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EFFECTS OF AN INHIBITOR OF NITRIC OXIDE PRODUCTION ON CELL CYCLE AND MICRONUCLEUS FREQUENCY IN IRRADIATED HUMAN BREAST ADENOCARCINOMA CELLS

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ABSTRACT

Breast adenocarcinomas are the most frequent malignant tumor (about 25% of cases), and its malignant outcome causes about 15% of all deaths of women with cancer. The production of nitric oxide (NO) by isoforms of nitric oxide synthases (NOS's) are related to increased malignancy and stimuli to metastatic progression of breast adenocarcinomas, but its presence in irradiated cells can lead to higher frequencies of DNA damage. The work used aminoguanidine, an inhibitor of isoform 2 of NOS (NOS2) to treat human breast cancer cells (MCF7) in non-toxic concentrations (1 and 2mM) before exposure to gamma irradiation (^{60}Co) to assess if reduction of intracellular NO can protect from, or induce radio induced cell damage, cell cycle disruption or death. Cells were treated and irradiated at 0, 0.5, 1, 2, 4 or 8Gy. Administration of aminoguanidine arrested the cell cycle in the synthesis (S) phase, altering the DNA repair capacity of cells. The higher concentration (2mM) led to less genotoxic damage (about 50%) in cells irradiated at 8Gy as observed using micronucleus scoring by flow-cytometry. Alternatively, 1mM of aminoguanidine increased genotoxic damage and induced a less significant increase of S-phase cells. Despite the findings, no significant alterations in cell proliferation rates were observed. Findings showed that aminoguanidine can modulate radio-induced effects on cells.

1. INTRODUCTION

The breast cancer is the most usual malignant tumor [1]. The connection between genetic susceptibility and the risk factors are the most significant factor of tumor growth induction [2]. Therapies against breast cancer can include surgery, chemotherapy, radiotherapy and hormone-based treatments [3]. The development of drug-resistance is the principal factor that can result in failure of chemotherapy [1]. Due to this, it is important to improve the current tools of diagnostic and treatments, and to find new treatments [4]. The metastatic potential of growth tumors depends on the escape of tumor cells from immune responses, migration through endothelium, cell survival and growth in target tissue [5]. The inflammation is an important component in the beginning of the cancer development. Relative fast cell growth results in internal hypoxia and suppression of nutrients in inner its regions [6].

Nitric oxide (NO) is a bioactive powerful molecule produced from nitric oxide synthase enzymes (NOS's) and it's present in several physiologic and pathologic mechanisms, like vasodilation and inflammation [5]. Increasing of NO amounts were found in blood of patients with breast cancer and when it's compared with a normal breast tissue or benign breast tissue, a higher activity of NOS was found in invasive breast tumors. The NO increases tumor blood flow and stimulate the angiogenesis, explaining the positive interaction between NO biosynthesis and a high-grade malignancy [4]

The NOS activation is involved in responses found in cells exposed to chemical agents or ionizing radiation [7]. After the exposure to ionizing radiation, high frequencies of DNA damage leading to cell death in large extensions of tissue can lead to liberation of inflammatory cytokines, that stimulate the NO generation through redox system [8].

The NO affinity with the superoxide anionic radical (O_2^-) results in high levels of peroxynitrite ($ONOO^-$) formation [9]. Therefore, the capacity of NO toxicity is to combine with superoxide anions to form peroxynitrite, a free oxidant radical that can induce DNA fragmentation and lipid oxidation [7]. The ONOO− is a powerful nitrosating molecule. The cleavage of DNA caused by ONOO− was observed in almost every nucleotide. ONOO− was also reported as an inactivator of some DNA-repair enzymes. These observations suggest additional ways in which ONOO− can be associated not just to high frequencies of DNA damage, but also to the capacity to impair DNA repair [9].

