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Caspase-3 activation and increased procollagen type I in irradiated hearts

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ABSTRACT

The caspase-3-cleaved presence was evaluated in this study in the heart of irradiated rats, during the decline of ventricular function. Female Wistar rats were irradiated with a single dose of radiation (15 Gy) delivered directly to the heart and the molecular, histological and physiological evaluations were performed at thirteen months post-irradiation. The expressions of procollagen type I, TGF-ß1 and caspase-3-cleaved were analyzed using Western blotting. Cardiac structural and functional alterations were investigated by echocardiography and electron microscopy. In the irradiated group, the levels of procollagen type I, TGF-ß1 and caspase-3-cleaved are increased. Significant histological changes (degeneration of heart tissue and collagen deposition) and functional (reduced ejection fraction) were observed. Data suggest that the cardiac function decline after exposure to ionizing radiation is related, in part, to increased collagen and increased caspase-3-cleaved.

Key words: apoptosis, caspase-3, fibrosis, heart, ionizing radiation, TGF-β1.

INTRODUCTION

Technological advancement in treatment of cancer combined with early detection and diagnosis have improved the survival of patients suffering from various types of cancers. And this also increases the chance of patients developing cancer treatment side effects (Giordano et al. 2005, Stewart et al. 2010, Offersen et al. 2011). Radiation therapy for breast cancer and Hodgkin`s lymphoma is associated with increased risk of heart disease and myocardial

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infarction. In clinical treatments part of the heart may be irradiated (Baker et al. 2009). Among other problems a diffuse myocardial fibrosis can be observed. It consists of diffuse proliferation of separating bands of collagen and/or replacing myocytes (Darby et al. 2010). Experimental studies show that this condition results in damage to the myocardial capillaries endothelium, causing a decrease in capillary density and can lead to fibrosis (Fajardo and Stewart 1973). If extensive, myocardial fibrosis may lead to congestive heart failure (Darby et al. 2010). An imbalance between vascular homeostasis and repair pathways, mediated in part by aberrant signaling by TGF-β1, has been implicated in this process (Stewart et al. 2010).

Loss of myocytes is a feature of the cardiomyopathic process that contributes to progressive decline in left ventricular function and congestive heart failure (Whelan et al. 2010). Studies have proposed that myocyte loss in cardiomyopathy can occur by apoptosis (Whelan et al. 2010). Apoptosis or programmed cell death is a highly regulated process, and as a final result of this gene regulation, it can be observed the chromatin condensation, cell shrinkage, nuclear and cellular fragmentation, ending with apoptotic bodies' formations, which are subsequently phagocytosed (Goldspink et al. 2003).

Apoptosis may be the consequence of prolonged growth stimulation of adult myocytes, which presents a limited capacity of division. Growth stimulation initially occurs as a compensatory effort to meet chronically altered hemodynamic demands on the failing myocardium and is mediated by systemic and/or local up-regulation of mediators of adrenergic or rennin-angiotensin axes (Goldspink et al. 2003, Schlüter and Wenzel 2008, Whelan et al. 2010). Similarly, certain cytokines, like TGF-β1 can induce growth as well as apoptosis (Schröder et al. 2006). The induction of apoptosis as a form of cell death distinct from necrosis is associated with activation of aspartate-specifc cysteine proteases such as caspase-3 (Goldspink et al. 2003).

Cardiomyocyte apoptosis plays an important role in the progression of many cardiovascular disorders including heart failure. However, there is little information about apoptosis involvement in the evolution of radiation induced heart disease (RIHD). Thus, the main aim of this study is to investigate the involvement of caspase-3 activation in RIHD, and the cardiac tissue ultrastructural analysis.

