Effect of Radiation on the Expression of CVD-related miRNAs, Inflammation and Endothelial Dysfunction of HUVECs

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Abstract. *Background/Aim:* Radiotherapy (RT) can lead to cardiovascular disease (CVD). Evidence suggests that radiation modulates miRNA levels. Our purpose was to assess the acute response to radiation-induced modulation of the expression of miRNA-146a, miRNA-155, miRNA-221, and miRNA-222, inflammatory response and endothelial dysfunction on endothelial cells. *Materials and Methods:* Human umbilical vein endothelial cells (HUVECs) were exposed to 2 Gy RT, and intracellular levels of selected miRNAs were measured by real-time polymerase chain reaction at 2 and 24 h. Cytokine and adhesion molecule release were also assessed. *Results:* Results showed that 2 Gy significantly increased the expression of miRNA-221 and miRNA-222, and reduced the level of miRNA-155 after 2 h; whereas miRNA-146a and miRNA-155 were significantly overexpressed and miRNA-222 was significantly down-regulated at 24 h. Interleukin-8 and soluble vascular cell adhesion molecule 1 levels were not affected by the studied RT. *Conclusion:* RT at 2 Gy modulated expression of selected miRNAs by endothelial cells after 2 and 24 h, which might be related to CVD development in patients who receive RT.

Breast cancer (BC) is the most frequent cancer in women, causing approximately half a million total deaths each year worldwide (1). Although preventative strategies reduce BC

risk, development cannot be avoided in low- and middle-income countries where BC is diagnosed in very late stages (2). The overall survival rate is 90% at 5 years, and many patients receive radiotherapy (RT), which is employed to cure localized BC, to provide local control or to relieve symptoms in locally advanced or disseminated cancer (3).

Despite RT reducing BC recurrence and mortality, both early (acute) and late (chronic) side-effects persist. While acute side-effects occur during treatment, chronic side-effects take place months or even years after treatment ends. In fact, it has been demonstrated that RT can lead to cardiovascular disease (CVD) due to exposure of the heart and vessels to high volumes and doses of radiation (3). The International Commission on Radiological Protection has listed CVD as a health risk of RT since 2012 and has reported that 0.5 Gy dose might lead to approximately 1% of exposed individuals developing CVD at 10 years post-exposure.

Due to the established relationship between CVD and RT in patients with BC, the radiation dose employed is limited by injury to the endothelial lining of blood vessels, in particular to arteries (4, 5). Endothelial cells integrate physical and neurohumoral signals from the blood and regulate vascular tone, cellular adhesion, inflammation, smooth muscle phenotype, proliferation and thromboresistance (6). At a molecular level, both radiation of endothelial cells and consequent oxidative stress evoke endothelial dysfunction, which is considered to be the first step of atherosclerosis, resulting in CVD development (7). An inflammatory response is induced by cytokine release from dysfunctional endothelium and from injured irradiated cells which activate defense mechanisms as a response to the toxic effects (8). Some cytokines, such as interleukin (IL)-1 and tumor necrosis factor α (TNF α), promotes the expression of adhesion molecules in endothelial cells, such as intercellular adhesion molecule 1

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(ICAM1), vascular cell adhesion molecule 1 (VCAM1) and E-selectin (9). Adhesion molecules promote leukocyte adhesion to endothelial cells, and can be liberated into the circulation (10). In fact, patients with CVD have increased circulating levels of these adhesion molecules (11, 12).

Recent evidence suggests that radiation modulates miRNA levels, thus altering miRNA-controlled pathways (13). In addition, miRNAs are important regulators of pathophysiological processes involved in the development of CVD, such as cellular adhesion, proliferation, lipid uptake and efflux, and recruitment of inflammatory mediators (14). miRNAs are a set of about 22 nucleotides which can silence or down-regulate expression of their targets by destabilizing or cleaving their target mRNA, or reducing the efficiency with which the mRNA can be processed. One miRNA may regulate as much as 60% of available mRNAs (15).

