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Active caspase-3 expression levels as bioindicator of individual radiosensitivity

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ABSTRACT

Several molecules and events involved in cell response to radiation-induced damage have been investigated towards a personalized radiotherapy. Considering the importance of active caspase-3 in the proteolytic cascade that ensures radiation-induced apoptosis execution, this research was designed to evaluate the expression levels of this protein as a bioindicator of individual radiosensitivity. Peripheral blood samples of 10 healthy individuals were gamma-irradiated (cobalt-60 source) with 1, 2 and 4 Gy (control: non-irradiated samples), and active caspase-3 expression levels were measured in lymphocytes, by flow cytometry, *ex vivo* and after different times of *in vitro* incubation (24, 48 and 72 hours). Short-term incubation of 24 h was the most adequate condition to evidence correlations between dose radiation and active caspase-3 expression. For each radiation dose, it was observed a significant inter-individual variation in active caspase-3 expression intensity, suggesting that this parameter may be suitable for evidence individual radiosensitivity. The methodology presented and discussed in this work may help to predict healthy tissues response to radiation exposure toward the better patient outcome.

Key words: active caspase-3, apoptosis, flow cytometry, ionizing radiation, lymphocytes.

INTRODUCTION

In searching for a biology-based personalized radiation therapy (RT), researchers have been focused on the better understanding of lethal and carcinogenic effects of ionizing radiation (IR), at the molecular, cellular and tissue levels. The contribution of those works on radiological protection is widely known and, more recently, radiation oncologists have shown great interest in such issues, aiming to improve the effectiveness of radiotherapy by protecting healthy tissues (Joubert et al. 2011, Choi and Cho 2016).

The field of major interest and impact in radiobiology is the cellular response to radiation-induced damage in the molecule of deoxyribonucleic acid (DNA), especially in terms of biochemical

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cascades initiated by DNA double-strand breaks (DSB), since this is the main type of damage related to carcinogenic and lethal effects of radiation (Rodemann and Wouters 2011, Selzer and Hebar 2012).

DSB triggers a sophisticated molecular circuit that involves proteins that are hypo- or hyper-regulated in order to execute the cellular response to DNA damage (Daub 2012, Agarwal and Miller 2016). These proteins can release biochemical signals acting on other proteins, commonly associated with progression or inhibition of cell cycle, or to repair system (Ross and Kaina 2013, Lavin et al. 2015). If the damage is not repaired, cells can accumulate mutations, chromosomal aberrations and changes in cell proliferation, or even, undergo cell death (Matt and Hofmann 2016).

Apoptosis is the principal cell death process in response to radiation stress, and it is triggered in order to eliminate seriously damaged cells, on inflammation-free processes (Eriksson and Stigbrand 2010, Maier et al. 2016). This programmed cell death involves a complex series of molecules, localized in various cellular compartments, that mediate the process through either p53-dependent or -independent mechanisms, related to mitochondria (intrinsic pathway) or even to membrane (extrinsic pathway). Regardless the initiation route, all events converge on a common signaling cascade involving a group of proteases called caspases (cysteinyl aspartate proteinases) (Maier et al. 2016, Matt and Hofmann 2016).

Caspases take part of a complex and highly coordinated event leading to biochemical changes, which in turn culminate in morphological and structural features that characterize apoptotic cells (Kuranaga 2010, Savistskaya and Onishchenko, 2015). In this proteolytic cascade, one active caspase activates a procaspase, amplifying the apoptotic signaling pathway to cell death in an apparently irreversible way. The final pathway of apoptosis consists of the execution phase, where caspase-3, -6 and -7 are the main actors (McIlwain et al. 2013, Bolkent et al. 2016).

