17

# Gamma Radiation Induces p53-Mediated Cell Cycle Arrest in Bone Marrow Cells

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## 1. Introduction

The hematopoietic system is organized in a hierarchical manner in which rare hematopoietic stem cells initiate the hierarchy and have the ability to self-renew, proliferate and differentiate into different lineages of peripheral blood cells as well as to intermediate hematopoietic progenitor cells. Most hematopoietic stem cells are quiescent under steady-state conditions and function as a stock population to protect the hematopoietic system from exhaustion due to various stressful conditions. In contrast, hematopoietic progenitor cells are rapidly proliferating cells with limited self-renewal ability. The proliferation and differentiation of hematopoietic progenitor cells fulfills the requirements of normal hematopoiesis allowing the hematopoietic system to react promptly and effectively to meet the demand for increasing the output of mature cells during hematopoietic crisis such as loss of blood, hemolysis, infection, the depletion of HPCs by chemotherapy and/or radiotherapy (Reya, 2003; Weissman *et al*., 2001; Walkley *et al*., 2005).

Reactive oxygen species (ROS) are produced in organisms due to radiation, biotransformation of dietary chemicals, some diet components, transient metal ions, inflammatory reactions and during normal cellular metabolism.

The effect of gamma radiation ionization affects the main components of biological material such as carbon, hydrogen, oxygen, nitrogen. Radiobiology is described as the action of ionizing radiation on living things. On the molecular and cellular levels direct ionizing radiation can affect molecules in cells, especially DNA, lipids and proteins, promoting breakage and / or modifications or indirectly acting on water molecules and generating excitation ionization products of water radiolysis which include free radicals present and reactive oxygen species (ROS). Both direct and indirect effects of radiation on cells and tissues have biological effects in the short or long term. Such effects can be mitigated or

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eliminated by the antioxidant system and cell repair system that work against oxidizable stress and / or cellular stress (Figure 1).

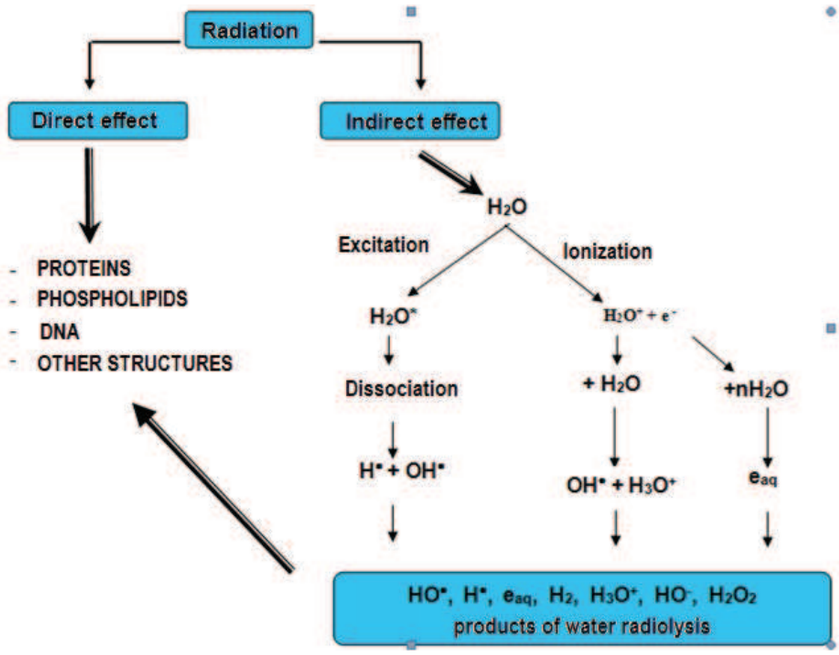



Fig. 1. Direct and indirect effect of radiation produced by free radicals of water radiolysis. Modified scheme of Stark, 1991

The pro-oxidant/antioxidant balance leads to a disturbance which, in turn, results in a condition of oxidative stress subsequently oxidizing cell components, activating cytoplasmic and/or nuclear signal transduction pathways, modulating gene and protein expression and changing both DNA and RNA polymerases activities. Normal cellular metabolism seems to be a primary source for endogenous ROS (such as the participation of oxidatively damaged DNA and repair in aging or cancer development). Oxidative damage to cellular DNA often causes mutagenesis as well as programmed cell death. While mutagenesis might result in carcinogenesis, programmed cell death often causes degenerative disorders (Nakabeppu *et al.*, 2007). Hydroxyl radicals generated from ionizing radiation attack DNA resulting in single strand breaks and oxidative damage to sugar and base residues. Hydroxyl radicals cause ionization of DNA bases as well as of other cellular components. Unsaturated fatty acids play an important role, since lipid peroxidation yields a plethora of stable derivates, which add to nucleic acids forming exocyclic DNA adducts of high miscoding potential, as well as DNA-DNA and DNA-protein cross-links (Bartsch *et al.*, 2004).

Damage of cells by ionizing radiation includes mainly modifications of DNA molecules, such as single and double-strand breaks. While single-strand breaks are quickly repaired in a process that requires poly-(ADP-ribose)-polymerase (PARP), double-strand breaks represent potentially lethal damage and their repair is complicated. Imperfect DNA repair causes mutations and contributes to genome instability. This is mostly manifested as chromosome aberrations, interchromosomal and intrachromosomal rearrangements (dicentric aberrations, translocations, or inversions). Detection of chromosomal aberrations in peripheral lymphocytes is an important indicator of obtained dose of radiation (Kozubek, 2000).

When the DNA is damaged an interconnected network of signaling is activated, resulting in damage repair, temporary or permanent cell cycle arrest or cell death. Cell cycle arrest allows time for DNA damage repair. If the repair is unsuccessful, the cells are removed by apoptosis, necrosis or their proliferation is permanently suppressed by initiation of stress-induced premature senescence (SIPS). Cmielová *et al*., 2011) reported that it is possible that the major mechanism of response of these tissues to irradiation is not apoptosis, but induction of SIPS. Both pathways often work together to induce replicative and premature senescence. In general, activation of p53 and upregulation of p21 in cells undergoing senescence are transient (Toussaint *et al*., 2000; Robles & Adami, 1998).

Increased p53 activity and p21 expression usually occur during the onset of senescence and then subside when the expression of p16 starts to rise. Before p16 upregulation, inactivation of p53 can prevent senescence induction in some cells. However, once p16 is highly expressed cell cycle arrest becomes irreversible simply by downregulation of p53 (Campisi *et al*., 2005; Beausejour *et al*., 2004; Narita *et al*., 2004). This suggests that while both p53 and p21 play an important role in the initiation of senescence, only p16 is required for the maintenance of senescence. In agreement with this suggestion, we found that IR-induced activation of p53 and upregulation of p21 occurred prior to the increased expression of p16 and p19 in murine BM HSCs (Meng *et al*., 2003; Neben *et al*., 1993; Wang *et al*., 2006)

Recent studies showed that a majority of murine BM hematopoietic cells including HSCs died by apoptosis after exposure to a moderate dose of IR *in vitro*. However, a subset of these cells survived IR damage up to 35 days in a long-term BM-cell culture although having lost their clonogenic function. These surviving cells exhibited an increased AS β gal activity, a biomarker for senescent cells, and expressed elevated levels of the proteins (p16Ink4a and p19Arf) encoded by the *Ink4a-Arf* locus, whose expression has been implicated in the establishment and maintenance of senescence by direct inhibition of various cyclin-dependent kinases (CDKs), (Meng *et al*., 2003; Dimri *et al*., 1995; Lowe *et al*., 2003; Sharpless *et al*., 1999).