The aminoguanidine is a selective inhibitor of nitric oxide synthase 2 (NOS2), which has a cytoprotective effect due to its antioxidant proprieties *in vitro* and *in vivo*. NOS2 also has many biologic effects with non-toxic function [10,11], such inhibition of the formation of reactive oxygen species (ROS), the lipid peroxidation and the induced apoptosis from oxidants [11,13]. The aminoguanidine was used in MCF7 cultures to observe the cytotoxic, alterations in clonogenic potential and genotoxic, in experimental situations that reported production alterations in NO [12].

This work studied the effect of aminoguanidine in irradiated MCF-7 cells, to observe a possible molecular mechanism involved in NOS inhibition and cellular repair from aminoguanidine activity.

2. MATERIAL AND METHODS

2.1. Cell culture and treatment

MCF7 cells (**ATCC, Manassas, VA**) were maintained in RPMI-1640 medium (**Gibco / BRL, Grand Island, NY**) with 10% fetal bovine serum (**GIBCO-Estados Unidos**) and mixture of antibiotics (1% penicillin / streptomycin, (**GIBCO, Grand Insland, NY**) under controlled atmosphere (37 ºC, 5% CO_2).

2.2. Aminoguanidine

Aminoguanidine (hydrochloride) (Sigma-Aldrich, CAS 1937-19-5) was diluted in RPMI 1640 medium in concentrations in 1mM e 2mM just prior to administration to cell cultures. Solutions were filter sterilized (0.22µm)

2.3. Irradiation

The cells were detached from culture flasks using trypsine (0,25%) solution. Cells from non-treated (control) cultures, as from cultures treated with 1 or 2mM of aminoguanidine were washed with sterile phosphate-saline buffered solution (PBS), and irradiated as suspensions in 2mL of PBS in sealed conical tubes (15mL).

The irradiations were made in a Cobalt-60 source (Gamma Cell 220, Canadian Atomic Energy Commission, Ltd) at The Center of Radiation Technologies – IPEN. The irradiation doses used was 0; 0,5; 1; 2; 4; 8Gy. A metallic lead attenuator was used corresponding 90% the attenuation. Through this shield, dose rate was not superior to 85Gy/h.

2.4. Genotoxicity – Micronucleus scoring by flow cytometry

Genotoxicity was assessed using a method described elsewhere [14]. Cells were treated and irradiated as above. After irradiation, the suspensions were centrifuged and the viable cell fractions were determined by the method of dye exclusion using trypan blue. The concentrations were adjusted to 50 0000 cells/mL per suspension, and 100 µL (5000 cells) were plated per well in 96-well plates in quadruplicates. After seeding, cells were incubated 72 hours with the same conditions written above.

Mitomycin (4µg/ML) and Colchicine (8µg/mL) were used as positive controls. A solution NaCl 0.9% was used (5µL/well) as negative control.

After 72 hours in cell culture, the cells were centrifuged in plates (1500 RPM in 10 minutes), and received a solution containing the fluorescent dye ethidium bromide monoazide (EMA, Thermo-Fisher Scientific, E1374) in 8,5 µg/mL concentration diluted in PBS supplemented with 2% fetal bovine serum. In this phase of the experiment, EMA can penetrate only through membranes of non-viable cells. Plates were opened and exposed for 30 minutes to a blue LED source (440-450nm, 30W) on ice bath to photoactivation, which associates the colouring agent in irreversible way to DNA from unviable cells. The objective of this procedure was to label dead the cells for further exclusion form micronucleus scoring, in addition to providing some measurement of cytotoxicity. After this step, the cells received PBS with 2% fetal bovine serum to remove the free dye.