Caspase-3 has been considered the primary executioner of the apoptotic process. Activated by any initiator caspase (-8, -9 or -10), caspase-3 acts directly cleaving or catalyzing the cleavage of hundreds of cellular protein substrates, mainly related to chromatin condensation and margination, DNA fragmentation and nuclear collapse (Elmore 2007, Palai and Mishra 2015). Indeed, caspase-3 inhibition has been associated with necrotic characteristics, indicating the crucial importance of the activity of this protein on the occurrence of apoptosis after exposure to IR (Coelho et al. 2000, Garcia-Belinchón et al. 2015). Depletion of the others executioner caspases (caspase-6 or -7) has shown minimal impact on the apoptotic process (Slee et al. 2001), and even caspase-7 cleavage seems to be mostly processed by active caspase-3 (Trisciuoglio and Bianchi 2009, Matt and Hofmann 2016).

On radiation cellular response investigation, blood is considered an easy resource to study genotoxicity and to estimate the effects of radiation. Lymphocytes have been the most widely employed model, due to its high radiosensitivity, accessibility and high concentrations in peripheral blood (Cozzarini 2015, Sridharan et al. 2016, Tewari et al. 2016). This kind of cell is particularly interesting for analysis of radiation-induced apoptosis due to its readiness to undergo apoptosis following irradiation (Jella et al. 2013, Ogawa 2016). Thus, the purpose of this research was to investigate radiation-induced apoptosis in quiescent (G0) human lymphocytes by flow cytometric measurement of active form caspase-3 expression, aiming to evaluate the expression levels of this protein as a bioindicator of individual radiosensitivity. To define the adequate incubation condition to perform such analyses, the active caspase-3 expression was analyzed in non-mitogen-stimulated lymphocytes, *ex vivo* and at different *in vitro* incubation time points.

MATERIALS AND METHODS

STUDY POPULATION AND ETHICAL ASPECTS

The present study was conducted with 10 healthy volunteers, 6 women and 4 men aged 19 to 44 years (mean 28.8 years). None of them underwent medical procedures involving IR or mutagenic chemicals, at least three months before this study. This research complies with the Declaration of Helsinki and was performed following recommendations of the Committee of Ethics in Research involving Humans at the Health Sciences Center of the Federal University of Pernambuco (n. 297/09). Informed consent form was obtained from all participants before sample collection and use of questionnaire data.

BLOOD SAMPLES AND IRRADIATION SETUP

From each volunteer, a sample of venous peripheral blood was collected in heparinized tubes. The total sample was divided into four aliquots: one was kept as non-irradiated control, while the three others were separately gamma-irradiated in a cobalt-60 source (GammaCell-220, Radionics Laboratory, USA - dose rate: 3.7 Gy/h), receiving respectively 1, 2 and 4 grays (Gy).

ISOLATION AND INCUBATION OF LYMPHOCYTES

Following the standard method recommended by the manufacturer of Ficoll Paque density gradient media (GE Healthcare, USA), each fresh blood aliquot was diluted in an equal volume of phosphate-buffered saline (PBS, pH 7.2-7.4), carefully layered over Ficoll gradient (diluted blood:ficoll, 3:1) and centrifuged at 400 x g, for 35 min. Mononuclear cells layer was recovered and washed with PBS, and then centrifuged (250 x g, 10 min). The final pellet was resuspended in Roswell Park Memorial Institute 1640 medium (RPMI-1640 medium) (Cultilab, Brazil) supplemented with 10 % heat-inactivated fetal bovine serum (Cultilab, Brazil) and cell viability was assessed by trypan blue exclusion test (Sigma-Aldrich, USA).

For *ex vivo* analyses, an aliquot of the cell suspension was directly processed for flow cytometry while for *in vitro* analysis, cells were seeded into flat-bottom 96-well plates with supplemented RPMI media (like described above) and incubated at 37 °C in a humidified 5 % CO_2 atmosphere, for 24, 48 and 72 hours (h). This procedure has been presented previously elsewhere (Santos et al. 2013).