MATERIALS AND METHODS

Animals and Irradiation

Female Wistar rats (n=14) were obtained at three months of age and weighing approximately 250 g. Rats were maintained on a 12-h light/dark cycle with food and water provided *ad libitum*. Animals were distributed into two groups (7 per group): control group and irradiated group (a single dose of irradiation of 15 Gy). Euthanasia was performed thirteen months post-irradiation. Before irradiation, animals were anesthetized with ketamine/xylazine (0.1 mg/kg) intraperitoneally injected (ip). Single dose of 15 Gy was calculated to be approximately equivalent to total fractionated doses of 30–50 Gy (2 Gy per fraction), according to the LQ model and an a/b ratio of 2:3 as described by Schultz-Hector (Schultz-Hector et al. 1992). Cardiac-specific irradiation was performed using a Varian Clinac 2100 C linear accelerator (Variant Medical Systems, CA, USA) with a 6 MV X-ray, and a dose rate of 240 centi-Gy/min. Individual rats were irradiated in the supine position using an anterior field size of 2x2 cm2 with bolus depth of 0.5 cm. The heart position was marked after CT simulation (HiSpeed CT/ Dual, GE Healthcare, USA). The study protocol was approved by the local ethical council (Universidade do Estado do Rio de Janeiro, UERJ, Brazil).

Echocardiography

Thirteen months after irradiation, the groups were analyzed by echocardiography under ketamine/xylazine anesthesia (0.1 mg/kg, i.p.).

Echocardiographic analyses were performed using a two-dimensional echo Doppler cardiogram (Esaote, model CarisPlus, Firenze, Italy) equipped with a 10 MHz transducer. Measures included septum wall thickness, posterior wall thickness, left ventricle (LV) internal diameters and ejection fraction (EF). LV cardiac output was calculated by the following equation: Stroke volume (determined from aortic integral flow velocity and aortic valve area) x heart rate (HR). HR was obtained indirectly in M-Mode by measuring the interval between the systolic LV contraction waves at the level of the papillary muscles in the transverse axis. The parameters cited were obtained in accordance with the American Society of Echocardiography (Lang et al. 2005).

Western Blotting

To extract total protein, LV samples were washed in phosphate buffer solution before homogenization in lysis buffer (1 mM $NaHCO_3$ pH 8.0, 1 mM phenylmethanesulfonyl fluoride, 1 mM ethylene glycol tetraacetic acid, 4% nonyl phenoxyl polyethoxy ethanol-40, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 2 μg/mL aprotinin and 3 mM Na_3VO_4). Lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8% or 15%) and subsequently transferred to nitrocellulose membranes. All chemicals were purchased from Sigma Aldrich (St Louis, MO, USA). The primary antibodies for proc type I (Santa Cruz Biotechnology, CA, USA), TGF-β1 (Santa Cruz Biotechnology), pro-caspase-3 (Santa Cruz Biotechnology, CA, USA) and α-tubulin (Clone b-5-1-1 mouse) (Sigma Aldrich) were in dilutions of 1:50, 1:500, 1:500 and 1:5,000, respectively. The α-tubulin was used as a protein loading control. The proteins were detected using the Luminol system (Santa Cruz Biotechnology). Band densitometry was obtained by the ImageJ 1.45 software. Proc type I, TGF-β1 and pro-caspase-3 expression were calculated relative to α-tubulin expression in order to rule out the effects of divergent protein loading.

Electron Microscopy

The left ventricle heart tissue of the animals at 13 months (controls and irradiated with 15Gy) was processed and analyzed qualitatively with the use of transmission electron microscope Zeiss EM10C. The heart muscle fragments were washed in PBS and fixed for 1 hour in a solution containing 2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M, pH 7.2 plus 3.5% sucrose. Then, the samples were washed for 10 min in the same buffer. This washing step was repeated 3 times. The material was post fixed for 1h with a 1% osmium tetroxide (OsO4) solution, in sodium cacodylate buffer 0.1 M, pH 7.2 plus 3.5% sucrose; dehydrated in acetone series (30, 50, 70, 90 and 100%) and embedded in Poly/Bed® 812 resin (Ted Pella, Inc). After polymerization, ultrathin sections were obtained and contrasted with uranyl acetate-lead citrate for ultrastructural observation.

Statistical Analysis

Echocardiogram and Western blotting parameters were compared using the Student's unpaired t-test. In all analyses, P values < 0.05 were considered statistically significant. All results are expressed as mean ± standard deviation (SD).

RESULTS

Alterations in the Heart Function of Rats Submitted to Irradiation.