A two-step lysis was performed to release nucleus and micronucleus from cells and to label DNA of them. The fist step lysis the cells was done in solution with sodium chroride (0,854 mg/mL), sodium citrate, (1 mg/mL), IGEPAL® (0,3 µg/mL), and 0,4 µM of green fluorescent colouring agent SYTOX® Green (Thermo-Fisher Scientific, S7020). This solution also had RNAse A (Sigma-Aldrich, CAS 9001-99-4), which eliminates RNA and thus the coupling of dye to residual RNA molecules. After lysis for 60 minutes (37°C), the plates were centrifuged and the biologic material received the second lysis solution (sucrose 85,6 mg/mL, citric acid 15 mg/mL and SYTOX® Green 0,4 µM). The second lysis solution was supplemented with latex beads (AccuCheck Counting Beads, Thermo-Fisher Scientific, PBC100). After incubation for 30 minutes in room temperature, evets were collected in a flow cytometer

(Accuri C6, BD Biosciences) at the Radiobiology Laboratory of the Center of Biotechnology (IPEN/CNEN-SP).

The test followed the methodology described at literature [14]. Briefly, the events marked with EMA were excluded from final counts. The events with SYTOX were assessed in accordance with its size (FSC) and fluorescence (FL1) to discrimination between nucleus and micronucleus. In each sample at least 20000 events were counted in nucleus area. Results were expressed in micronucleus amounts (negative EMA and positive SYTOX), in relation with the control and the comparation with the control wells (not irradiated cells, untreated). Nuclear-to-bead ratios were used to analyse changes in cell proliferation rates.

2.5. Cell Cycle

Prior to irradiation, cells were synchronized through serum starvation to arrest cells in G0/G1 phase. After 24h, the cells were treated with aminoguanidine diluted in normal (serum-supplemented) media (1mM and 2 mM). The cells were maintained in this way for more 24h. In the third day the cells were trypsinized and the cellular suspension transferred to conical tubes with PBS without EDTA and irradiated.

After irradiation, cells were plated in 6-well plates (10^6 viable cells/well) and let to growth for additional 24h, and the were trypsinized, suspended in PBS and slowly pipeted in 15mL conical tubes containing 5mL of 70% EtOH (-20ºC). After gentle agitation, suspensions were kept at -20ºC for no more than 1 week until analysis for fixation.

For analysis of DNA content, fixed cells were centrifuged and resuspended in a solution containing PBS, fetal bovine serum (1%), Triton X-100 (0.1%), RNAse A (200µg/mL) and 7AAD (50ng/µL), followed to incubation (37ºC) for 30 minutes in dark. The solution permeabilized cells, allowing 7AAD association to DNA and eliminated RNA.

Flow cytometry analysis using the Accuri C6 acquired at least 10^5 relevant events in FL3. Analysis of DNA content of events allowed to discriminations between cell populations at G0/G1, S or G2/M phases.

2.6. Statistical Analysis

Results from micronucleus analysis were expressed as fold-ratios of controls. Groups were compared with one-way ANOVA and Bonferroni post-test.

3. RESULTS E DISCUSSION

3.1. Micronucleus assay for cytometry flow

Micronucleus frequencies of MCF7 cells treated with 1mM aminoguanidine and irradiated with doses between 0,5 and 8 Gy are shown in Figure 1.