FLOW CYTOMETRY

Intracellular staining

Active caspase-3 staining protocol was performed following manufacturer's instructions (PE active caspase-3 apoptosis kit, BD Pharmingen). Harvested cells were transferred into 5 mL polystyrene tubes (6 x 10^5 cells/tube) and the cell culture medium was removed by centrifugation (300 x g, 5 min). Cells were washed once with ice-cold PBS (300 x g, 5 min, for solution removal), and then fixed and permeabilized with buffer containing paraformaldehyde 4 % and saponin 0.1 % (BD Cytofix/Cytoperm, USA) during 20 min, on ice. After buffer removal (400 x g, 5 min), cells were washed twice with a solution containing saponin, fetal bovine serum and sodium azide (BD Perm/Wash 1X, USA – referred here as permeabilizing/washing solution), centrifugating at 400 x g, 5 min. Then, cells were resuspended in this same solution for labeling with monoclonal antibodies.

Non-irradiated and irradiated cells were labeled with anti-active caspase-3-phycoerythrin (PE) (BD Pharmingen, USA, clone C92-605). For initial setup of cytometer and detection of unspecific fluorescence, additional aliquots of non-irradiated cells were maintained without labeling and labeled with anti-immunoglobulin G (IgG)-PE (BD Phosflow, USA), respectively. Incubation was performed in the dark, for 30 min, and antibody

excess was removed by washing of cells with permeabilizing/washing solution, followed by centrifugation (400 x g, 5 min). Finally, cells were resuspended in this same solution, maintained at 4 °C until reading.

Acquisition and analysis of data

Data acquisition was performed on Gallios flow cytometer (Beckman Coulter) equipped with a blue laser (488 nm), using software specific for this device (Gallios Software, Beckman Coulter). Equipment acquisition setup: forward scatter (FSC) and side scatter (SSC), with linear amplification and FSC threshold set; and yellow-orange fluorescence (FL2), with logarithmic amplification. At least, 50,000 events were acquired from each sample.

Data files were analyzed with Kaluza software (Beckman Coulter), using two-dimensional density plots and histogram. Lymphocytes were identified based on FSC and SSC parameters, and total protein expression was measured in percentage values, from this cell population.

STATISTICAL ANALYSIS

Analyses of active caspase-3 expression data were performed using BioEstat version 5.3 software (Miramauá Institute). All data were expressed as mean ± standard deviation (SD). Differences between means were evaluated by one-way analysis of variance (ANOVA) with post-hoc Student's T-test. Statistically significant differences were accepted for calculated probability $p < 0.05$.

RESULTS

The protein-based assays have been developed to characterize radiation-induced cellular response, and to analyze the potential of such proteins as biomarkers of exposure, effect or susceptibility to IR (Bennett and Waters 2000, Kim et al. 2013). In the present work, the expression of active caspase-3 was evaluated for characterization of radiation-induced apoptotic cell death and then to analyze possibilities of employment on practices benefited by advances on radiobiology, particularly to a personalized RT.

Blood aliquots were separately exposed to 1, 2 and 4 Gy, which are dose levels with therapeutic relevance. Although conventional radiotherapy protocols employ 2 Gy daily fractions, new schedules aiming to preserve normal tissues or overcome tumoral repopulation may involve hyper- or hypo-fractionation, in order to deliver smaller or higher doses per fraction, respectively (Marcu 2010, Deloch et al. 2016).

Regarding post-radiation exposure, the expression level of active caspase-3 was analyzed throughout the time range of 0-72 h, over 24 h intervals.

MULTIPARAMETRIC ANALYSES FOR LYMPHOCYTE IDENTIFICATION AND CASPASE-3 EXPRESSION

Figure 1 shows two representative graphics obtained from flow cytometric analyses of 24 h incubated samples from one individual. The 2D-density plot (Figure 1-a) presents the mononuclear cells, with the lymphocyte population delimited on gate – differentiation between lymphocyte and monocyte populations was based on correlation of FSC and SSC parameters (that indicate cell size and internal complexity, respectively). The histogram (Figure 1-b) gathers data about active caspase-3 expression levels from control (green line), 1 Gy (orange line), 2 Gy (blue line) and 4 Gy-irradiated samples (red line).