The main function of mitochondria is ATP production, which occurs during mitochondrial oxidative phosphorylation (ox-phos). In several cell types, mitochondria also act as a very efficient Ca2+ buffer, taking up substantial amounts of cytosolic Ca2+ at the expense of mitochondrial membrane potential (ΔΨm). The pathways of Ca2+ entry into mitochondrial matrix are known as the mitochondrial calcium uniporter (MCU), the "rapid mode" mechanism, and the mitochondrial ryanodine receptor. The main role of mitochondrial Ca2+ is the stimulation of the ox-phos enzymes. In addition to ox-phos, mitochondria are central players in cellular Ca2+ signaling by shaping and buffering cellular Ca2+ signals. As a consequence of Ca2+ uptake, mitochondria can suffer Ca2+ overload, triggering the opening of the permeability transition pore (PTP) which is associated with apoptosis *via* the mitochondrial pathway or necrosis due to mitochondrial damage. PTPs have been shown to be promoted by thiol oxidation and inhibited by antioxidants, supporting a role of ROS in pore opening. In addition, it has been demonstrated that mitochondrial Ca2+ uptake can lead to free radical production. From a thermodynamic point of view, however, it has been noted that Ca2+ uptake occurring at the expense of membrane potential should result in a decrease in ROS production (Crosstalk signaling between mitochondrial Ca2+ and ROS) (Brookes *et al*., 2004). Mitochondrial PTP is formed from a number of proteins within the

matrix, and mitochondrial inner and outer membranes (Ishas & Mazat, 1998; Brookes *et al.*, 2004). One of the processes through which mitochondria contribute to cell death is through PTP opening (Bratton & Cohen, 2001). The PTP precise composition remains unclear, but it is evident that this is a multi-subunit protein channel that spans the mitochondrial inner and outer membrane. Critical components appear to include the mitochondrial VDAC (voltage-dependent anion channel), the ANT (adenine nucleotide translocase), and cyclophilin D (Ishas & Mazat, 1998). It has also been shown that pre-treatment of isolated mitochondria with pro-oxidants can lower the threshold at which the PTP opening occurs (Brookes & Darley-Usmar, 2004). It seems that the opening of this $Ca^{2+}$-dependent channel plays an important role in controlling the commitment of the cell to death through apoptotic or necrotic mechanisms (Kokoszka *et al.*, 2004; Baines *et al.*, 2005).

## 2. Objective

The purpose of this work is to evaluate ionizing radiation-induced apoptosis in mice bone marrow cells and the role of p53, p21 and Ca++ in this process, cell cycle alterations and indirect determination of reactive oxygen specimens (ROS).

## 3. Methods

### 3.1 Animals

Mice C57BL/10 (3 months) were provided by the Instituto Nacional de Farmacologia (located at Rua Três de Maio 100, Vila Clementino, SP, Brazil). The animals were maintained on standard mouse feed and water *ad libitum*.

### 3.2 Irradiation

Gamma irradiation was carried out with an Alcyon II $^{60}CO$ teletherapy unit with the mice at a distance of 80 cm from the source. The dose rate was 1.35 Gy/min. Animals in batches of ten were placed in a well–ventilated acrylic box with an individual cell for each mouse and exposed whole – body to 7Gy (Segreto *et al.*, 1999).

### 3.3 Preparation procedure of bone marrow cells

The mice were killed by cervical dislocation 4 hours after gamma irradiation, and both femurs were removed from each mouse. After cutting of the proximal and distal ends of the femurs, the bone marrow cells were gently flushed out with 5ml suspension using phosphate buffered saline (PBS) (Segreto *et al.*, 1999).

### 3.4 Intracellular reactive oxygen species

Intracellular peroxides were determined by incubating $2X10^6$ cells/ml in medium (defined above) with 5 nM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) for 30 min at 37°C, then analyzed using a Becton Dickinson (BD) Bioscience Flow Cytometer - model FACScalibur (San Jose, CA) equipped with an argon laser emitting at 488 nm (Jagetia & Venkatesh, 2007). BD CellQuest Pro software was used for fast reliable acquisition, analysis and presentation of information.

### 3.5 Measurement of intracellular Ca++

Calcium was measured after incubation of the bone marrow cells with the fluorescence indicator Fura- 2/AM in the form of acetoxymethyl ester (AM). The bone marrow cells at concentration of $10^6$ cells/ml were suspended in 2.5 ml of Tyrode's solution (137 mM NaCl, 2.68 KCl, 1.36 mM CaCl2_2H2O, 0.49 mM MgCl2_6H2O, 12 mM NaHCO3, 0.36 mM NaH2PO4, and 5.5 mM D-glucose) for 30 min. The suspension was then centrifuged at 100 g for 4 min, the supernatant was aspirated, and the result pellet was suspended in 2.5 ml of Tyrode and transferred to the quartz cuvette of a SPEX fluorometer (AR CM System) for fluorescence determination. Measurements were made at alternated 340- and 380-nm excitation wavelengths, with emission at 505 nm. The autofluorescence ratio was less than 10% and therefore was not subtracted from the fluorescence measurements before calculation of the fluorescence ratio. The cells were incubated with 0.01% pluronic 127 detergent and 2M Fura-2/AM, and the cuvette was transferred to a PerkinElmer Life Sciences spectrofluorometer (LS 5B, Buckinghamshire, UK) to determine the fluorescence spectrum of the indicator in the excitation range of 300 to 400 nm, with emission at 520 nm. In the esterified form, maximum fluorescence was observed at 390 nm. As the indicator Fura- 2/AM was transformed into the acid form, the fluorescence peak shifted to 350 nm within an average period of 2 h, thus indicating the maximum amount of indicator incorporation into the cell suspension. At that time the cell suspension was washed with 15 ml of Tyrode and centrifuged at 100*g* for 4 min. The supernatant was discarded, and the pellet was suspended in 2.5 ml of Tyrode and transferred to a SPEX fluorometer programmed for excitation at two wavelengths (340 and 380 nm) with emission at 505 nm, under constant stirring at 37 °C. The first reading of this phase corresponded to basal calcium. At the end of each experiment, a control was performed using 50 mM digitonin, 1mM MnCl2, and 2 mM EGTA. The results are calculated by the relative ratio of 340 and 380 nm, considering the reading for digitonin to be 100% (*R*max) and 0% for EGTA (*R*min). Using the ratio *R*max/*R*min, calcium concentration was estimated by the formula of (Grynkiewicz *et al.*, 1985).

### 3.6 Apoptosis assay

The bone marrow cells were labeled with annexin V-FITC (Roche), binding to phosphatidylserine at the cell surface of apoptotic cells and propidium iodide (PI) provided by Sigma, is used as a marker of cell membrane permeability following manufacturer's instructions. Samples were examined by fluorescence-activated cell sorter (FACS) analysis, and the results were analyzed using Cell-Quest software (Becton Dickinson Model: Facscalibur, San Jose, CA) (Vermes *et al*., 1995).