At thirteen months after irradiation, 15 Gy group showed reduced septum wall thickness and increased LV diameter (systole) in relation to control group (Table I). Functional analysis also revealed decrease in the EF, heart rate and cardiac output, featuring a picture of decompensated heart failure.

Cardiac TGF-β1 and Proc Type I Proteins Were Increased in Rats Submitted to Irradiation.

Molecular alterations of TGF-β1 and proc type I proteins were evaluated by western blotting in

TABLE I
Effect of single dose irradiation (15 Gy) on cardiac examinations using echocardiography.

Parameters	Control group 13m (*n*=7)	Irradiated group 15Gy 13m (*n*=7)	p value
LV septum diastolic thickness (cm)	0.15±0.03	0.17±0.04	0.3033
LV posterior wall diastolic thickness (cm)	0.16±0.02	0.19±0.01	0.1131
LV end-diastolic diameter (cm)	0.47±0.07	0.50±0.06	0.2035
LV septum systolic thickness (cm)	0.28±0.05	0.23±0.05*	0.0490
LV posterior wall systolic thickness (cm)	0.27±0.03	0.28±0.04	0.6569
LV end-systolic diameter (cm)	0.18±0.04	0.23±0.05*	0.0250
Ejection fraction (%)	93±3.0	84±9*	0.0294
Heart rate (beats/min)	419±30	375±35***	0.0001
Cardiac output (L/min)	0.10±0.03	0.05±0.02***	0.0001

Values are mean ± SD. LV: left ventricle; 13m: 13th month. Each point represents the mean of seven animals. Statistical significance was accepted when $p < 0.05$ (*), using student's t-test.

irradiated heart tissue. As shown in Figure 1A and 1B, proc type I and TGF-β1 proteins expressions in the 15 Gy group were significantly increased compared to the control group.

Pro- Caspase-3 Levels Decrease in Rats Submitted to Irradiation.

The results demonstrated that the irradiated group showed reduced levels of protein pro-caspase-3 and concomitant increase in protein cleaved-caspase-3 (Figure 2). These results seem to indicate activation of this protein in the progression of heart failure caused by irradiation.

Morphological Alterations in the Cardiac Tissue After Irradiation.

In control group (Figure 3A), it is possible to note the presence of myofibrils parallel to each other

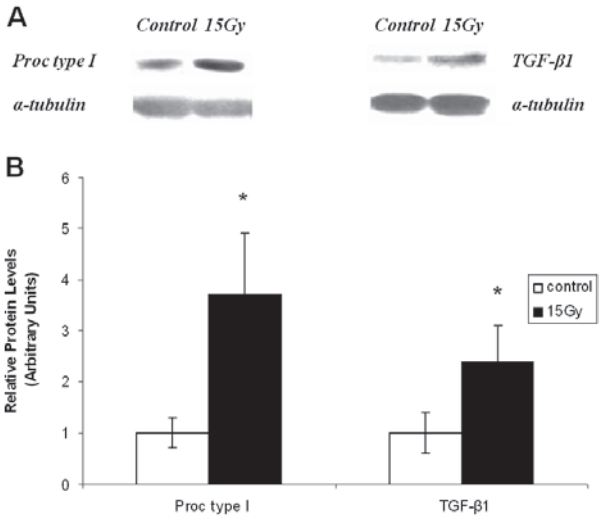

Fig. 1 - Difference in expression of the proc type I and TGF-β1 proteins in the LV.
Panel A, representative Western blotting. of proc type I and TGF-β1. Panel B, densitometric analyses of the proc type I and TGF-β1 blots. White bars represent control and black bars represent irradiated group. *$p < 0.05$.