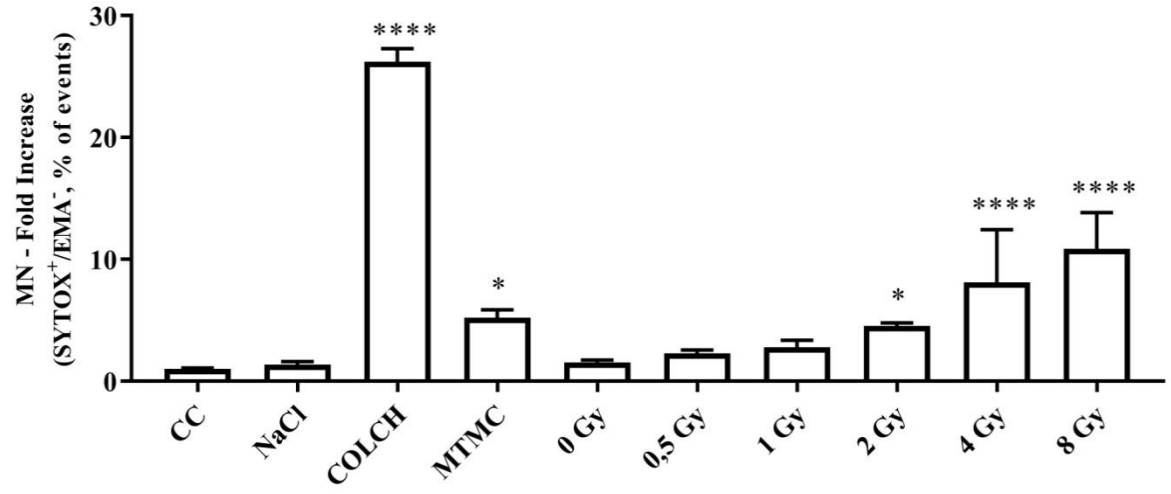

Figure 1: Micronucleus frequency of MCF7 cells treated with 1mM of aminoguanidine (MTMC): positive control, Mitomycin. (COLCH): positive control, Colchicin. (NaCl): negative control, Sodium Chloride. (*) = p < 0.05; (****) =p < 0.0001.

A dose of at least of 2Gy was required to induce significant increase of the micronucleus frequencies in cells treated with 1mM of aminoguanidine.

Micronucleus frequencies of MCF7 cells treated with 2mM aminoguanidine and irradiated with doses between 0,5 and 8 Gy are shown in Figure 2.

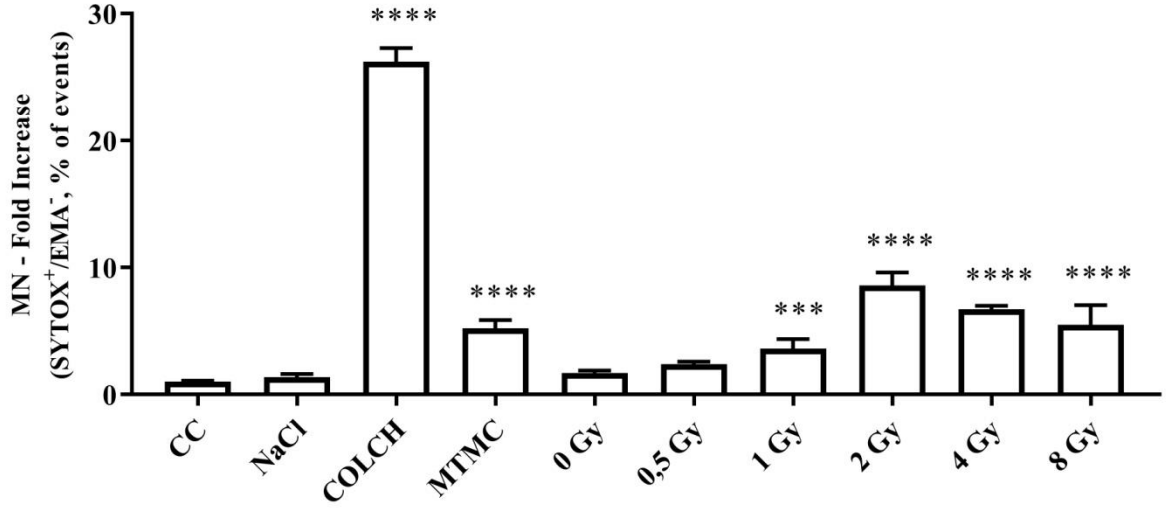

Figure 2: Micronucleus frequency of MCF7 cells treated with 2mM of aminoguanidine (MTMC): positive control, Mitomycin. (COLCH): positive control, Colchicin. (NaCl): negative control, Sodium Chloride. (***) = p <0.001; (****) = p <0.0001

In this case, even a 1Gy dose induced increase of micronucleus frequency.