### 3.7 Cell cycle analysis

For cell cycle analysis the cells were washed with cold PBS and fixed with 70% ethanol. For detection of low-molecular-weight fragments of DNA, the cells were incubated for 5 min at room temperature in a buffer (1.92 ml: 0.2 mol/L $Na_2HPO_4$ + 8 ml: 0. 1 mol/L citric acid, pH 7.8) and then stained with PI in Vindelov's solution for 40 min at 37 °C. The measurements were performed in a Becton Dickinson Flow Cytometer model FACScalibur (San Jose, CA); data were analyzed using Cell Quest software (Vindelov & Christensen, 1990)

### 3.8 Flow cytometric analysis of p53 and p21

For analysis of p53 and p21 expression and activation, cells were washed once in phosphate-buffered saline (PBS) containing 0.1% sodium azide (Sigma), centrifuged at 1.4G, fixed in 2% paraformaldehyde for 10 minutes at 4°C, washed 3 times in PBS, and washed twice in PBS with 50 mmol/L $NH_4Cl$. Cells were then permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C. The first primary antibody incubation was performed in PBS containing 10% normal bovine serum and 0.1% saponin. Aliquots were then incubated for 60 minutes with anti-p53 and anti-p21 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), final dilution 1:800, or rabbit IgG as a control, followed by washing in PBS containing 0.1% saponin 3 times for 5 minutes at 22°C. Cells were then incubated with the first fluorochrome-conjugated secondary antibody Alexa 488 and 594, final dilution 1:1600, for 40 minutes at 37°C in the dark (Danova *et al.*, 1990).

### 3.9 Immunocytochemistry

After cutting off the proximal and distal ends of the femurs, the bone marrow cells were gently flushed out with 5ml suspension using phosphate buffered saline (PBS) (Segreto *et al.*, 1999). Bone marrow cells were washed 3 times with phosphate-buffered saline (PBS), cytofuged onto glass slides, fixed in 4% paraformaldehyde for 10 minutes at 4°C, washed 3 times in PBS, and washed twice in PBS with 50 mmol/l $NH_4Cl$. Cells were permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C. The first primary antibody incubation was performed in PBS containing 10% normal bovine serum and 0.1% saponin. Slides were incubated with anti-p53 and anti-p21 antibodies at the dilution 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours, followed by the nuclear staining dye DAPI (4_6-diamidino-2-phenylindole) 1:10000 (catalog #D1036; Molecular Probes, Invitrogen, Carlsbad, CA ) which was diluted in the preparatory solution A of Slowfade Antifade kit (Molecular Probes, Eugene, OR) for a final dilution 1:100 and the fluorescent secondary antibody Alexa 594 and 647, final dilution 1:500 (Invitrogen). Neither one used lectin conjugated with Alexa fluor 488 for the cellular membrane. After an initial primary and secondary antibody staining, the procedure was repeated for the second primary and secondary antibody staining, and the slides were then mounted in the preparatory solution A containing Dapi. Each fluorochrome was analyzed individually by means of an inverted confocal laser scanning Fluorescence Microscope (LSM; Zeiss, Germany).

### 3.10 Ethics

The present study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was approved by the Research Ethics Committee of the Federal University of São Paulo, Brazil (CEP N° 1248/01).

### 3.11 Statistical analysis

The results obtained were analyzed using a one-way analysis of variance (ANOVA) followed by the Student – Newman – Keuls Multiple Range Test.

## 4. Results and discussion

Cell exposure to DNA-damaging agents can result in timely repair of the damage and maintenance of genetic fidelity in daughter cells, cell death, or the development of heritable genetic changes in viable daughter cells through replication of damaged DNA or segregation of damaged chromosomes before repair (Nakabeppu *et al.*, 2007; Christine *et al.*, 1995; Szymczyk *et al.*, 2004). The last one could generate the genetic changes that contribute to the development of a malignant phenotype. As a result, the gene products controlling timely repair and appropriate cell death after DNA damage are expected to be critical determinants of neoplastic evolution. Moreover, as most antineoplastic agents are DNA-damaging agents, these same gene products probably influence response and cure rates during the treatment of human tumors (Christine *et al.*, 1995).

The number of cells in apoptosis was determined using Annexin-V and cell viability by propidium iodide using the technique of flow cytometry, Figure 2. These results indicated the loss of transport function or structural integrity of the cytoplasmic membrane, which is crucial to distinguish viable from unviable cells, given the wide variety of cell viability assays using different cationic markers such as (PI, 7 -AAD) or (Hoechst 33342O). These fluorochromes determine the transport characteristics of the plasma membrane (Ormerod *et al.*, 1992, 1993; Poot *et al.*, 1997). In addition, flow cytometry has identified the viability of bone marrow cells (Figure 2) i.e. the number of non-viable cells that characterize death by late apoptosis (Vermes *et al.*, 1995). Contour diagram of FITC-Annexin V/PI flow cytometry of bone marrow cells after 7 Gy irradiation. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrants (7.0 ± 1.2) % contain the non-viable, necrotic cells, positive for FITC-Annexin V binding and for PI uptake. The lower right quadrants (50.1 ± 1.2) % represent the apoptotic cells, FITC-Annexin V positive and PI negative demonstrating cytoplasmic membrane integrity.

Ionizing radiation induces G2/M cell cycle arrest and triggers a p53-dependent signaling pathway that, in turn, may induce apoptotic cell death (Szymczyk *et al.*, 2004). Nonetheless, this response may vary and be cell type-dependent because p53 response causes cell cycle arrest in untransformed cells (Bates *et al.*, 1999) and apoptosis in transformed cells (Bates & Vousden, 1999). Cell cycle distribution was determined by propidium iodide staining for both the control group and irradiated group, the latter with cells exposed to 7 Gy radiation (GI). An increase in the percentage of apoptotic cells was observed in the irradiated group 4h after irradiation (Figure 3B) when compared to the control group (Figure 3A). Cells in G2 are more susceptible to ionizing radiation, as this is the moment when the cell must be repaired prior to entering mitosis. It is possible that the increased activity of protein kinase ATM (ataxiatelangiectasia) modifies the p53 levels thereby altering the cell cycle distribution (Figure 3).