TIME COURSE ANALYSIS OF RADIATION-INDUCED CASPASE-3 EXPRESSION

Figure 2 presents the results of active caspase-3 mean expression levels for control (non-irradiated) and irradiated cells under *ex vivo* and *in vitro* conditions.

In the *ex vivo* analyses (immediately after irradiation), regarding active caspase-3 expression,

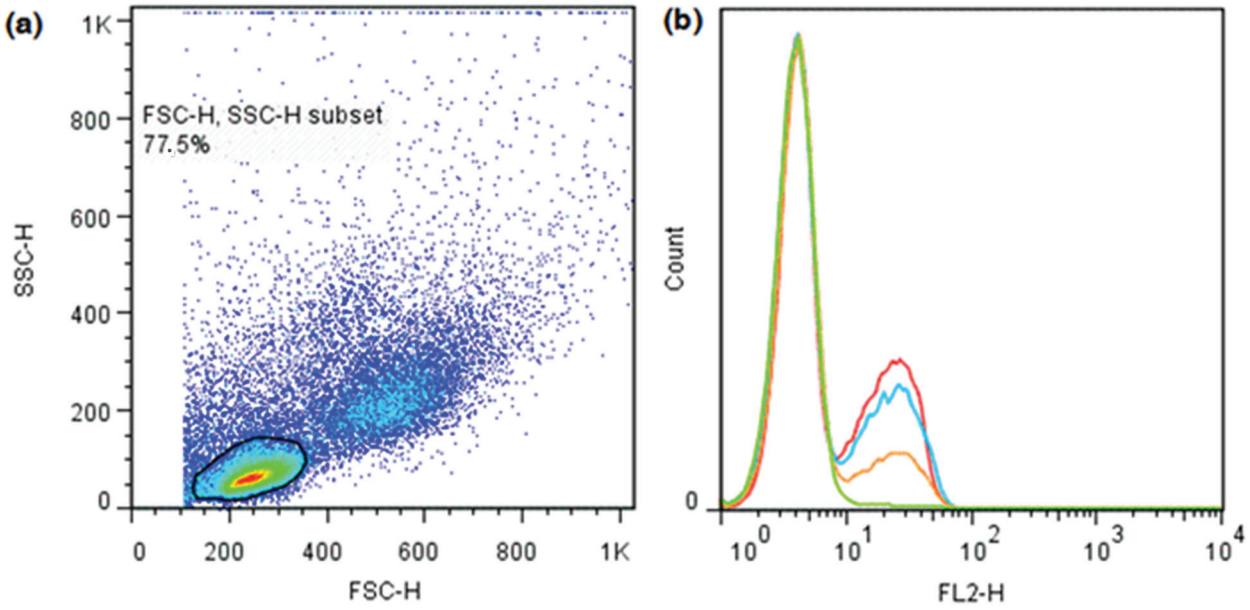

Figure 1 - (**a**) FSC/SSC 2D-density plot, with gate drawn around the lymphocyte population (**b**) FL2 histogram corresponding to active caspase-3 expression from gated lymphocytes, gathering data of non-irradiated (green), 1 Gy- (orange), 2 Gy- (blue) and 4 Gy-irradiated samples (red). The first peak (lower 10^1) represents cells negative for active caspase-3 expression, and the second peak (higher 10^1), those positive to this parameter. The second peak height indicates positivity intensity, in a way that non-irradiated lymphocytes (green) were primarily negative for the presence of active caspase-3, whereas irradiated lymphocytes were positive for active caspase-3 staining, increasing for higher doses.