Previous data supported the association of *miRNA-146a*, *miRNA-155*, *miRNA-221*, and *miRNA-222* with endothelial dysfunction and adhesion, and also with infiltration of inflammatory cells into the endothelial space (16, 17). *miRNA-146a* reduces lipid uptake and cytokine release in activated macrophages, exhibiting an atheroprotective and anti-inflammatory role (18), despite its absence leading to atherosclerosis suppression in mice (19). *miRNA-155* promotes lipid uptake in monocytes and macrophages, and regulates the immune and inflammatory response (20). Furthermore, both *miRNA-221* and *miRNA-222* induce apoptosis of endothelial cells, whereas they promote proliferation and migration of smooth muscle cells, as well as inhibit their apoptosis (21).

Therefore, our purpose was to assess the radiation-induced modulation of the expression of these miRNAs, as well as inflammatory response and endothelial dysfunction as possible miRNA-mediated mechanisms of action, by assessing the release of cytokines and adhesion molecules, respectively, by endothelial cells. We decided to work with human umbilical vein endothelial cells (HUVECs) because they are commonly used as *in vitro* models of the endothelium. Firstly, we wanted to determine dose and post-radiation time-points by measuring cell cytotoxicity and viability at both 2 and 10 Gy. An RT dose of 2 Gy was selected as clinical patients usually receive multiple fractions of this dose over several weeks until achieving total radiation doses of 50 to 60 Gy when treated with RT (22), and 10 Gy has been widely used on HUVECs (23-25). Furthermore, several time-points from 1 to 48 h were chosen as we wanted to assess the acute response. Then, with selected dose and time-points, the experiments were performed.

Materials and Methods

HUVEC culture and RT procedure. HUVECs at the fifth passage (C-015-5C; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) were cultured in Medium 200 (Life Technologies, Thermo Fisher Scientific) supplemented with Low Serum Growth Supplement (Life

Technologies, Thermo Fisher Scientific) containing fetal bovine serum (2% v/v), hydrocortisone (1 µg/ml), human epidermal growth factor (10 ng/ml), basic fibroblast growth factor (3 ng/ml) and heparin (10 µg/ml) with gentamicin/amphotericin B solution (Life Technologies, Thermo Fisher Scientific). Cells were cultured at a density of 2,500 cells/cm² in a humidified atmosphere with 5% CO₂ at 37°C. At 80% confluence, cells were irradiated.

Radiation was performed using a Clinac 2100C linear accelerator (Varian Medical Systems, Alcobendas, Spain). Doses rate of 4.5 Gy/min (400 MU/min) were employed for radiations at doses of 2 and 10 Gy. Control samples were placed into the instrument for the same time without being irradiated.

Firstly, single experiments were performed in order to determine RT dose and time-points by performing cytotoxicity and viability tests of RT at 2 and 10 Gy on HUVECs from 1 to 48 h post-radiation. Then HUVECs were irradiated at a selected dose to test the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222*, as well as inflammatory response and endothelial dysfunction (three-replicate experiments). Endothelial dysfunction was assessed by measuring the release of the following adhesion molecules in cell supernatant: sICAM1, sVCAM1 and sE-selectin.

Cytotoxicity to HUVECs of irradiation at doses of 2 and 10 Gy. Cytotoxicity of irradiated HUVECs at both 2 and 10 Gy after 0, 1, 3, 6, 24 and 48 h was assessed using lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Roche, Sant Cugat del Vallès, Barcelona, Spain), which measures LDH content in supernatant of cell culture. HUVECs were removed from culture supernatant by centrifuging for 5 min at 1,000 × g. Then, 100 µl of cell-free supernatant was mixed with 100 µl of reaction mixture and the sample was incubated for up to 30 min at room temperature, protecting from light. Finally, absorbance at 490 nm was measured using a Synergy HT instrument (Biotek, Winooski, VT, USA). The percentage LDH release was calculated as sample optical density (OD)/control OD at the respective time × 100.