Cell cycle progression and cell fate are determined by the dynamic balance between different p53 downstream effectors, including p21 (Waf1/Cip1) (Waldman *et al.*, 1996). DNA damage-induced cell cycle arrest is regulated by the tumor suppressor p53 by direct stimulation of $p21^{WAF1/CIP1}$ expression, an inhibitor of cyclin-dependent kinases (Cdks). Working with the cyclin proteins, Cdks ensure, for example, that DNA replication in the S phase follows from the $G_1$ resting phase. $p21^{WAF1/CIP1}$ then, inhibits both the $G_1$/S and the

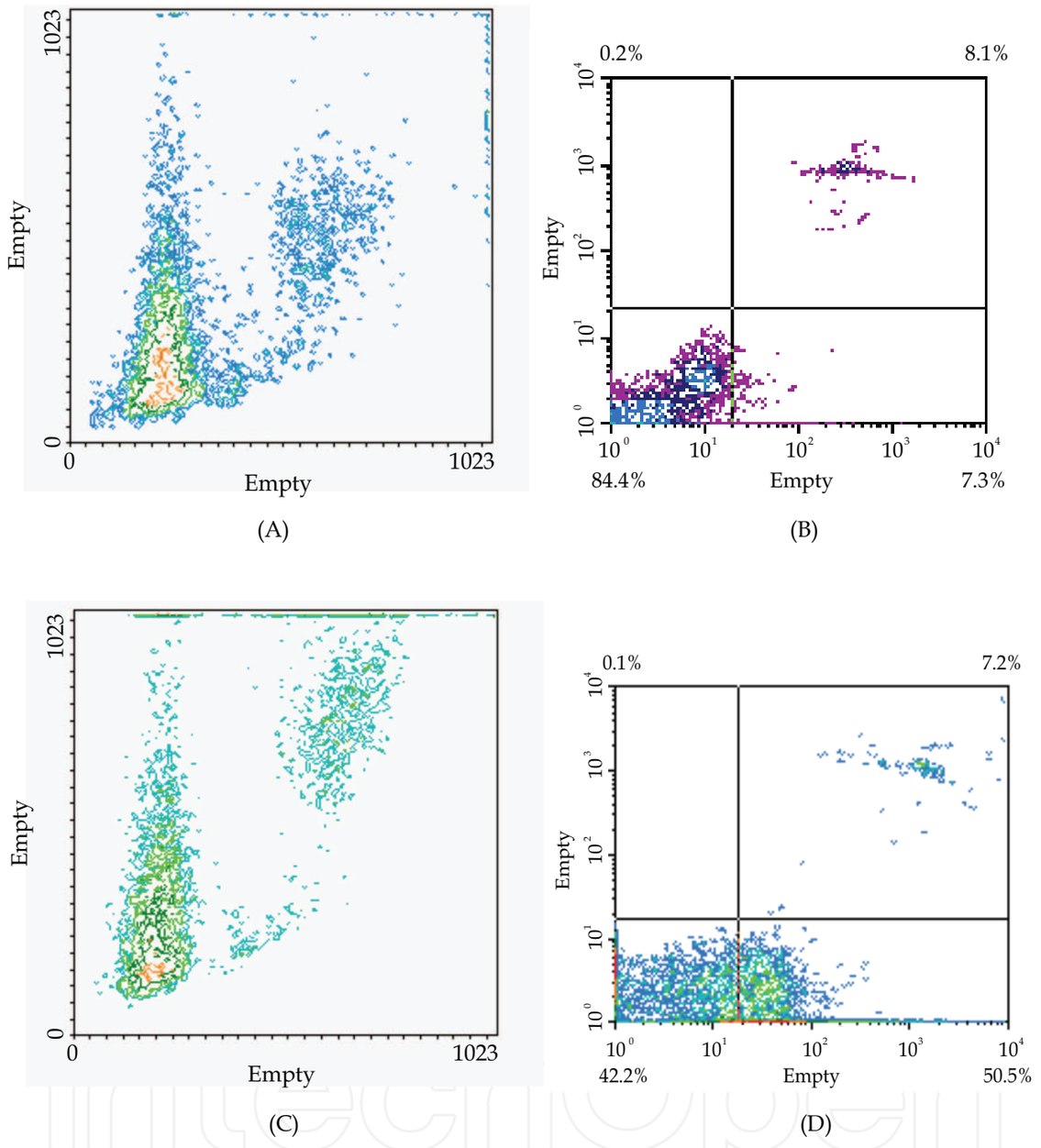
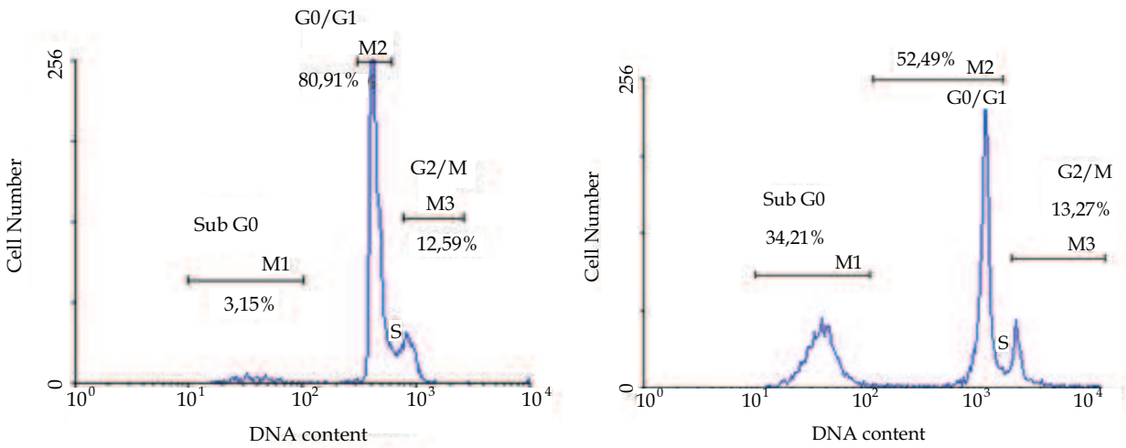



Fig. 2. Flow Cytometric Analysis of control (A and B) and irradiated (C and D) groups to demonstrate the basis for the gating of viable, apoptotic and necrotic cells. The mice were killed by cervical dislocation 4 hours after gamma irradiation. The exposure time was 5.18 minutes in order to achieve 7 Gy of radiation. On Figure 2, A and C, axis X refers to forward scatter and axis Y refers to side scatter. According to the control samples, the following quadrants of the cytograms were defined: - the lower left quadrant showing viable cells; the lower right quadrant representing early apoptotic cells, binding annexin V, but still retaining their cytoplasmic integrity; the upper right quadrant representing nonviable, late apoptotic/necrotic cells, positive for annexin V and the upper left quadrant showing nonviable necrotic cells/nuclear fragments with no annexin V

$G_2$/mitosis (M) transitions, by exerting its negative effects on various Cdks. For example, according to current theories, by inhibiting cyclin B/Cdc2 (Cdk1) $p21^{WAF1/CIP1}$ induces $G_2$/M phase arrest. Even without being required for initiating $G_2$/M cell cycle arrest, p53 and $p21^{WAF1/CIP1}$ seem to be critical for sustaining it as, for instance, cells lacking p53 and $p21^{WAF1/CIP1}$ leave the $G_2$/M cell cycle arrest prematurely and either enter mitosis or reinitiate DNA replication, causing genomic instability and possibly leading to accumulation of oncogenic mutations (Bork *et al.*, 2009). Changes in the cell cycle in response to ionizing radiation were evaluated using the p53 protein (phosphorylated - serina15). The p53 protein activation is the major route of cellular response after DNA damage in many cell types, as described by Yu & Zhang, 2005. We demonstrated that this machinery is activated when apoptosis is induced by radiation. The signaling pathway after p53 activation leads to an increased expression of p21, which consequently affects the profile of the cell cycle and also leads to cell death by apoptosis. Phosphorylation of serine 15 is a modification that is due to DNA damage induced by radiation. This was evaluated 4h after irradiation, using specific antibodies against p53 and p21 (Figures 4 and 5). We observed the increased expression of p53 and p21 by confocal fluorescence microscopy. These data corroborate the method presented by flow cytometry, which showed an increase in fluorescence intensity of




| Control group | Irradiated group |
|---|---|
| Sub G0 = 3.3 ± 0.6% | Sub G0 = 32.5 ± 4.1% |
| G0/G1 = 77.4 ± 5.3% | G0/G1 = 49.2 ± 3.7% |
| G2/M = 13.6 ± 4.4% | G2/M = 12.1 ± 5.3% |