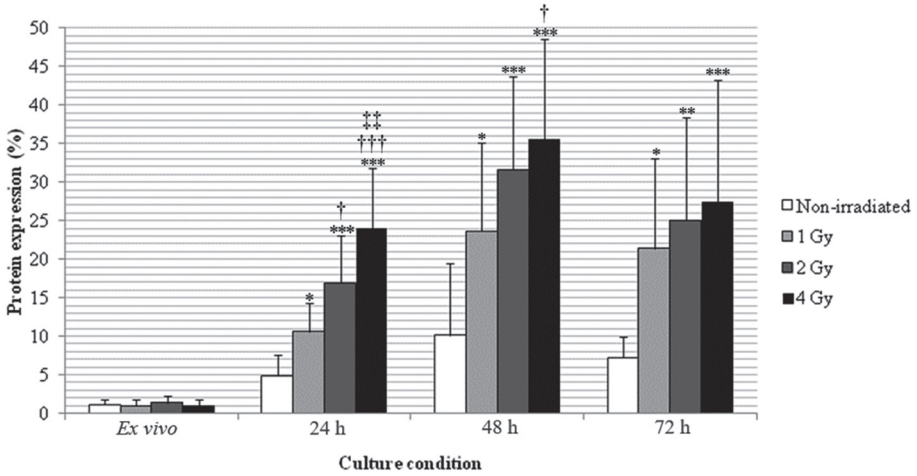

Figure 2 - Expression level of active caspase-3 on lymphocytes through time, for ex vivo or in vitro protocols. Bars show mean ± SD. Symbols: *, for comparison with control group, † for comparison with 1 Gy-irradiated, and ‡ for comparison with 2 Gy-irradiated. One symbol: $p \leq 0.05$; two symbols: $p \leq 0.01$; three symbols: $p \leq 0.001$.

irradiated cells could not be distinguished from non-irradiated ones. However, when these cells were incubated *in vitro*, it was observed a significant increase in the expression level of this protein in non-irradiated cells ($p < 0.01$), for all analyzed time points. The active caspase-3 expression became higher after radiation exposure ($p < 0.0001$).

For the 24 h time point, it was possible to distinguish non-irradiated cells from irradiated ones, for all evaluated dose levels. Additionally, the

active caspase-3 expression allowed differentiating among the three evaluated dose levels.

Considering the lymphocytes incubated for 48 h, the results presented significant differences in active caspase-3 expressions for non-irradiated cells when compared with those irradiated. However, considering the irradiated cells, significant differences were only observed between the samples irradiated with 1 Gy and 4 Gy.

After 72 h incubation, significant differences of active caspase-3 expression were found only in terms of non-irradiated and irradiated cells.

INDIVIDUAL VARIATION

Considering all *in vitro* analyses, a high standard deviation was observed, indicating that, beyond to dose-response relations, there is a wide difference among individuals on the magnitude of active caspase-3 expression levels ($p < 0.001$). To illustrate this interindividual evidence, Figure 3 presents data dispersion for the 24 h time point, which was the time point where the results allow a better fit between active caspase-3 expression and radiation doses.

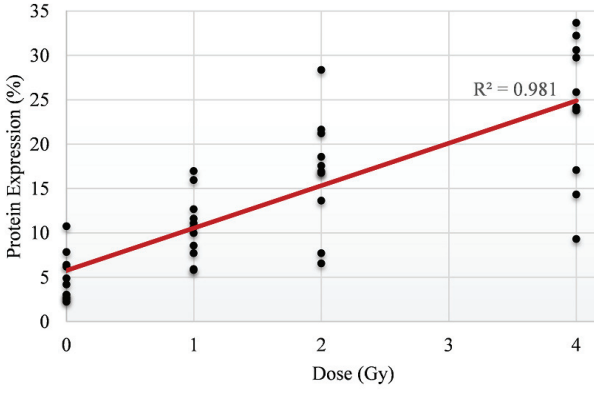

Figure 3 - Dispersion of active caspase-3 expression for lymphocytes incubated for 24 h. Black dots symbolize individual data for each radiation dose (n = 10), and red dots, mean values. The red line represents the linear trendline, displayed with coefficient of determination (R^2).

