radicals, such as hydroxyl, that damage the cellular DNA, since live cells consist of approximately 85% water, while these molecules are the principal target of IR (MORRIS; DOBSON, 2007). Furthermore, the response of live cells and of tissues to IR depends on the dose or dosage rate of the radiation applied, and on the radiosensitivity of the cellular population. The majority of dividing cells are more sensitive to IR than differentiated ones.

However, we emphasize that cells in the mitotic phase of the cellular cycle are more radiosensitive, whereas those in the DNA replication phase are more resistant as are cells undergoing growth and preparation for mitosis (NORTH; BANKS, 2009; TAUHATA, et al. 2014). The OSA cells are considered historically resistant to IR, due to the utilization of conventional radiation therapy; for this reason, the use of elevated doses is recommended. Among the protocols available in the literature, the most utilized is the incidence of cobalt-60 at an intensity of 8 to 10 Grays (Gy) applied in three sessions (MORAES, 2009; FRACASSO, 2012; PIMENTA, et al. 2013; MANTOVANI, et al. 2016). In this context, the present study aimed to evaluate the noxious effect of different intensities of IR in canine OSA cells, by working with cells from a primary cellular

culture since, in relation to the tissue of origin, they possess identical morphology and maintain the same physiological characteristics, besides presenting diverse neoplastic clones, which makes them representative of the natural conditions of the organism and, therefore, a good *in vitro* model in therapeutic oncology (FRESHNEY, 2010; BERSANO, 2011; MIGITA; OLIVEIRO, 2013; SIGMA-ALDRICH, 2017). This study is also intended to contribute to research in comparative and multidisciplinary oncology, serving as an incentive for seeking new therapeutic foci.

2 I METHODS

2.1 Origin of study material

The primary cell cultures of osteosarcoma canine were obtained from the tumors of five dogs (one German Shepherd, two Rottweilers, one Irish Setter and one of undefined breed) of medium and large size afflicted in bones of the appendicular skeleton. The cell culture belongs to the São Paulo State University (UNESP) and was characterized by flow cytometry with the markers osteocalcin (AB13418), osterix (AB22552) and osteopontin (SC73631) (BERSANO, 2011), and cytogenetic analysis, in which acrocentric and submetacentric chromosomes are identified. The count obtained a mean of 69 (± 2.31) chromosomes (Figure 1), while the dog karyotype is equal to 78.

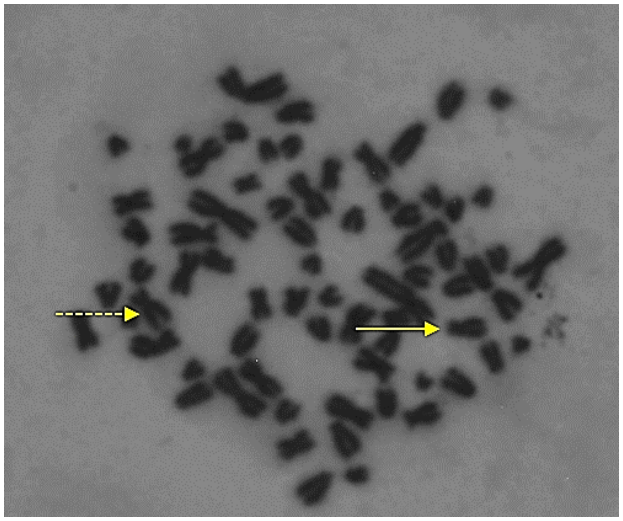

Figure 1. Cytogenetic characterization of canine osteosarcoma primary cell culture – OLYMPIOS® CX31 (100X). Chromosomes submetaccentric (-------►) and acrocentric (——►). Images obtained in the Laboratory of Animal Genetics of the Department of Genetics - UNESP, Botucatu, São Paulo, Brazil.