Viability test of HUVECs irradiated at doses of 2 and 10 Gy. As adenosine triphosphate (ATP) indicates the presence of metabolically active cells, ATP quantification of irradiated HUVECs at both 2 and 10 Gy after 0, 1, 3, 6 and 24 h was performed. The number of viable cells was assessed using CellTiter-Blo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Briefly, 100 µl of assay reagent was added to cell cultures and samples were mixed on an orbital shaker for 2 min to induce cell lysis. After 10-min incubation in the dark at room temperature, luminescence, which was proportional to the amount of ATP, was recorded in a Synergy HT (Biotek). ATP release was calculated as sample luminescence/control luminescence at respective time × 100.

miRNA isolation of irradiated HUVECs. To allow miRNA isolation, HUVECs were washed with PBS and lysed by adding 350 µl of Lysis Solution. After collection, the sample was mixed with 200 µl of 100% ethanol. The content was vortexed and transferred onto the assay column and the next steps were performed by following the miRCURY RNA Isolation Kit (Exiqon, BioNova, Madrid, Spain). Sample was centrifuged for 1 min at 3,500 × g. Flowthrough was discarded and the column was then washed three times with 400 µl of Wash Solution. Subsequent centrifugation of 1 min at 14,000 × g was performed and the flowthrough was discarded. The column was centrifuged for 2 min at 14,000 × g to dry it. After adding 50 µl of Buffer Elution onto the column, the sample was finally centrifuged

twice for 2 min at $200 \times g$ and 1 min at $14,000 \times g$. The flowthrough containing miRNA was stored in RNA-free tubes at -80°C until use.

cDNA synthesis. To synthesize cDNA, miRCURY LNATM Universal RT microRNA polymerase chain reaction (PCR), polyadenylation and cDNA synthesis kit II (Exiqon) was used. Briefly, 2 μl of miRNA sample was mixed with 1 μl reverse transcriptase enzyme, 2 μl reaction buffer and 5 μl H_2O . The sample was then incubated employing the GeneAmp PCR System 2700 Thermal Cycler (Applied BiosystemsTM, Thermo Fisher Scientific) using the following thermocycler program: 60 min at 42°C for reverse transcription, 5 min at 95°C to inactivate the reverse transcriptase and a final phase at 4°C maintenance. Finally, samples containing cDNA were stored at -20°C .

Real-time PCR. Real-time PCR assays were performed in order to determine the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222*; employing *miRNA-U6* as housekeeper. Briefly, the reactions were performed in a final reaction volume of 10 μl , adding 3.5 μl cDNA sample (previously diluted 1:40 with RNase-free water) and 6.5 μl mix containing 5 μl ExiLENT SYBR Green Master Mix (Exiqon), 1 μl tested primer (miRCURY LNATM uniRT PCR primer mix; Exiqon), 0.2 μl ROX (passive reference RT-PARE-03; Exiqon) and 0.3 μl RNase-free water. Negative controls were also run for each assay. To run real-time reactions, 7900 HT Fast Real-Time PCR System (Applied BiosystemsTM, Thermo Fisher Scientific) was employed activating dsDNA denaturation for 10 min at 95°C and then amplifying samples with 40 cycles of 10 s at 95°C followed by 1 min at 60°C . Finally, results were analyzed using 2.4 SDS Software (Applied BiosystemsTM, Thermo Fisher Scientific) and RQ Manager 1.2.1 (Applied BiosystemsTM, Thermo Fisher Scientific). For the statistical analysis, cycle threshold (Ct) was measured for each sample and deltaCt (DCt) of selected miRNAs was calculated as $\text{Ct}_{\text{sample}} - \text{Ct}_{\text{median U6}}$, in order to normalize miRNA expression. The miRNA expression as fold-change in DCt of selected miRNAs was calculated as sample DCt/control DCt at the respective time.