The latex beads quantities in wells were analysed. Its amount among wells not varied too much (1054±40,48, median ± standard error of mean, data not shown). Therefore, it was possible to calculate the relation between amount of nuclei and beads in each sample. Variations on this ratio comparing to controls were interpreted as representative of changes in cell mitotic rate.

Effects of 1mM of aminoguanidine in cultures are shown in Figure 3.

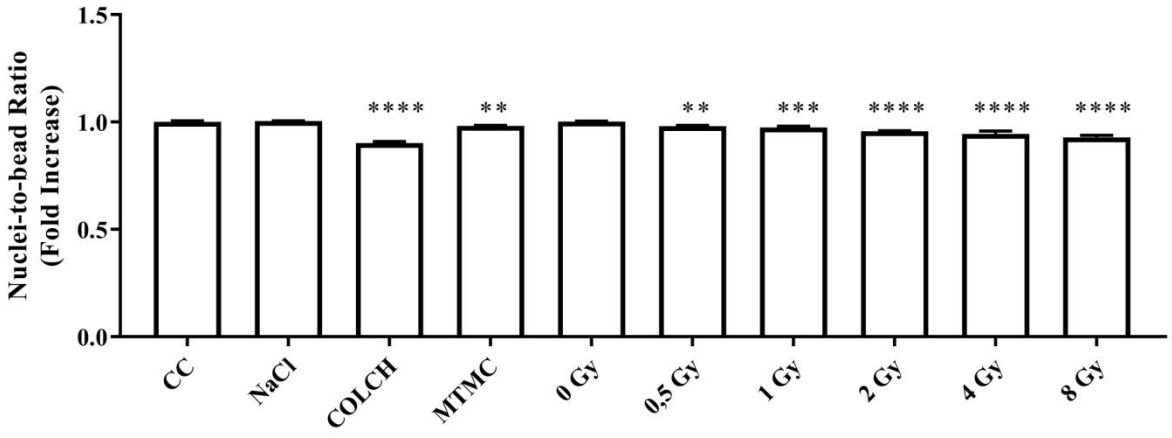

Figure 3: Nuclei-to-bead ratios of MCF7 cells treated with 1mM of aminoguanidine. (MTMC): positive control, Mitomycin. (COLCH): positive control, Colchicine. (NaCl): negative control, Sodium Chloride. (**) = p < 0.01; (****) = p < 0.0001.

For cultures treated with 1mM of aminoguanidine, at least 0.5Gy was required to reduce cell proliferation rates.

Effects of 2mM of aminoguanidine in cultures are shown in Figure 4.