2.2 Cell culture

OSA cells were cultured in DMEM high glucose (GIBCO 11995–065) medium containing 10% fetal bovine serum (FBS – GIBCO 12657–029) and 1% antibiotic antimitotic solution (GIBCO, 100x) in a humidified incubator at 37 °C and 5% CO_2. The concentration utilized of the study with 96-well plates was equal to $0.5x10^5$ cells per well. For 12-weel, the concentration was equal to $1x10^5$ cells per well and there

were wells occupied with round coverslips treated with PLL (Poli L. Lisina), following the protocol of SIGMA-ALDRICH (SITTERLEY, 2016). The treatments, carried out in triplicate, were performed with the cells in the fifteenth passage.

2.3 Radiation therapy

Cells were arrayed in 96- or 12-well plates (according to the test realized) 24 hours before being treated. OSA cells were divided into control and treated groups, in the following doses: 5 Gy, 10 Gy and 30 Gy. After this period, the plates containing the respective cells proposed in the study were removed from the incubator and transported at ambient temperature to the Sector for Radiation Therapy, where the treatments were performed utilizing the Unit of Cobalt therapy (GE – *General Eletric Company Medical System*, USA; CGR MeV, ALCYON, Nº: 1008), with an irradiation field of 30 cm x 30 cm, source surface distance (SSD) of 80.0 cm and depth of 0.5 cm (to obtain a percentage of depth dose (PDP) of 100%).

The experimental design of this study was based on the protocol of Mantovani et al. (2016), in which the treatment table and gantry were positioned at 0º, five acrylic plates were placed under the sample treated to avoid the retrospreading of ionizing radiation and an acrylic plate was placed on the sample to eliminate the GAP of air (spacing or distancing between the fields of beams, on their entry surface), in order to homogenize the dose absorbed.

The temperature, relative air humidity and atmospheric pressure of the command and treatment rooms of the sector were measured three times (SETTING, Certif.: PS-11-006/14, TP-10-562/14, TB-01); subsequently the mean of these values was calculated. The exposure time (t), in minutes, of the treated groups was calculated according to the following equation:

$$t = \frac{\text{Dose}}{(\text{PDP})x(\text{Fc})x(\text{Rend})},$$

in which PDP is the percentage of the percentage of depth dose, Fc is the field factor (equal to 1.109) and Rend is the output from the radioactive source (Co-60). Fc consists of the ratio of the dose for one given field in relation to the dose for the field of reference (10 cm x 10 cm), at the depth of the maximum dose. PDP denotes a percentage relation of the dose at a determined depth in relation to the maximum dose depth (electronic equilibrium) and is elevated with the area of the field, since with the increase of volume irradiated there is a greater quantity of radiation spread.

2.4 Analysis of cellular survival

2.4.1 MTT Assay

The cells were divided in 96-well plates 24 hours before being irradiated into one control group and five groups that were treated. After 24 hours, the cells were submitted to the protocol of the test of cytotoxicity with methylthioltetrazolium (MTT – SIGMA® M 2128) to verify the cellular viability (mitochondrial) of each group. In this assay 100 μL of MTT was placed in each well of the plate and, after 3 hours of incubation, the MTT was removed and the same quantity of dimethyl sulfoxide (DMSO – DINAMICA) was added into the wells. Next, the plates were read at 540 nm through an Elisa reader (BIOTEK ELx800®).

2.4.2 AO/EB staining

The cells were divided in 12-well plates 24 hours before being irradiated into one control group and three groups that were treated. Among the wells was placed a round glass coverslip treated with PLL. The test was carried out 24 and 72 hours after the treatment, in which the cells were stained with a solution composed of two fluorescent dyes, acridine orange (AO – $C_{17}H_{19}N_3$ – SIGMA® 158550-25G) and ethidium bromide (EB – $C_{21}H_{20}BrN_3$ – SIGMA® E7637-1G), both at the concentration of 100 μg/mL, diluted in a phosphate-saline solution (PBS – GIBCO, 1x, pH 7.2).