Inflammatory response of irradiated HUVECs. The inflammatory response of HUVECs to radiation was assessed by measuring the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (INF γ), IL1 β , IL2, IL4, IL5, IL6, IL8, IL10 and TNF α by employing the Human Cytokine Magnetic 10-Plex Panel (InvitrogenTM, Thermo Fisher Scientific). All steps were performed protecting plates from light. Briefly, 25 μl of antibody beads was added to wells. After washing twice with 200 μl of wash solution, 50 μl of incubation buffer, 50 μl of assay diluent and 50 μl of cell supernatant sample were added. The plate was covered and incubated for 2 h at room temperature under agitation on an orbital plate shaker. Next, liquid was decanted and the plate was washed twice. Subsequently, 100 μl of biotinylated detector antibody was added, and incubation at room temperature on an orbital shaker in the dark for 1 h was performed. After liquid decantation and two washes, 100 μl of streptavidin-RPE solution was added. The plate was shaken for 30 min at room temperature away from light. Then liquid was decanted and the plate was washed three times. Finally, 150 μl of Wash Solution was added and after 10 min shaking, the plate was read in xPONENT 4.2 for MAGPIX Software User (IVD, Luminox, Austin, TX, USA). Standard curves were also produced. Concentration of cytokines were given by the program and fold-change in cytokine release was calculated as sample release (pg/ml)/control release (pg/ml) at the respective time.

Determination of markers of endothelial dysfunction. sICAM1 and E-selectin: To determine sICAM1 and sE-selectin in cell culture supernatant, Human Interleukin Adhesion Molecule 1 Enzyme-Linked Immunosorbent Assay (ELISA) Kit and Human E-Selectin ELISA Kit (both from Cusabio[®], Houston, TX, USA) were used, respectively. Briefly, 100 μl of cell supernatant was added to each well of the ELISA plate which was then incubated for 2 h at 37°C . Liquid was then removed and 100 μl of biotin antibody was added. The plate was incubated for 1 h at 37°C and after removal of liquid, the plate was washed three times using 200 μl of Wash Buffer. Avidin (100 μl) was added to each well and the plate was incubated for 1 h at 37°C . The plate was washed five times as before and 90 μl of TMB substrate was added. Finally, the plate was incubated for 15-30 min at 37°C protecting from light and 50 μl of stop solution was added until reading the sample OD.

sVCAM1: ELISA kit for Vascular Cell Adhesion Molecule 1 (Cloud-Clone Corp., Katy, TX, USA) was employed in order to determinate sVCAM1. Briefly, 100 μl of cell supernatant was added to the plate which was then incubated for 1 h at 37°C . After removal of liquid, 100 μl of detection reagent A was added and the plate was incubated for 1 h at 37°C . Then liquid was aspirated and three washes were performed using 350 μl of wash solution. Detection reagent B (100 μl) was then added and after incubation for 30 min at 37°C , the plate was washed five times before adding 90 μl of substrate solution and incubating for 10-20 min at 37°C protected from light. Finally, 50 μl of stop solution was added and sample OD was measured.

The measurement of sICAM1, sVCAM1 and sE-selectin levels were performed by reading the OD at 450 nm and correcting to 540 nm by employing a Synergy HT (Biotek) reader. Fold-change in adhesion molecule release was calculated as sample release (ng/ml)/control release (ng/ml) at the respective time.

Statistical analysis. All data were expressed as the mean $\pm$ standard error of the mean (SEM). Gaussian distribution was assessed by Kolmogorov-Smirnov test. ANOVA followed by the Bonferroni's *post-hoc* test was executed to evaluate cytotoxicity and viability, whose variances were homogenous, whereas Welch's unpaired one-tailed *t*-test was used to test the expression of selected miRNAs, inflammatory response and endothelial dysfunction. The level of statistical significance for all tests was established at $p < 0.05$. All data were analyzed by GraphPad Prism version 5.01 for Windows (GraphPad Prism, San Diego, CA, USA).

Results

Determination of radiation dose and time-points: Assessment of cytotoxicity and viability of irradiated HUVECs at doses of 2 and 10 Gy. In order to determine optimal dose and post-radiation time points for the experiment, cytotoxicity and viability tests were assessed at different times after radiation at 2 and 10 Gy.