Fig. 3. Analysis of cell cycle distribution: A) Control group and B) Irradiated group. Progression through the cell cycle was monitored 4 h after ionizing radiation. Bone marrow cells were analyzed for DNA content by FACS using PI staining. The histogram was generated using CellQuest software (Alam *et al.*, 2004). Representative histograms are shown from *n*=4 independent experiments. Table show cell percentages quantified by the mean (±SD) of four different peak analyses. Statistical analysis: Anova - Newman Keuls. **$p < 0.05$**

these proteins when compared to the control group (Figures 4 and 5). Overlapped images for immunocytochemistry of p53 and p21 proteins and cellular structures (Nuclei: blue-fluorescent DNA and cell membrane: green-fluorescent Lectin). In the irradiated group there is an increase in fluorescence intensity and a change in the distribution of p53 and p21 when compared to the control group (Figures 6 and 7).

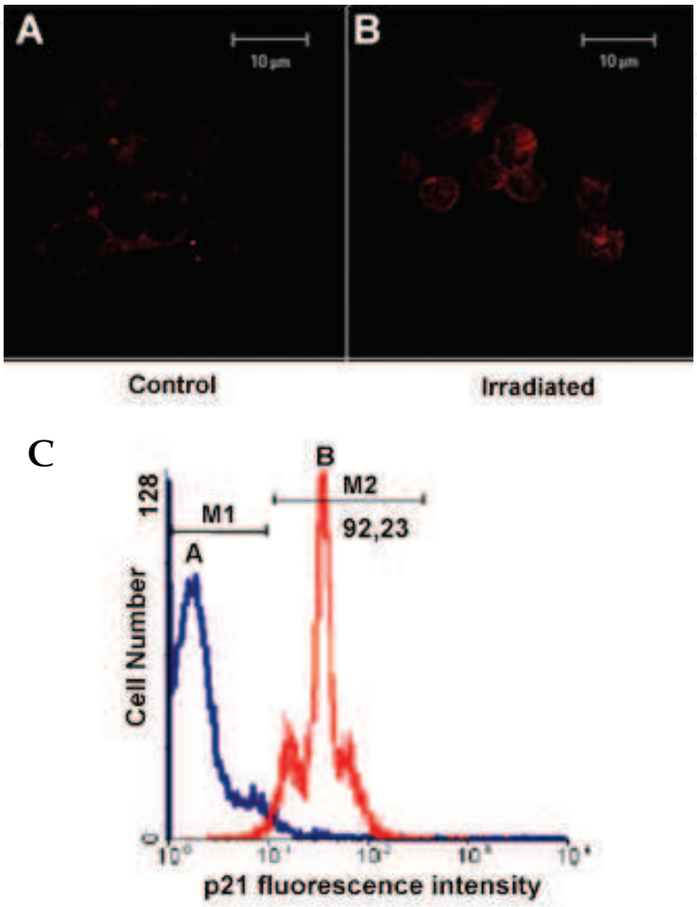



Fig. 4. (above – A and B) Confocal microscopy analysis: bone marrow cells of mice were immunostained in red-fluorescent. Specific staining using antibody p21 (red-fluorescent Alexa Fluor® 594). The irradiated group (B) shows an increase in fluorescence intensity and a change in p21 distribution when compared to the control group (A). (graph below-C) Flow cytometric analysis of p21 protein in paraformaldehyde-fixed cells harvested from mice femurs. Control group A (left) and irradiated group B (right). The bone marrow cells were analyzed 4h after radiation. The cells were pooled, washed, permeabilized with saponin, and stained with anti-p21 antibody and then analyzed by flow cytometry. Histograms were generated and analyzed using CellQuest cell cycle software. Percentage of cells showing fluorescence intensity. Histogram related to gated p21 positive bone marrow cells of GC and GI (89.8 ± 5.3). Statistical analysis: Anova - Newman Keuls. **$p < 0.05$, $N = 4$.**

Cells with not completely repaired DNA damage entering SIPS instead of being removed by apoptosis cause the selection of a resistant population, creating a risk of developing a

population of cells with dangerously damaged genome. Stem cells are currently exhaustively studied due to their self-renewal capability, ability to produce a broad spectrum of cell types and high proliferation potential and they can be used in bone marrow transplantation and peripheral blood stem cell transplantation for patients who had radiation treatment or high dose of chemotherapy in treatment of cancer. Nonetheless, their reaction to DNA damage as a response to genotoxic stress is not widely known, such as ionizing radiation used in the treatment of many cancer types. Hematopoietic stem cells react to irradiation mostly by apoptosis induction, and sometimes by senescence induction. (Vávrová *et al.*, 2002; Meng *et al.*, 2003). Even though mesenchymal stem cells isolated from bone marrow do not die by apoptosis after irradiation, they are notable to proliferate anymore. The irradiation with the dose 2.5-15 Gy do not destruct the cells, but induces telomere shortening, stops cell division and increases the activity of senescence-associated β-galactosidase increases (Serakinci *et al.*,