For analysis of the results, the coverslips were taken out of the wells and washed with PBS, in order to remove any residue from the culture medium, since it contains phenol red, which may interfere in the analyses. Next 30 μL of solution containing the two stains was placed on each coverslip and subsequently placed on the glass slides for microscopy and incubated for 20 minutes in the dark at ambient temperature. Subsequently, the slides were analyzed through a fluorescent microscope (ZEISS®) with 400X magnification and 3-FITC filter. From each group, 200 cells were counted and the images were captured and analyzed by the software Axion vision Rel 4.7. The graphs were constructed in the software OriginPro® version 8E.

Cells were identified according to the following criteria: viable cells are stained in green; cells in early apoptosis are stained in green and present condensation of chromatin; cells in late apoptosis are stained in orange; and cells in necrosis are stained in red, presenting nuclear morphology similar to the viable cells (RIBBLE, et al. 2005; KASIBHATLA, et al. 2006; RENJU, et al. 2014; GARCÍA, et al. 2015; LIU, et al. 2015). AO is a vital dye that stains both viable and inviable cells, while EB, an intercalant of double-stranded DNA, stains only the cells that have lost membrane integrity, being that the cells in late apoptosis incorporate only the EB (KASIBHATLA, et al. 2006).

2.5 Statistical analysis

Statistical analysis of MTT assay consisted of the analysis of variance (ANOVA), considering the significance level of the statistical tests to be 5% ($p<0.05$). The analysis and construction of graphs were performed via the software GraphPad Prism® version 5.01, with the absorbance values being stored in a table of the software Gen5™

(Microplate Reader and Imager Software – BIOTEK®) version 2.0, whose values subsequently were expressed as percentages of mitochondrial viability by the dose of ionizing radiation received.

As to the double-staining test with AO/EB, the percentage of cells in apoptosis was calculated by the following equation adapted from Renju et al. (2014):

$$\text{Cells in apoptosis (\%)} = \frac{\text{cells in apoptosis x 100}}{\text{viable cells + cells in apoptosis}}$$

3 I RESULTS

In the present study no influence was observed from temperature alteration, relative air humidity or atmospheric pressure on the cellular death of treated cells when compared with the control group, since the viability of the latter remained very close (97.27% – control group at 24 hours after AO/EB test) or equal to 100% (in MTT assay), as displayed in Figures 2 to 4.

The mean values of temperature, relative air humidity and atmospheric pressure in command and treatment rooms were, respectively: 17.67ºC, 44% and 686.75 mmHg (command room) and 18ºC, 43% and 687 mmHg (treatment room) for the treatments in which the MTT assay was subsequently performed, versus 20ºC, 55.67% and 685.06 mmHg (command room) and 20ºC, 54.34% and 685.31 mmHg (treatment room) for the treatments in which the AO/EB test was later performed.

By the MTT assay, we practically no significant cellular death was observed at the 5 Gy dose, because the viability was 95.74% (Figure 2). Cellular viability at the 30 Gy dose was 9.33% and 80.99% with 10 Gy. It was also verified that cellular death between 24 hours and 72 hours was practically unaltered at the dose of 5 Gy, given that this level was 2.31% (Figures 3 and 4).

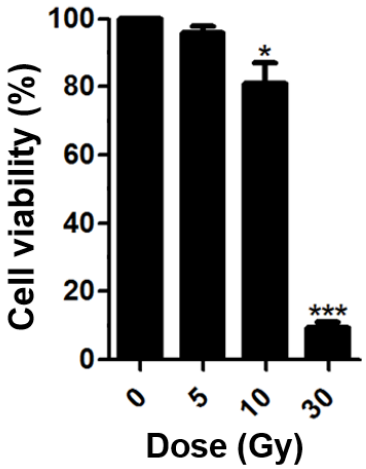

Figure 2. Cell viability (%) observed with the MTT assay 24 hours after treatment versus the dose (Gy) of ionizing radiation received (5 Gy, 10 Gy e 30 Gy). ANOVA test ($p<0.05$). Statical difference of $p<0.01$ (*) and $p<0.0001$ (***).

However, it was observed in these figures that for the doses of 10 and 30 Gy, there were respective increases of 20.36% and 15.0% in cellular death. This elevation is a result of both the late effects of ionizing radiation and the duration time of the cell cycle.