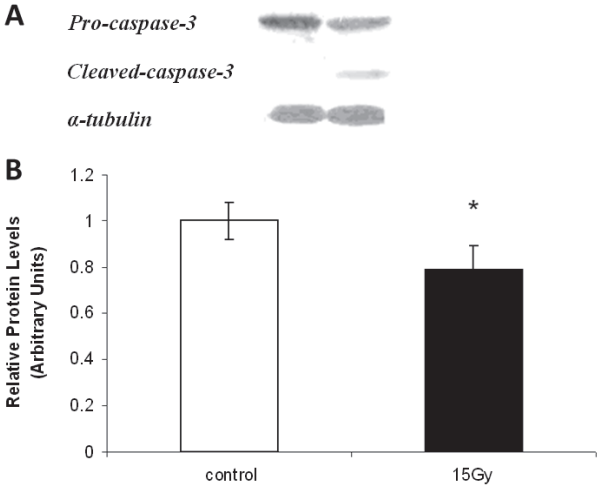

Fig. 2 - Difference in expression of the pro-caspase-3 proteins in the LV. **Panel A**, representative Western blotting. of pro-caspase-3. **Panel B**, densitometric analyses of the pro-caspase-3 blot. White bar represent control and black bar represent irradiated group. *$p < 0.05$.

and their preserved sarcomeres. The myofibrils alternating with the mitochondria can also be seen, a feature common in striated cardiac tissue. Also in control group (Figure 3B), the mitochondria electron-dense matrix and its external and internal membrane