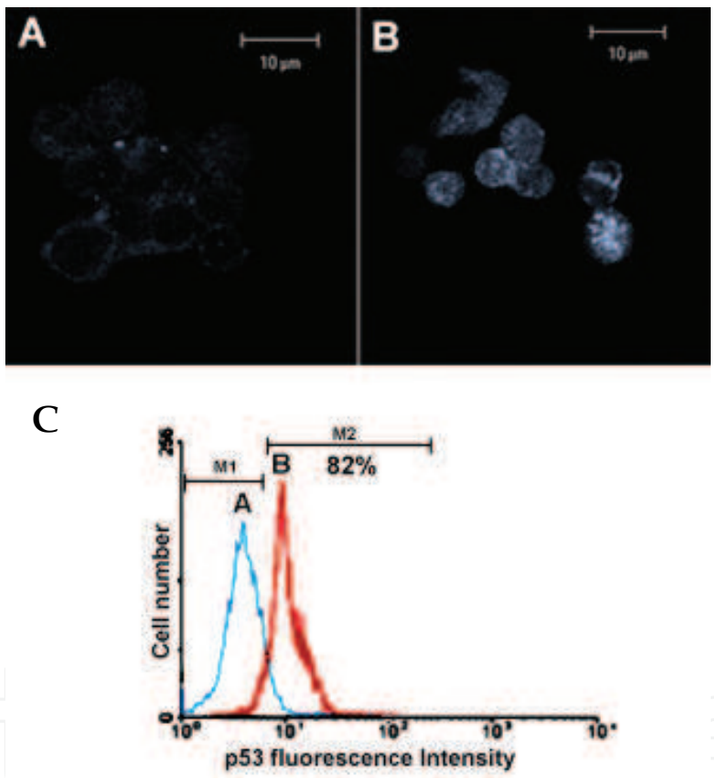



Fig. 5. (above – A and B) Confocal microscopy analysis of mice bone marrow cells. Specific staining using p53 antibody (blue-fluorescent Alexa Fluor® 647). The irradiated group (B) showed an increase in fluorescence intensity and a change in p21 distribution when compared to the control group (A). (graph below - C) Flow cytometric analysis of p53 protein in paraformaldehyde-fixed cells harvested from mice femurs. Control group A (left) and irradiated group B (right). The bone marrow cells were analyzed 4h after radiation. The cells were pooled, washed, permeabilized with saponin, and stained with anti-p53 antibody and then analyzed by flow cytometry. Histograms were generated and analyzed using CellQuest cell cycle software. Percentage of cells showing fluorescence intensity. Histogram related to gated p53 positive bone marrow cells of GC and GI (87.6 ± 5.3). Statistical analysis: Anova - Newman Keuls. **$p < 0.05$, $N = 4$**

2007). Induction of premature senescence have implicated two major pathways: the p53-p21Cip1/Waf1 or p19Arf-Mdm2-p53- p21Cip1/Waf1 pathway, triggered by DNA damage; and the p16Ink4a-Rb pathway, activated by the Ras-mitogen-activated protein kinase cascade (Livak & Schmittgen, 2001; Takano *et al.*, 2004; Cheng *et al.*, 2000). Activation of either pathway is sufficient to induce senescence, but they frequently work together causing premature senescence. Induction of apoptosis or premature senescence, or both, in HSCs and progenitors can result in inhibition of their hematopoietic function. (Wang *et al.*, 2005).

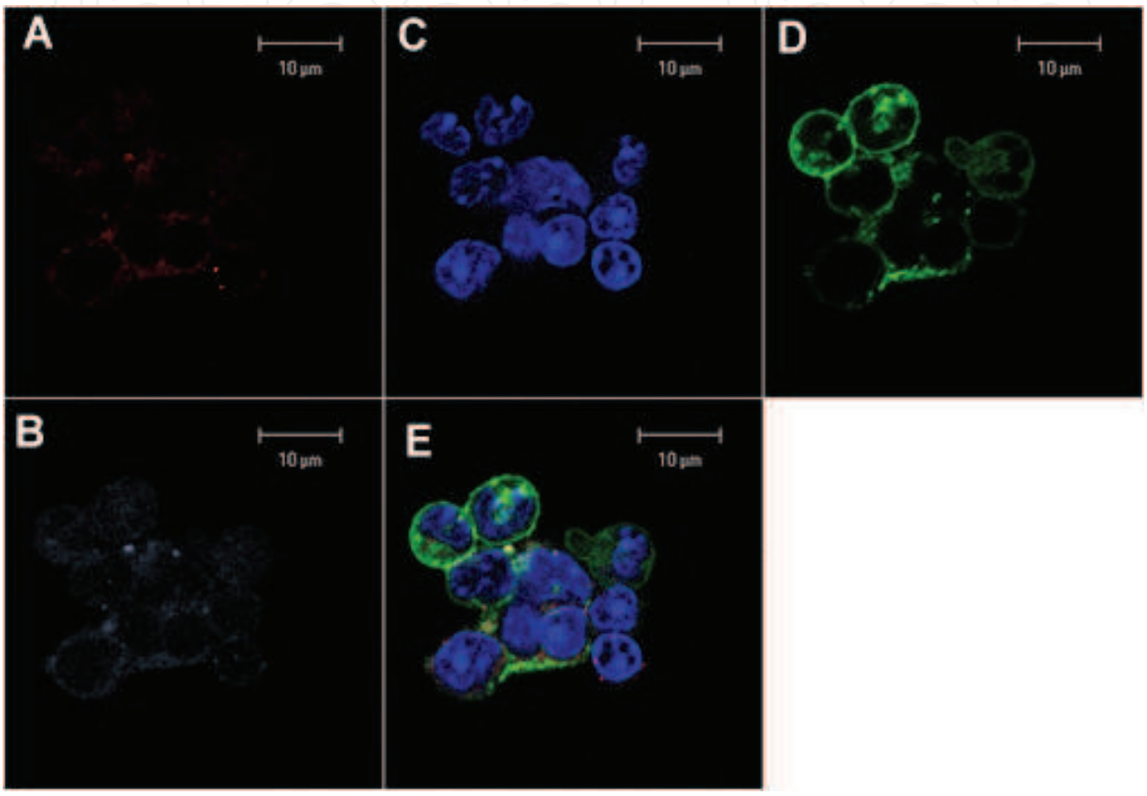



Fig. 6. Laser confocal images of bone marrow cells of the control group mice, were stained by immunofluorescence with combinations of the four fluorophores (red, green, blue and pseudo-colored gray): A) Red channel showing a specific staining using antibody anti-p21 (Alexa fluor 594). B) Pseudo-colored gray channel showing a specific staining using antibody anti-p53 (Alexa fluor 647). C) Blue channel showing nuclei counterstained with the blue-fluorescent DNA stain DAPI (diamidino-2-phenylindole) which is a blue fluorescent probe that fluoresces brightly upon selectively binding to the minor groove of double stranded DNA. D) Green channel showing a Lectin staining using the conjugated with green - fluorescent Alexa fluor 488. Lectin staining using the conjugated with green - fluorescent Alexa fluor 488. E) Specific combination of the four channels reveals overlapped images A, B, C and D. These findings suggest that the bone marrow is more highly susceptible to oxidative damage by radiation ionizing, it also induces alteration in intracellular calcium levels and cell death apoptosis. In summary, the present work demonstrates that radiation induced apoptosis is increase when p53 or p21 responsive G2 arrest being suggestive induction of premature senescence may represent a novel underlying mechanism for radiation