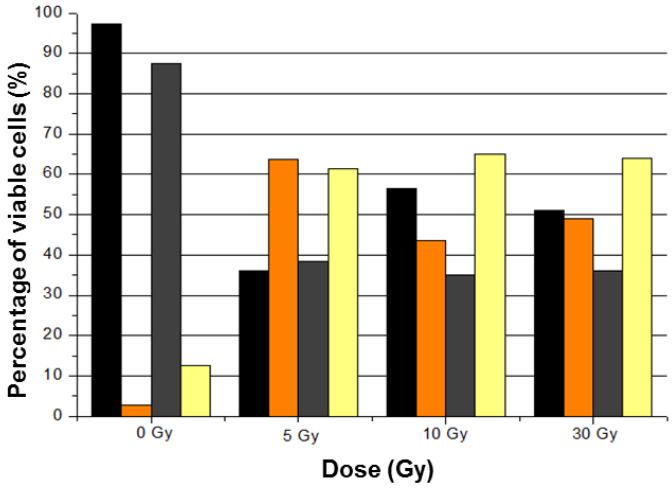

Figure 3. Percentage of viable cells and in apoptosis (%) 24 and 72 hours after the treatment with ionizing radiation by AO/EB staining. Black bars (24 hours) and gray bars (72 hours) refer to viable cells, while orange bars (24 hours) and yellow bar (72 hours) refer to cells in apoptosis.

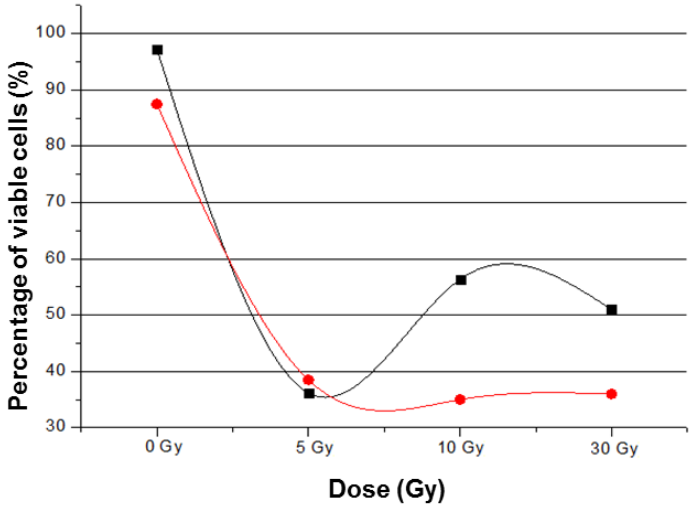

Figure 4. Comparative among the percentage of viable cells (%) versus the dose of ionizing radiation received (Gy) 24 hours (black curve) and 72 hours (red curve) after the treatment with the doses of 0 Gy (control group), 5 Gy, 10 Gy and 30 Gy with the AO/EB staining.

The analysis of slides verified that EB stained the nucleus of cells that had lost integrity of the plasmatic membrane, as was described by Kasibhatla et al. (2016) and in agreement with these authors and with Ribble et al. (2005), Liu et al. (2015), García et al. (2015) and Renju et al. (2014), the cells undergoing apoptosis presented staining that revealed a nucleus with a more orange coloration due to the incorporation of AO.