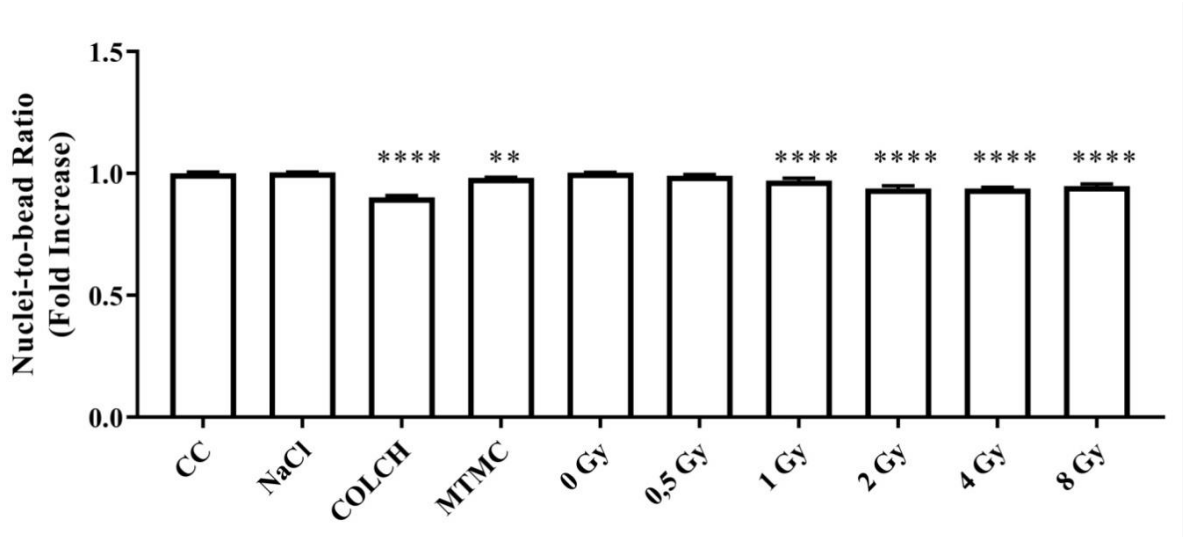

Figure 4: Nuclei-to-bead ratios of MCF7 cells treated with 2mM of aminoguanidine. (MTMC): positive control, Mitomycin. (COLCH): positive control, Colchicine. (NaCl): negative control, Sodium Chloride. (**)=p < 0.01, (***)=p<0.001 and (****)=p<0.0001.

It was required at least 1Gy to significantly reduce cell proliferation rate in MCF7 cells treated with 2mM of aminoguanidine.

The MN assay has been frequently used to evaluate the genotoxicity since 1970. The micronucleus frequency depends on the chromosomic rupture rate, about the damage and the frequency of cellular division. The micronuclei are present in almost every dividing cells. The amounts of genetic alterations and epigenetics in protooncogenes and suppressor genes of tumor developing an important role in neoplasia. The hypermetilation occurs in genes promoter regions which involves the development, suppressor tumor gene, DNA repair genes, cell cycle control genes, in other words, when this critic elements were deregulates, it corroborates with a tumor development or tumor progression. In addition, the hypomethylation of repetitive sequences from DNA can contribute a chromosomic instability. [20]

The excessive production of free radicals (for example, ROS/ RNS) induce severe damage to DNA, and additionally, this NO toxic effect can increase with the reaction of superoxide radical, that results in anion formation of the peroxynitrite highly reactive ($ONOO^-$). [21]

There was a reduction of proliferation in positive controls. In 1mM-treated cultures, doses as low 0.5 Gy reduced proliferation. In 2Gy, also had the same reduction in proliferation positive controls, but the minimum dose to reduce cell proliferation was found to be 1 Gy.

Similar work [12] along with other studies [15–17] showed that aminoguanidine has a effect to arrest cell cycle in G2/M phases. The irradiation can stop the cell cycle in S and G2 phase, which turns the DNA and the cells more sensitive to clastogenic effects.

3.2 Cell cycle analysis

Distribution of cell cycle phases (G0/G1, S and G2/M) in cultures are shown in Figure 5.

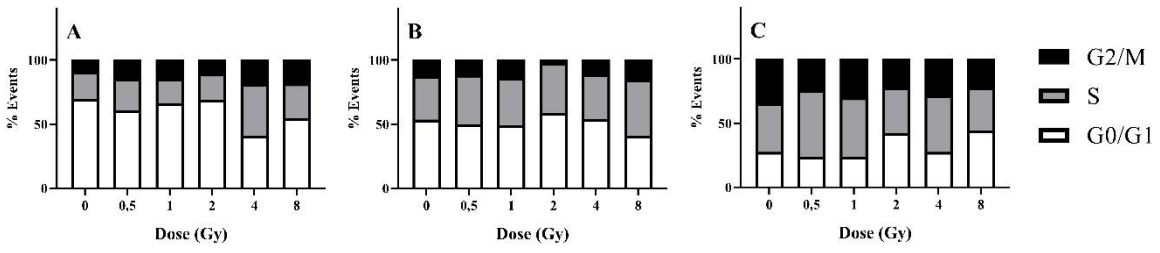

Figure 5: Distribution of cell cycle phases found in non-treated (A), and treated with 1 (B) or 2mMM (C) of aminoguanidine.