The trendline evidences positive linear relationship between the expression of active caspase-3 and radiation doses. However, a significant spread out of individual data occurred around mean values ($p < 0.001$), particularly at the higher radiation doses (2 and 4 Gy).

A better visualization of the radiation-induced response by each individual for 24 h-analysis is shown in Figure 4.

DISCUSSION

Active caspase-3 expression levels were measured from lymphocytes incubated during 24 up to 72 h without phytohemagglutinin (PHA) stimulation, to avoid negative influence of this mitogenic stimulus on expression of the protein at issue. Ulsh (2010) defends that suppression of apoptosis is an effect of PHA, and this evidence is supported by the research of Kornacker et al. 2001, where following 48-96 hours of cell culture with mitogens like PHA occurs upregulation of survivin, a member of the inhibitors of apoptosis protein family that binds (and inhibits) the terminal effectors of caspase-3 and -7.

The significant increase of basal expression of active caspase-3 on cultures without PHA indicates that the *in vitro* incubation itself contributes to enhance active caspase-3 expression levels in lymphocytes. This evidence may support two possibilities: (i) the lymphocytes were not able to adapt to *in vitro* incubation conditions and triggered cell death by apoptosis – process in which the role of active caspase-3 is indispensable (Palai and Mishra 2015); (ii) active caspase-3 participates on cell death-unrelated processes.

About this second possibility, Galluzzi et al. 2008 defend that any protein specifically implicated in apoptosis might have a phylogenetically conserved apoptosis-unrelated function, probably as part of an adaptive response to cellular stress. About this issue, Shalini et al. 2015 address multiple functions of caspases outside apoptosis.

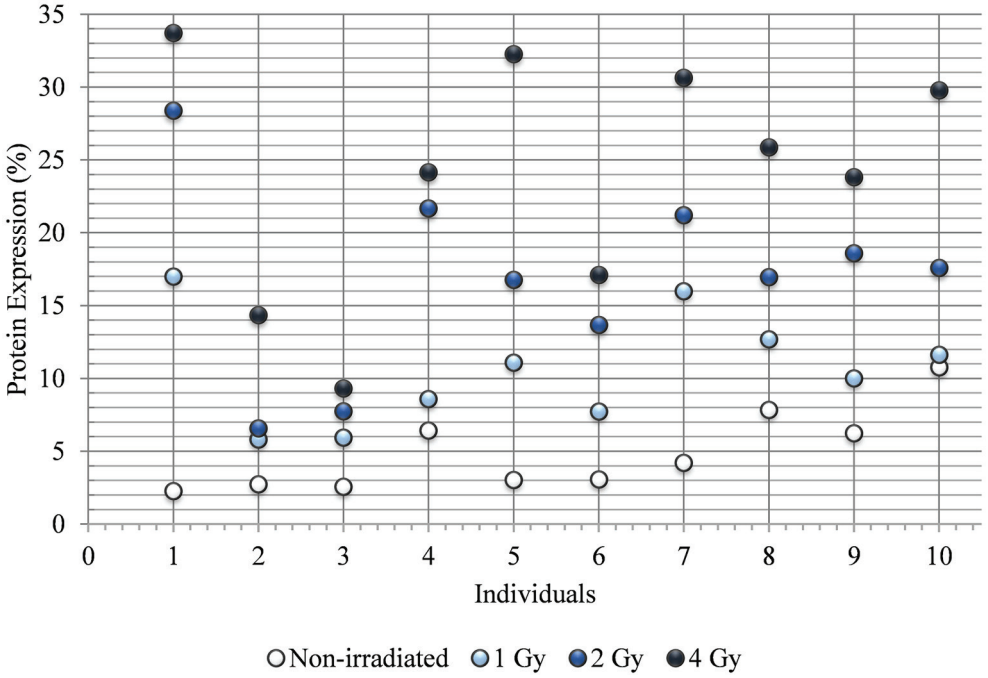

Figure 4 - Individual active caspase-3 expression on lymphocytes incubated for 24 h, for non-irradiated and 1 Gy-, 2 Gy- and 4 Gy-irradiated cells.