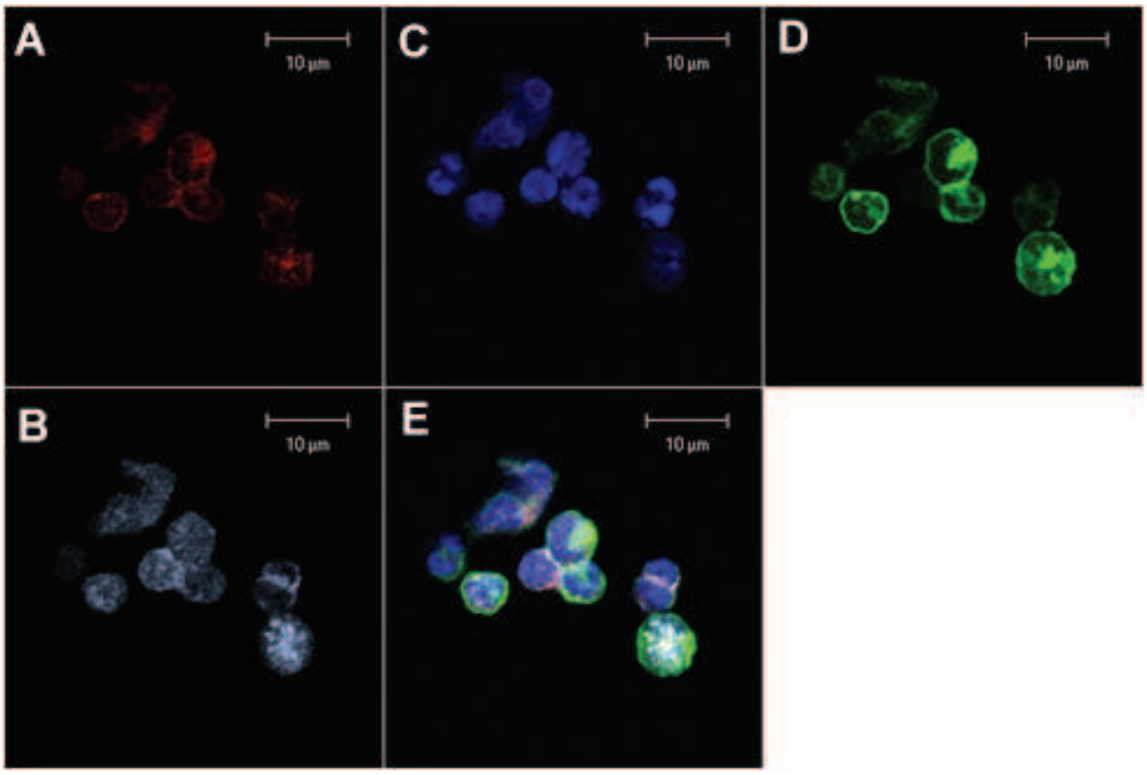



Fig. 7. Laser confocal images of bone marrow cells of the irradiated group mice, were stained by immunofluorescence with combinations of the four fluorophores (red, green, blue and pseudo-colored gray): A) Red channel showing specific staining using antibody anti-p21 (Alexa fluor 594). B) Pseudo-colored gray channel showing a specific staining using antibody anti-p53 (Alexa fluor 647). C) Blue channel showing nuclei counterstained with the blue-fluorescent DNA stain DAPI (diamidino-2-phenylindole) which is a blue fluorescent probe that fluoresces brightly upon selectively binding to the minor groove of double stranded DNA. D) Green channel showing a Lectin staining using the conjugated with green - fluorescent Alexa fluor 488. Lectin staining using the conjugated with green - fluorescent Alexa fluor 488. E) Specific combination of the four channels reveals overlapped images A, B, C and D. In the irradiated group there is an increase in fluorescence intensity and a change in the distribution of p53 and p21 when compared to the control group

In many circumstances, changes in cytosolic Ca2+ concentration may be observed. A surplus of cellular Ca2+ is dangerous for cell life, since it activates many proteases and phospholipases. This Ca2+ surplus can, thus, lead to necrotic cell death. Apoptotic cell death also relies on increased Ca2+ concentrations (Murgia *et al.*, 2009). Both deaths are mediated by endoplasmic reticulum (ER) calcium release and by capacitative Ca2+ influx through Ca2+ release-activated Ca2+ channels (Pinton & Rizzuto, 2006). In Figure 8, we observed the radiation-induced biological effects on cell membranes and the resulting damage which can raise the cytosolic calcium levels triggering cell death by apoptosis, as described by other authors (Starkov *et al.*, 2004). The elevation of cytosolic calcium results in activation of a variety of enzymes sensitive to this ion, such as phosphatases, caspases and calpains, which can be activated from the mitochondria by signaling molecules (Hajnóczky *et al.*, 2003). So it was relevant to determine calcium levels as an indicator of oxidative stress and smooth

endoplasmic reticulum stress. Our results showed a significant increase of basal levels of calcium after irradiation (GI). The baseline values of calcium are associated with various cellular responses such as death by necrosis, apoptosis and cell proliferation (Hirota *et al.*, 1998; Anza *et al.*, 1995).

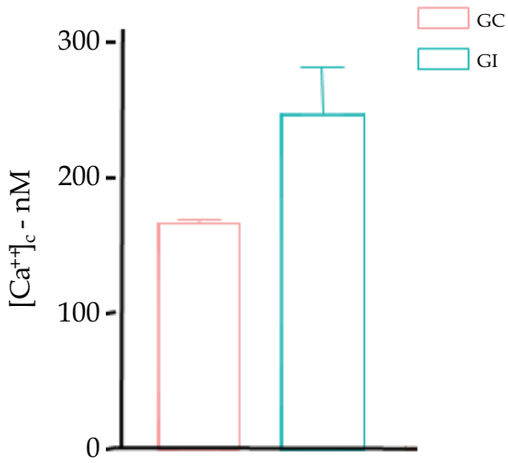
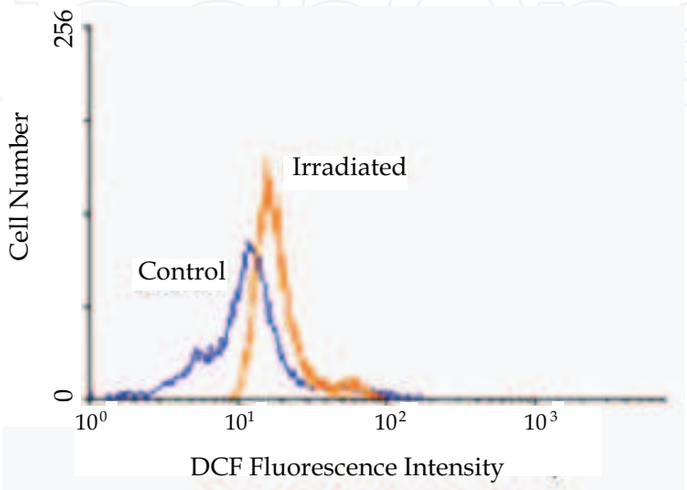


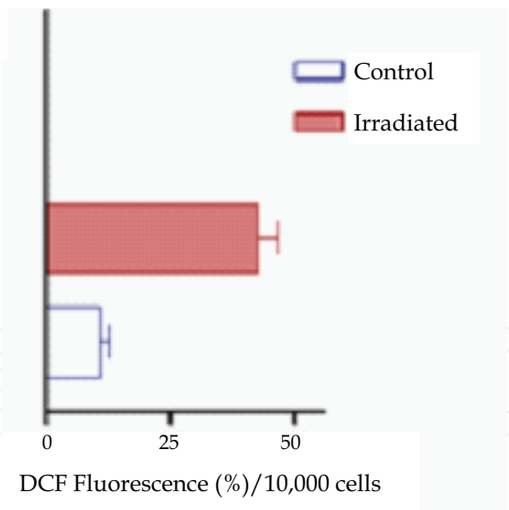

Fig. 8. Effect of γ radiation on basal intracellular $Ca^{2+}$ concentration in mice granulocyte of the control (GC) and irradiated (GI) groups with doses of 700cCy – measured by fura-2 fluorophore. Statistical analysis: Newman Keuls. **$p < 0.05$, N = 4**