Cytotoxicity, as increase in LDH release, of irradiated HUVECs at both doses at 1-48 h post-radiation is depicted in Figure 1. The results indicate that irradiation at both levels led to significantly greater LDH release than the control at 1 h post-radiation, whereas this significance was lost afterwards. In fact, 2 Gy irradiation led to significantly less cytotoxicity at 24 h than

Figure 1. Cytotoxicity after 1, 3, 6, 24 and 48 h as a change in percentage of lactate dehydrogenase (LDH) release at doses of 2 and 10 Gy. LDH release was calculated as sample OD/control OD at a respective time $\times 100$. Data are the mean $\pm$ SEM (single experiment). Significantly different at * $p < 0.05$ and ** $p < 0.01$ from the control at the respective time by ANOVA test and Bonferroni's post-hoc test.

the control. This indicates that irradiation did not exhibit cytotoxic effects on HUVECs at measured times at these doses.

HUVEC viability, as increase in intracellular ATP, of irradiated HUVECs at doses of 2 and 10 Gy after 1, 3, 6 and 24 h is shown in Figure 2. Results showed that ATP was significantly increased in both irradiation doses at 3 and 24 h, indicating that viability was not altered by the effect of 2 and 10 Gy doses.

In summary, these results showed that both 2 and 10 Gy did not induce cytotoxicity or viability failure in HUVECs. We then assessed the effects of 2 Gy RT at two time-points, 2 and 24 h, on the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222*, inflammatory response and endothelial dysfunction in these cells.

Comparison of the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222* of irradiated HUVECs at 2 Gy. In the current study, *miRNA-155* and *miRNA-221* presented the lowest expression, indicated by the highest fold-change DCt, at 2 and 24 h respectively. When compared to this miRNA, the expression of *miRNA-146a*, *miRNA-221* and *miRNA-222* was significantly greater at 2 h (Figure 3A). In addition, *miRNA-222* expression in comparison to *miRNA-221* was statistically higher at 24 h (Figure 3B).

Effect of radiation at 2 Gy on the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222* in HUVECs. The effect of 2 Gy RT on the expression of selected miRNAs is depicted in Figure 4. We observed that 2 Gy RT induced a statistical increase in the expression of *miRNA-221* (Figure 4C) and *miRNA-222* (Figure 4D), whereas *miRNA-155* was

statistically reduced at 2 h (Figure 4B). At 24 h, the level of *miRNA-221* was restored and *miRNA-222* expression was statistically reduced (Figure 4D). Furthermore, we found overexpression of *miRNA-146a* (Figure 4A) and *miRNA-155* (Figure 4B) relative to the control at this time-point.

Effect of radiation at 2 Gy dose on inflammatory response of HUVECs. Fold-change in measurable levels of IL8 in supernatant from control and irradiated HUVECs at 2 Gy after 2 and 24 h is shown in Figure 5. The results showed no significant effects. Under our experimental cell conditions, supernatant levels of analyzed cytokines GM-CSF, INF γ , IL1 β , IL2, IL4, IL5, IL6, IL10 and TNF α were too low to allow valid quantification (data not shown).

Effect of radiation at 2 Gy dose on sVCAM1 in HUVECs. Our results showed that the release of sVCAM1 into the supernatant, reflecting endothelial dysfunction did not differ from that of control and irradiated HUVECs at 2 Gy neither at 2 nor 24 h (Figure 6). On the other hand, sICAM1 and sE-selectin were not quantifiable as their levels were too low to quantify (data not shown).

Discussion

In the present study, we planned to assess the effect of RT at acute phase on the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222*, which are involved in endothelial dysfunction and formation of atherosclerotic plaques, as well as on inflammatory response and endothelial dysfunction as possible action mechanism of these miRNAs in endothelial cells.

Figure 2. Viability after 1, 3, 6 and 24 h as a change in percentage of intracellular adenosine triphosphate (ATP) at doses of 2 and 10 Gy. Intracellular ATP was calculated as sample luminescence/control luminescence at respective time $\times 100$. Change in intracellular ATP, calculated as intracellular ATP (%)–100, is shown. Data are the mean $\pm$ SEM (single experiment). Significantly different at $**p<0.01$ and $***p<0.001$ from the control at the respective time by ANOVA test and Bonferroni's post-hoc test.