Treatment with 2mM reduced G0/G1 populations, and increased S and G2/M. During the S phase, there is a DNA duplication and during the M (metaphasis) there is segregation of every cellular compounds between daughter cells.

The proliferation of malignant cells become self-sustainable and able to doubling indefinitely. Cancer cells doesn't respond to external signals which controls the transition G1/S, and because of it, the cells remain cycling [18]. The exit from cell cycle facilitates the maturation and differentiation, and these mechanisms also are prejudicated during tumorigenesis. If some problem is detected in G1/S phase, there is activation of p53 protein, in order to repair DNA errors, allowing that the proliferation cells occurs in ideal conditions. The literature suggests that p53 protein is expressed in damage/stress damage suffered for DNA, involving several ways to cell repair [19].

In the present study, the MCF-7 cells treated with 1mM aminoguanidine exhibited an increase in the cells proportion in S phase. In the stress genotoxic proteins are activated which have relation with the break double strand of DNA, the same proteins are expressed in some S and G2 phases at cell cycle. But the activity of this protein is the most important near the G1/S transition.

The ^{60}Co gamma radiation has the possibility to cause damage/stress to MCF-7 cells because of high energy and high penetration capacity. In these conditions, it has an increased probability to raise p53 to the promoter p21 which induces the cell cycle stop and MCF-7 cells apoptosis. The aminoguanidine combination with radiation can promote an inhibitory effect in cellular proliferation through the p21 active mechanism.

4. CONCLUSIONS

Inhibition of nitric oxide production can be an important adjuvant to radiotherapy against breast cancer.
Aminoguanidine, when used in 2mM, can reduce the minimum radiation doses required to induce genotoxic damage (1Gy) or reduction of cell proliferation rate (0.5Gy), probably because this concentration also led to a cell cycle arrest in G2/M, which are more sensitive to radiation than S or G0/G1 phases.

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REFERENCES

1. Zhou Q, Song C, Liu X, Zhang hao Q lixia MX. Peptidylarginine deiminase 4 overexpression resensitizes MCF-7 / ADR breast cancer cells to adriamycin via GSK3 β / p53 activation. Dove Med Press. 2019;10:625–36.
2. Qing D, Xuezhi Z, Xin Z, Ming W, Hongmei X, Shilei W. Propofol inhibits proliferation and epithelial-mesenchymal transition of MCF-7 cells by suppressing miR-21 expression. Artif Cells, Nanomedicine, Biotechnol [Internet]. 2019;47(1):1265–71. Available from: https://doi.org/10.1080/21691401.2019.1594000