As cell culture itself imposes a state of oxidative stress on cells (Halliwell 2003, 2014), this relevant increasing on basal expression levels of active caspase-3 may be a response to this condition, but its activation does not lead to death. It remains unclear how this caspase-3 activation does not lead to cell death - however, it has been proposed that, in parallel to caspase activation, cells may express antiapoptotic molecules (e.g. inhibitors of apoptosis protein family) or execute mechanisms like Ras GTPase activating protein (RasGAP) cleavage (Yang et al. 2004) and be able to tolerate low caspase activity in response to mild stresses (Launay et al. 2005, Khalil et al. 2012).

Radiation doses were chosen based on the context of radiation oncology as well as for radioprotection. The dose levels employed in this work can be associated to accidental overexposures (Pinto et al. 2010) as well as doses administered per session in conventional radiotherapy schedules (2-Gy fraction) and altered regimens (< 2 Gy and > 2 Gy, for hyperfractionated and hypofractionated radiotherapy schedules, respectively) (Marcu 2010, Deloch et al. 2016).

Ex vivo analyses did not evidence any significant alteration of active caspase-3 levels by irradiation and so does not permit any inference about radiation-induced apoptosis on G0-lymphocytes. Changes on expression levels of active caspase-3 were only observed in lymphocytes incubated for 24 hours minimum. This evidence is in accordance with other works, which assert that the apoptotic event occurs between 24 hours and 1 week after irradiation in gamma-irradiated human G0-lymphocytes exposed to the same dose levels here employed (Torudd et al. 2005, Belyaev 2010). Although most of the works investigate apoptosis by other biological endpoints, such as surface exposure of phosphatidylserine and cytoplasmic membrane permeability, they also did not recommend *ex vivo* or short-time analysis (e.g. 6 hours) for such purpose (Vokurková et al. 2006, Tavakoli et al. 2015). On our case, *ex vivo* analyses were employed as a control for *in vitro* conditions.

For *in vitro* conditions, active caspase-3 expression allowed differentiation between non-irradiated and irradiated groups, for all doses (1, 2 and 4 Gy), indicating a close relation between active caspase-3 and radiation-induced response of lymphocytes. Incubation during 24 hours was considered the better condition to visualize this relation, since just at this time point was possible to distinguish each dose within the dose range, evidencing the influence of IR exposure (Figure 2), with a positive trend (Figure 3). For 48 h-incubation samples, it was not possible to distinguish between doses closer within the range (1 Gy from 2 Gy; 2 Gy from 4 Gy). For 72 h-incubated cells, no statically significant difference of active caspase-3 expression levels was found comparing any irradiated samples.

Similar researches indicate that human G0-lymphocytes present a general trend to saturation of apoptosis-related dose response following gamma-ray irradiation and incubation at 48 and 72 hours. Belyaev 2010 emphasized that this behavior seems to be a trend of this kind of cell rather than a peculiarity of a specific time point or specific end-point. After 24 hours, the plateau has been observed from 5 up 10 Gy (Vral et al. 1998, Torudd et al. 2005). Data of the present work confirm this evidence and reinforces incubation during 24 h as the better condition to evaluate radiation-induced apoptosis on gamma-irradiated G0 lymphocytes for dose range up to 4 Gy, based on the expression of active caspase-3.