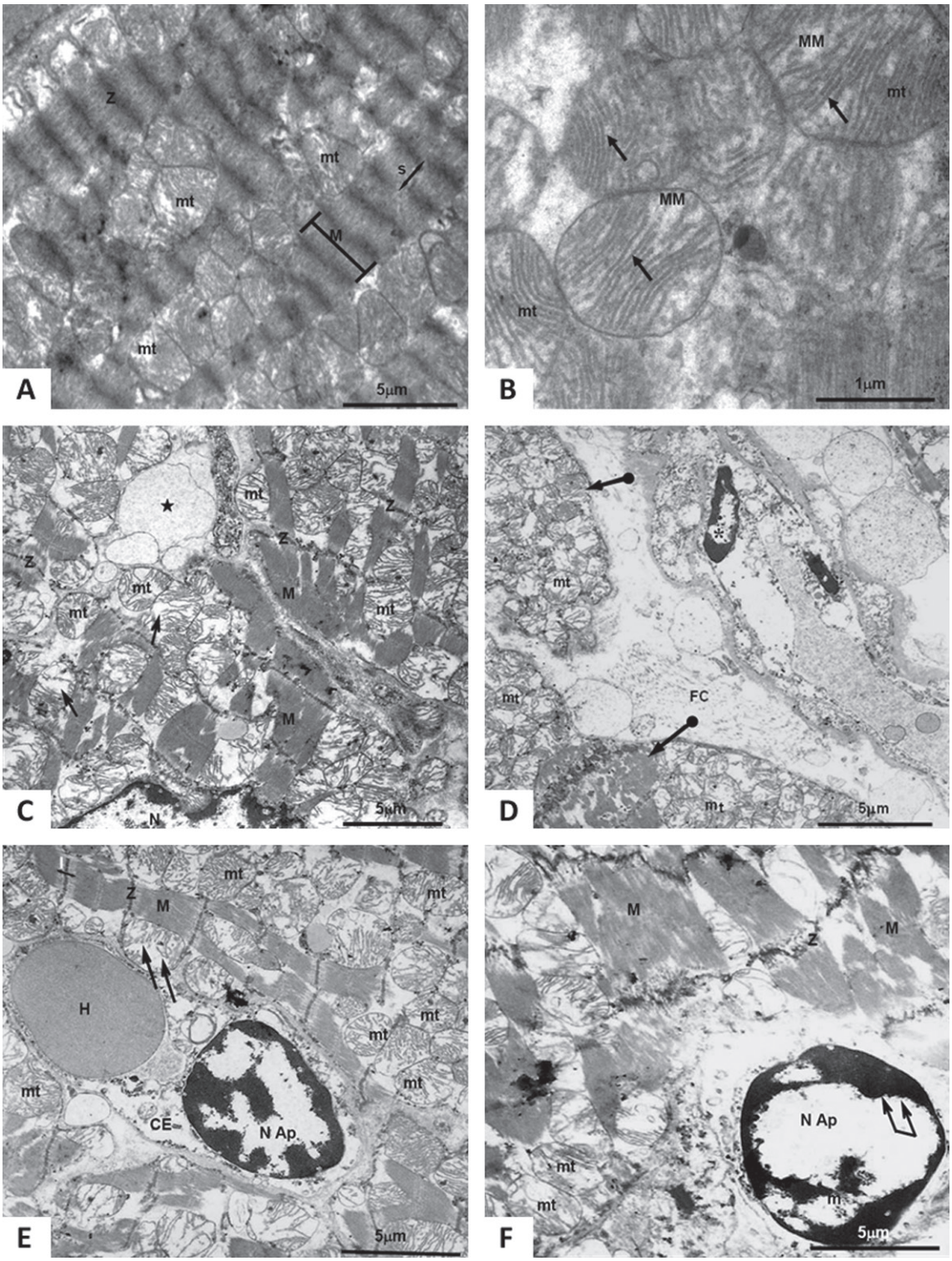

Fig. 3 - Transmission electron microscopy. **(A)** and **(B)** Control group; **(C)**, **(D)**, **(E)** and **(F)** Irradiated group.
mt: mitochondria, s: sarcomeres, one arrow: cristae, MM: mitochondrial matrix, M: myofibrils, asterisk: vacuoles containing cytoplasmic material, N: nucleus, arrow with end ball: cardiac muscle fibers, FC: collagen fibers, H: erythrocytes, Nap: apoptotic nuclei, CE: endothelial cell, double arrow: highly condensed chromatin.

(cristae mitochondrial) are intact. In the irradiated group, ultra structural changes of heart tissues are evident. Figure 3C demonstrates the existence of the extracellular matrix between the cardiomyocytes. Disorganized and degenerated myocytes are noted (Figure 3D). There was no default in alignment of cardiomyocytes myofibrils, leading to a structural impairment in contractile units. Another feature often observed in the irradiated group myocytes was the loss of mitochondrial integrity. These organelles are presented swollen, with disrupted membranes, highlighting the reduction of mitochondrial cristae and loss of electron-dense matrix. Besides the mentioned alterations, endothelial cells (Figure 3E) and myocytes (Figure 3F) were observed, displaying highly condensed chromatin associated with the nuclear envelope. This characteristic is an indication that they may be in the apoptosis process.

DISCUSSION

Increased interstitial fibrosis is an important event in the RIHD (Darby et al. 2010, Boerma and Hauer-Jensen 2010). This has been observed in patients exposed to radiation in the thoracic region and in heart irradiated animal studies. Tissue fibrosis is the excessive accumulation of collagen and other extracellular matrix (ECM) components following breakdown in the normal balance of ECM synthesis and degradation (Darby et al. 2010, Yarnold and Brotons 2010). Many experimental studies have demonstrated the involvement of TGF-β1 in this process. TGF-β1 is a cytokine involved in (myo) fibroblasts proliferation and activation. Its increased expression is followed by increased collagen deposition in irradiated tissue (Krüse et al. 2001, Boerma et al. 2002, Wynn 2008, Ferreira-Machado et al. 2010). TGF-β1 can also be the primary mediator for apoptosis (Schröder et al. 2006), but this relationship is unclear in the irradiated heart.

Animals or patients, studies have demonstrated increased apoptosis pathway, evidenced particularly by the presence of cleaved-caspase-3, especially in the following heart diseases: ischemic cardiomyopathy, dilated cardiomyopathy, myocardial infarction, myocarditis, and arrhythmias (Condorelli et al. 1999, Feuerstein and Young 2000, Philipp et al. 2004, Kunapuli et al. 2006, Ferrari et al. 2009). Researches were driven by the intention of identifying the stimuli that induce cardiomyocyte apoptosis. Various stimuli can lead to myocytes apoptosis, and many can coexist in an advanced heart failure (Kang et al. 2000).

In this study, the expression of TGF-β1 and proc type I are concomitantly increased in irradiated group. These events coincide with increased cleavage of caspase-3 in cleaved-caspase-3 and the observation of cardiomyocytes with apoptotic characteristics. These data suggest that apoptosis in irradiated cardiac tissue is followed by collagen increases. These alterations, taken together, appear to contribute to the cardiac function decline, as it could be seen with the ejection fraction, cardiac output and heart rate reduction.