Either necrosis or apoptosis is activated depending on the range of the insult, and consequently on the amount of Ca2+ increase. Ca2+ can establish local concentrations within the cell, due to its low rate of diffusion, when compared to other second messengers and on the dynamic sequestration of this ion by several organelles (Rizzuto & Pozzan, 2006). This unique property allows the cell to decipher various signals, triggering different consequences through a single molecule. ER is crucial to regulate calcium concentration and the sensitivity to apoptosis. Ca2+ accumulated in the ER can be released upon apoptotic stimuli coupled to IP3, and being detected by mitochondria. This process is regulated by Bcl-2 family proteins, strategically located at the ER and mitochondria surfaces. Cells that overexpress Bcl-2 showed a considerable decrease in Ca2+ levels within the ER and the Golgi apparatus. Consequently, reduced Ca2+ concentration increases upon stimuli coupled to IP3 generation were detected both in the cytosol and in the mitochondria (Pinton *et al.*, 2000). In cells in which the pro-apoptotic members of Bcl-2 family, Bax and Bak, were deleted, the same effect was observed. On the other hand, Bax and Bak double knockout cells are protected against apoptotic stimuli (Scorrano *et al.*, 2003). In these cells, ER Ca2+ values were restored to control level through silencing of Bcl-2 (Danial & Korsmeyer, 2004). Stimuli that produce cytosolic Ca2+ increase bring out large Ca2+ influxes in the mitochondria matrix, regardless of the low affinity of mitochondrial Ca2+ carriers for this ion. This is explained by the existence of mitochondria-ER contacts, where microdomains of high Ca2+ concentrations are found and trigger fast accumulation of Ca2+ in the matrix (Hayashi *et al.*, 2009). A variety of responses is caused by mitochondria Ca2+ uptake, from stimulation of metabolism (ATP production) - when they are subject to a transient stimuli, to apoptosis - in case of a more persistent or excessive Ca2+ increase. Additionally, mitochondria Ca2+ accumulation triggered by apoptotic stimuli causes swelling and fragmentation, followed by cytochrome c release. The opening of permeability transition pores (PTP) was

also observed upon ceramide-induced apoptosis, which sensitizes mitochondria to the otherwise physiological IP3-mediated Ca2+ signal (Szalai *et al.*, 1999). In which concerns ER calcium concentrations, Bcl-2 family members control this apoptotic pathway. In particular, upon apoptotic stimuli, Bax and Bak localize at the outer mitochondria membrane triggering its permeabilization and release of apoptotic factors in the cytosol (Szalai *et al.*, 1999). Apoptotic cell death may also be caused by mitochondrial malfunction due to ROS-induced mtDNA damage, lipid peroxidation or protein oxidation (Migliaccio *et al.*, 1999).




(a)




(b)

Fig. 9. Measurement of reactive oxygen species (ROS) induced by ionizing radiation in bone marrow cells. The cells were loaded with DCFH-DA (10μM) for 30 minutes. After conversion of the ester to DCFH by intracellular esterases, the number of cells exhibiting increased fluorescence of oxidized DCF was evaluated by flow cytometry. A- Histograms represent four independent experiments in bone marrow cells for groups control and exposed to ionizing radiation. B- Statistical analysis for groups control and irradiated: Anova - Newman Keuls. **$p < 0.05$, N = 4**

ROS cellular levels induced by ionizing radiation were evaluated by measuring DCFH-fluorescence. DCFH is permeable to membrane cell where it is rapidly hydrolyzed by esterases, thus the production of ROS leads to increased DCF fluorescence. We observed an increase of about 50% in DCF fluorescence induced by radiation in bone marrow cells (Figure 9A) in the irradiated group and with flow cytometry we were able to distinguish differences to this type of analysis (Figure 9B).

However, since the caspases are dependent on a reduced thiol group in their active site to cleave their substrates, apoptosis progression is inhibited by excessive ROS, or reactive oxygen species production (Hengartner, 2000). Once more, it should be pointed out, that a pro-oxidant state is induced in mitochondria by CaCl2 through indirect action, since this molecule did not present an intrinsic capacity to induce oxidative stress directly. As previously reported, the redox effects induced by CaCl2 is very likely to be consequence of CaCl2-dependent MPTP induction (Zamzami & Kroemer, 2001).

## 5. Conclusion

There is controversy in the literature regarding the use of antioxidants as inhibitor of apoptotic response. In some systems it is almost certain that free radicals and ROS are produced, and in these cases, antioxidants have been shown to reduce or delay apoptosis. Oxidative stress induced by gamma radiation can induce to clonogenic death or apoptosis. Although physical and chemical agents can promote cytotoxicity or genotoxicity action leading to apoptosis independent of production of free radicals, oxidative stress induced by radiation can, without any doubts, elicit cell death, and mild oxidative stress which in turn initiate apoptosis. ROS can cause oxidative stress even though not being essential for the apoptotic processes. A very interesting hypothesis is that perturbation of cellular redox homeostasis may control these key events. p53 protein is widely regarded as an important sensor of genotoxic damage in cells, and mutations in p53 are the most frequent observed in human cancers. After radiation induced damage, a protein expression such as p53 occur, which takes to p21 transcription, leading to cell cycle arrest in G0/G1 in order to repair the DNA damage, irreversible growth arrest, terminal differentiation, or apoptosis. Major processes resulting from oxidative stress include alteration of metabolic pathways, lipid peroxidation and the loss of intracellular calcium homeostasis. Thus, the lethality of gamma radiation is well known as much as cellular radiosensitivity. The syndrome of acute radiation may occur by failure of the bone marrow, gastrointestinal tract or central nervous system. The management of patients suffering from acute radiation syndromes still remains a major challenge. Survival of radiation induced bone marrow failure depends on the dose of radiation received. At radiation doses of 3 to 8 Gy, morbidity and lethality are primarily caused by hematopoietic injury. Our results suggest that bone marrow is highly susceptible to oxidative damage by the gamma radiation dose of 7 Gy, whose mechanism of action is directly related with changes in the intracellular calcium levels, increasing ROS and cell death apoptosis. It was observed that major processes resulting from oxidative stress include alteration of metabolic pathways, lipid peroxidation and the loss of intracellular calcium homeostasis. We demonstrated that this machinery is activated when apoptosis is induced by radiation. The signaling pathway after p53 activation leads to an increased expression of p21, which consequently affects the profile of the cell cycle and also leads to

cell death by apoptosis. This confirms the hypothesis that perturbation of cellular redox homeostasis may control these key events in the process of cell death by apoptosis. In summary, our work demonstrates that radiation induced apoptosis is increased when p53 or p21 responsive G2 arrest occur, suggesting induction of premature senescence which may represent a novel underlying mechanism for radiation and may be able to contribute to the treatment of cancer by radiotherapy.

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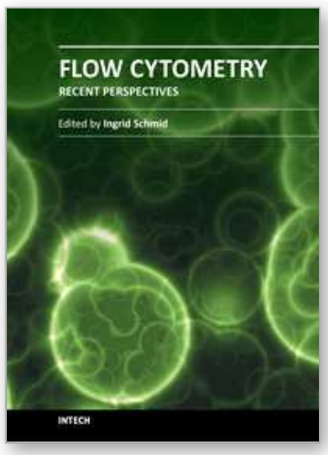


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