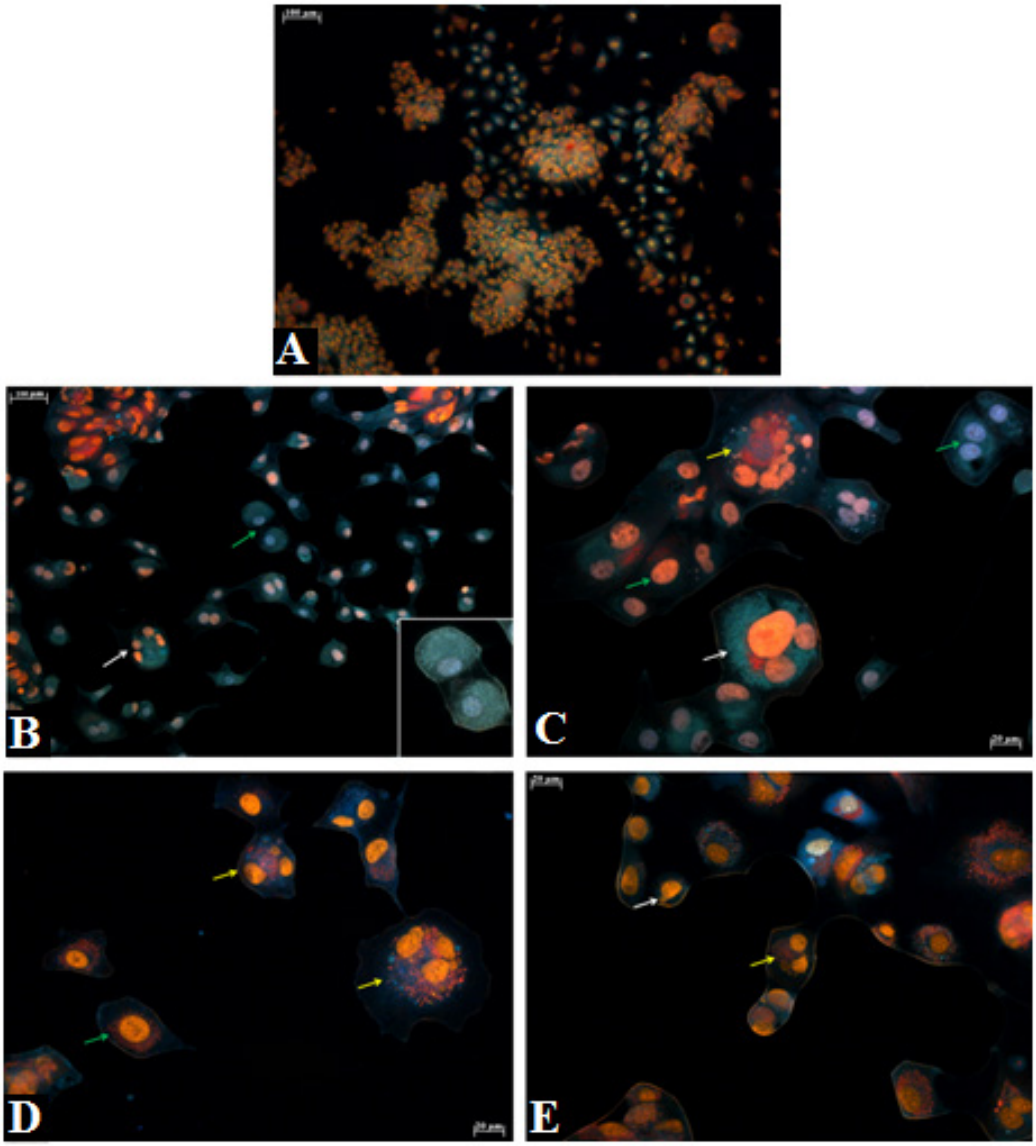

Figure 5. Fluorescent microscopy of AO/EB staining – Axion vision Rel 4.7. Viable cells (green arrows), cells in apoptosis (white arrows) and cells in late apoptosis (yellow arrows). A: control group; B: treatment with 5 Gy, highlighting two viable cells; C and D: treatment with 10 Gy; E: treatment with 30 Gy.

The viable cells presented uniformly green staining, but also cells with an orange-colored intact nucleus, which is attributable to the AO stain, as described by Kasibhatla et al. (2016), this stain colors both viable and inviable cells. In addition, the formation of apoptotic antibodies was observed whereas none of the groups presented cells in necrosis. The cells in late apoptosis, as observed in Figure 5 in C and D (also present in F, but not so evident), present a contrast between the nucleus and cytoplasm and their fragmentation with the presence of various apoptotic bodies.

In all the assays realized, despite not having observed an influence on temperature change, atmospheric pressure or relative air humidity in relation to the viability of cells. The control group presented an unviability of 12.5% after 72 hours (Figures 3 and 4).