3. Hosseini S, Chamani J, Reza M, Ebadpour N, Hojjati AS, Hasan M, et al. Nano-curcumin ' s suppression of breast cancer cells (MCF7) through the inhibition of cyclinD1 expression. Dove Med Press. 2019;11:137–42.
4. Avtandilyan N, Javrushyan H, Petrosyan G, Trchounian A. The Involvement of Arginase and Nitric Oxide Synthase in Breast Cancer Development : Arginase and NO Synthase as Therapeutic Targets in Cancer. Biomed Res Int. 2018;2018:9.
5. Gauthier N, Lohm S, Touzery C, Perette B, Reveneau S, Inserm D. Tumour-derived and host-derived nitric oxide differentially regulate breast carcinoma metastasis to the lungs. 2004;25(9):1559–65.
6. Heinecke JL, Ridnour LA, Cheng RYS, Switzer CH, Lizardo MM, Khanna C. regulation of NOS2 in breast cancer progression. Natl Acad Sci United States Am. 2014;111(17):6323–6328.
7. Han W, Wu L, Chen S, Bao L, Zhang L, Jiang E, et al. Constitutive nitric oxide acting as a possible intercellular signaling molecule in the initiation of radiation-induced DNA double strand breaks in non-irradiated bystander cells. Oncogene. 2007;26(16):2330–9.
8. Farhood B, Goradel NH, Mortezaee K, Khanlarkhani N, Salehi E. Intercellular communications-redox interactions in radiation toxicity ; potential targets for radiation mitigation. J Cell Commun Signal. 2019;13(1):3–16.
9. Korkmaz A, Oter S, Seyrek M, Topal T. Molecular , genetic and epigenetic pathways of peroxynitrite-induced cellular toxicity. Interdisc Toxicol. 2009;2(4):219–28.
10. Shaalan A, Carpenter G, Proctor G. Nitric Oxide Inducible nitric oxide synthase-mediated injury in a mouse model of acute salivary gland dysfunction. Nitric Oxide [Internet]. 2018;78(May):95–102. Available from: https://doi.org/10.1016/j.niox.2018.06.001
11. Uang ENGENH, Eng MDF, Ang HW, In IUIL, Ang KUDY. BIOLOGY CONTRIBUTION AMINOGUANIDINE ALLEVIATES RADIATION-INDUCED SMALL-BOWEL DAMAGE THROUGH ITS ANTIOXIDANT EFFECT. Int J Radiat Oncol Biol Phys. 2009;74(1):237–44.
12. Fonseca D.C., Ocampo I.Z. VDP. Genotoxic and anti-proliferative effects of aminoguanidine on gamma- irradiated MCF-7 breast cancer cells. Brazilian J Radiat Sci. 2018;7(1):1–20.
13. Liu DC, Gao MJ, Huo Q, Ma T, Wang Y, Wu CZ. Design , synthesis, and apoptosis-promoting effect evaluation of novel pyrazole with benzo [d] thiazole derivatives containing aminoguanidine units. J Enzyme Inhib Med Chem [Internet]. 2019;34(01):829–37. Available from: https://doi.org/10.1080/14756366.2019.1591391
14. Bryce SM, Bemis JC, Avlasevich SL, Dertinger SD. In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutat Res Toxicol Environ Mutagen [Internet]. 2007 Jun;630(1–2):78–91. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1383571807000745
15. Sugiyama T, Shimada H, Nomurab M. Aminoguanidine inhibits cell proliferation by prolongation of the mitotic phase. Toxicol Lett. 1993;69:213–8.
16. Giunta S, Belotserkovskaya R, Jackson SP. DNA damage signaling in response to double-strand breaks during mitosis. J Cell Bio. 2009;190(2):197–207.
17. Tsuchida E, Kaida A, Pratama E, Ikeda M, Suzuki K. Effect of X-Irradiation at Different Stages in the Cell Cycle on Individual Cell – Based Kinetics in an Asynchronous Cell Population. PLoS One 10. 2015;(6):1–17.
18. Aninditha, K. P., Weber, K. J., Brons, S., Debus, J., &Hauswald, H. In vitro sensitivity of malignant melanoma cells lines to photon and heavy ion radiation. Clinical and translational radiation oncology. (2019). Available from: ttps://doi.org/10.1080/21691401.2019.1594000

19. Da Cruz, Adriana Taveira. Components of the cell cycle along the genesis of melanoma and its possible regulators. Federal University of São Paulo - Paulista Medical School. 2010; (9) 28-144.
20. Ahmad, A., Jan, B. L., Raish, M., Rachamalla, H. K. R., Banerjee, R., Mukhopadhyay, D., &Alkharfy, K. M. (2019). Evaluation of the in vivo genotoxicity of liposomal formulation for delivering anticancer estrogenic derivative (ESC8) in a mouse model. Saudi Pharmaceutical Journal, 2019.
21. Ghate, N. B., Chaudhuri, D., Sarkar, R., Sajem, A. L., Panja, S., Rout, J., & Mandal, N. (2013). An antioxidant extract of tropical lichen, Parmotremareticulatum, induces cell cycle arrest and apoptosis in breast carcinoma cell line MCF-7. PloS one, 8(12), e82293.