Considering that the heart muscle cells are rarely replaced, cell death indicates an irreparable damage, with consequent loss of function. In addition to apoptosis pathway, the caspase-3 can act in myofilaments components, as troponin C, α-actin, α-actinin (Philipp et al. 2004). The cleavage of these proteins by caspase 3 can directly affect the activation of the myofilaments and contractile function (Philipp et al. 2004, Communal et al. 2002). By electron microscopy, the ultra structural analysis revealed that in control group the sarcomeres are organized and exhibits structured Z-bands, and their mitochondria have well-defined cristae between the myofibrils, as described in the literature (Real and Meirelles 1991). In the irradiated group the presence of degenerated myocytes are noted with total disorganization of sarcomeres and morphological alterations of their mitochondria. These organelles are showed scattered in the cell. The mitochondria are organelles that have a vital role in cell energy metabolism and are essential for cell survival, especially when it comes to

a muscle cell. When this organelle is altered, it may represent an energy deficit in the cell. This way, there may be a decline in myocyte function, consequently provoking a deficit in the heart functioning. In addition, this remodeling present in the mitochondrial cristae might lead to the release of cytochrome C, an important factor in the activation of procaspase in many models of apoptosis (Kang et al. 2000, Heath-Engel and Shore 2006).

The evidences of apoptosis, confirm the molecular assay findings, which the conversion of pro-caspase-3 to cleaved caspase-3 is noted. Although the electron microscopy did not reveal a constant chromatin condensation and nuclear fragmentation (cell death), the cardiomyocyte are showed very deteriorated (Communal et al. 2002). This could indicate that the myocytes are in a preapoptotic stage, while the morphologic nuclear changes do not happen (Narula et al. 1999). Dogan et al. (2010) also identified in rats, positive apoptotic cardiomyocytes in the heart irradiated with a dose of 20 Gy (Dogan et al. 2010), and in our study it was corroborated by western blotting , where it is possible to observe an increase cleaved caspase-3.

In the RIHD, the endothelial injury, appear to be the main event responsible for triggering late damage. Evidence shows that microvascular lesions (endothelial dysfunction) may be responsible for sustaining the chronic nature of the fibrosis induced by irradiation. Because of low blood perfusion, late myocardial degeneration has been observed by some authors and it is associated with progressive increase of fibrosis and cardiomyocytes apoptosis (Wang et al. 2007, Jelonek et al. 2011). That is why authors suggest that cleaved caspase-3 is more related to consequences of heart damages generated after long period of radiation therapy.

Besides the procollagen increase, this study showed an increased level of caspase-3 cleaved. Continued loss of myocytes may lead to myocardial dysfunction. Strategies that reduce the biological signals responsible for myocyte loss and chamber remodeling should improve clinical outcomes, as decreased systolic function is a powerful predictor of adverse outcome in congestive heart failure.

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RESUMO

Foi avaliada neste estudo a presença de caspase-3-clivada no coração de ratos irradiados durante o declínio da função ventricular. Ratas Wistar foram irradiadas com uma dose única de radiação (15 Gy) diretamente no coração e as análises molecular, histológica e fisiológica foram realizadas treze meses pós a irradiação dos animais. As expressões de procolágeno tipo I, TGF-ß1 e caspase-3-clivada foram analisadas usando Western blotting. Alterações cardíacas estruturais e funcionais foram investigadas por meio de ecocardiografia e microscopia eletrônica. No grupo irradiado, os níveis de procolágeno tipo I, TGF-ß1 e caspase-3-clivada estão aumentados Alterações histológicas importantes (degeneração do tecido cardíaco e deposição de colágeno) e funcionais (fração de ejeção reduzida) foram observadas. Dados sugerem que o declínio da função cardíaca após a exposição à radiação ionizante está relacionado, em parte, com aumento do colágeno e da caspase-3-clivada.

Palavras-chave: apoptose, caspase-3, fibrose, coração, radiação ionizante, TGF-β1.

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