4 I DISCUSSION

The works performed utilizing primary cultures to evaluate therapies, besides being simpler biological models, are efficient by virtue of the fact that the cells maintain morphology and physiological characteristics of their tissue of origin unlike a continuous cell line, as described by Migita & Oliveira (2012), Bersano (2006), and Freshney (2010). The temperature variation of cells in the CO_2 incubator for the command and treatment rooms did not influence their cellular death, since this variation would not cause the cellular viability of the control group to be reduced significantly, as observed in the two viability tests. In this manner, the experimental model utilized in this study was demonstrated to be efficient to evaluate treatments and can be compared to *in vivo* models. The MTT assay was proven efficient in the evaluation of cell survival analysis after the treatment with ionizing radiation, since this assay is capable of measuring the cellular respiration, which is proportional to the quantity of Formazan produced, and, consequently, to the number of viable cells, that is, with an intact mitochondrial membrane, as described by Bertollo (2010).

The results of this test showed that the efficiency of the treatment with a final dose of 20 Gy is similar to that whose final dose is 30 Gy, in concordance with the extant protocols that employ three sessions of 8 Gy, in which the final dose is close to 20 Gy, as described by Moraes (2009), Fracasso (2012) and Mantovani *et al*. (2016).

Furthermore, the interaction of ionizing radiation with the cells promotes direct and indirect effects, leading to their death, while according to North and Banks (2009), in Veterinary Medicine, the greatest effect is the indirect one due to the production of free radicals and to the fact that the cells consist of approximately 85% water. In addition, cell survival depends on the integrity of cytoplasm and on the mitochondrial and plasmatic membranes.

Matovani et al. (2016), reported in their studies that canine osteosarcomas are radio-resistant tumors, thus necessitating the use of elevated doses to obtain a satisfactory response. This resistance was observed in our study, since the cellular unviability was significant only in the groups treated with more than 10 Gy. However, elevated doses can pose a disadvantage due to the toxic effects caused. In the study of these authors, the viability at the dose of 10 Gy was close to 70% in two of the three lineages utilized, while in our study, with the MTT assay, this level was 80.99%. The difference observed between our study and those of Matovani et al. (2016) is due to the fact that the analyses of these was performed 72 hours after the treatment and not at 24 hours, and that immortalized lineages were utilized rather than a primary culture, as in the present work. Another reason for this difference is due to the administration of Erlotinib in the culture of these authors, an anticancerogenic compound responsible for inhibiting action of the enzyme Tirosinoquinase, which blocks cellular multiplication. Furthermore, according to Morris et al. (2007), the fraction of cellular population growth is related to the radiosensitivity of this population, so that the majority of dividing cells

are more sensitive to ionizing radiation than differentiated cells, that is, those not in constant division, such as bone cells. This fact is one reason for the cellular viability being elevated despite the incidence of high doses of ionizing radiation.

With the AO/EB test, the increase of cellular unviability from 24 to 72 hours results from late effects of ionizing radiation and is related to the duration time of the cell cycle, which varies from 14 to 36 hours, as presented by Tauhata et al. (2014). In addition, cellular death equal to 12.5% observed in the control group in the analysis at 72 hours is due to competition of cells for space and nutrients.

5 I CONCLUSION

In this study it was possible, through two different assays, to evaluate the survival of cells after treatment. Although the unviability of cells did not decrease linearly, the MTT assay revealed a reduction that is a function of the dose of ionizing radiation received as well as the test of double staining. Furthermore, the results of this assays confirmed the radioresistance of this neoplasia, as reported by Matovani et al. (2016) and that it should be used by co-adjuvant treatment. These results, associated with the fact that the primary cultures of the cells present a response highly similar to that of the tissues of their origin, as described by Freshney (2010), Bersano (2011) and Migita &Oliveira (2012), should serve to incentivize studies to investigate the mechanisms involved in the resistance to radiation therapy and improvement of the extant therapies up to the present.

6 I FUNDING INFORMATION

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8 I CONFLICT OF INTEREST

The authors declare no conflict of interest.

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