Concerning the dose-dependent kinetic, apoptosis in peripheral blood lymphocytes has been suggested as a potential biological dosimeter. Most of these studies are based on the morphological appearance of the lethal process, like fragmentation and condensation of DNA or superficial phosphatidylserine exposure/cytoplasmic membrane permeability (Boreham et al. 1996, Menz et al. 1997, Vokurková et al. 2006, Faraj et al. 2010). Unlike them, the present study extends apoptosis evaluation to an enzymological criterion, employing the same analytical tool, namely flow cytometry. The flow cytometry has emerged as a powerful analytical tool due to its rapidity and high sensitivity during analysis of a large number of cells (Adan et al. 2016). On the measurement of active caspase-3 expression realized on the present work, we confirm the high efficacy of this technology on the determination of protein expression levels at few minutes after sample acquisition.

Focusing on one day-time post-irradiation analyses, it was identified that, despite all individuals had evidenced the influence of IR on active caspase-3 expression, individual results presented a high dispersion around mean values, more evident at higher doses (Figure 2). Analyzing separately each subject, differences on the magnitude of protein expression in response to the same dose levels are clearly significant (Figure 3), suggesting that the response (active caspase-3 expression) to radiation-induced damage is dose-dependent, but varies significantly from person to person and so is determined by individual characteristics. This inter-individual variance has been related by several researches (Erasmus et al. 2005, Schnarr et al. 2007, Pinar et al. 2010).

The research line that defends the use of apoptosis assay as a rapid screening test for clinical prediction of late normal tissue toxicity following radiotherapy is still stronger than those indicating its employment for biodosimetric evaluations (Tavakoli et al. 2015). Effects on normal tissue often limiting clinical use of radiation involves delayed cycles of inflammation, tissue dysfunction, atrophy, and/or fibrotic remodeling (Panganiban et al. 2013). Studies point out that low apoptotic response is associated with risk for development of late side effects, which occur around 6 months to many years after exposure and, thus, demanding follow-up relatively long. Besides this, this kind of effect can be irreversible (Erasmus et al. 2005,

Ozsahin et al. 2005, Bordón et al. 2009, Schnarr et al. 2009, Pinar et al. 2010).

The identification of radiation-sensitive group may orientate alterations on a therapeutic protocol that would involve reduction of radiotherapy dose or adoption of alternative therapy (Barnett et al. 2009, Marcu 2010, Deloch et al. 2016). Applying these evidence to normal subjects group analyzed in this work, individuals 2 and 3 may require a differentiate care during situations involving radiation exposure. Both exhibited low levels of radiation-induced expression of active caspase-3 that may indicate a higher risk for late side effects after a radiotherapy session, for example.

Concerning radiation protection management, works of Schnarr et al. 2007 and Cavalcanti et al. 2015 have pointed out the individual radiosensitivity as an important parameter to professionals and public at large. Today, dose limits are set to ensure exposure at safe levels, assuming that people have similar responses to IR. However, accepting that higher individual sensitivity increases the probability of development of long-term side effects of radiation exposure, maybe radiation protection regulations would consider dose limits tailored for such people.

On the other hand, there are some evidence of the unsuitability of such analysis to predict individual clinical radiosensitivity (Barber et al. 2000, Greve et al. 2009), indicating the need for further studies, particularly clinical trials, to well-establish the aspects here observed. Validation of tests for early detection of exposure and prediction of individual radiosensitivity may be hereafter implemented in fields benefited by radiobiological advances, like medical and occupational practices and accidental exposures.

CONCLUSIONS

Our work contributes with a methodology for measuring active caspase-3 expression levels in human lymphocytes, stressing that apoptosis-related events require a minimal time to be triggered after gamma-radiation exposure of normal resting (G0) human peripheral blood lymphocytes. Short-term incubation of 24 h appeared as the more suitable condition to investigate apoptosis induced by high-dose IR exposure based on active caspase-3 analyses, providing insights into general behavior and individual response. Thus, the proposed assay could be used as a tool for detecting differences in radiation sensitivity of patients before they undergo radiotherapy, in order to adjust the dose regimen to the patient response or even to prescribe an alternative therapy. Naturally, further studies are needed with a larger number of individuals to define the interval limits representative of a population for the same set of doses employed in this work.

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