Figure 3. Fold-change DCt of miRNA-146a, miRNA-155, miRNA-221 and miRNA-222 at 2 Gy after 2 h (A) and 24 h (B). Fold changes in comparison to miRNA-155 at 2 h and to miRNA-221 at 24 h, which had the highest fold-change DCt (lowest expression) are indicated. Fold-change in DCt of selected miRNAs was calculated as sample DCt/control DCt. Data are the mean $\pm$ SEM (three-replicate experiments). Significantly different at $*p<0.05$ and $***p<0.001$ by Welch's one-tailed unpaired t-test.

First, we determined the optimal RT dose, 2 or 10 Gy, and post-radiation time-points, from 1 to 48 h by assessing cytotoxicity and viability on HUVECs. We observed that cytotoxicity, characterized by an enhancement of LDH

release, was not impaired by RT at either dose. Results showed greater increases in LDH release by both doses after 1 h, but this increase then fell at later time-points. Indeed, a decrease at 2 Gy was observed after 24 h. Thus, neither RT

Figure 4. Comparison of fold-change DCt of miRNA-146a (A), miRNA-155 (B), miRNA-221 (C) and miRNA-222 (D) at 2 Gy after 2 and 24 h. Fold-change in DCt of selected miRNAs was calculated as sample DCt/control DCt. Data are the mean $\pm$ SEM (three-replicate experiments). Significantly different at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Welch's one-tailed unpaired t-test.

dose had consistent cytotoxic effects on HUVECs. In contrast to our results, Yang (26), who also worked with HUVECs, observed LDH increase after RT at 2, 4, 6 and 8 Gy. However, time-points assessed after RT was not defined, which may be the reason for observing differences between their and our study. Furthermore, cytotoxicity to other endothelial cell lines has been assessed in previous studies. For instance, similarly to our results, Vorotnikova *et*

al. (27) found that RT at 20 Gy did not induce cytotoxicity in bovine capillary endothelial cells after 2 to 72 h. Nevertheless, many studies performed in the 1980s reported that at same post-RT times, LDH release increased in bovine aorta endothelial cells after single doses of 0.5 to 10 Gy (28-31). The observed discordance may be due to variability in RT dose and cell lines. In addition, we measured viability, which is associated with high amounts of ATP, and we

Figure 5. Fold-change in interleukin-8 (IL8) release at 2 Gy after 2 and 24 h. Fold-change in IL8 release was calculated as sample IL8 release (pg/ml)/control IL8 release (pg/ml). Data are the mean $\pm$ SEM (three-replicate experiments). Welch's one-tailed unpaired t-test was used for statistical analysis.

Figure 6. Fold-change in soluble vascular cell adhesion molecule 1 (sVCAM1) release at 2 Gy after 2 and 24 h. Fold-change in VCAM1 release was calculated as sample sVCAM1 release (ng/ml)/control sVCAM1 release (ng/ml). Data are the mean $\pm$ SEM (three-replicate experiments). Welch's one-tailed unpaired t-test was used for statistical analysis.

observed that the increase in ATP release was enhanced after both 2 and 10 Gy at 3 and 24 h. This indicated that viability was not impaired by these RT doses on HUVECs. To our knowledge, the effects of RT on ATP release in HUVECs has not been assessed, although it has in other cell line types. For example, Lindgard *et al.* (32) found no differences in ATP concentration in human monocytes at the end of irradiation for 2 min at 25°C using photons at 634 nm. In addition, Hietanen *et al.* (33) studied the effects of single radiation doses at 1 to 60 Gy alone and combined with heating on natural killer cells, and they found ATP decrease after 72 h for all doses. In fact, Hietanen *et al.* (34) had suggested that ATP production was stimulated by low-dose RT and inhibited by doses over 10 Gy for this cell type. They observed that 1 to 8 Gy RT of natural killer cells led to an ATP enhancement after 0 to 6 h and a decrease from 8 to 72 h post-radiation; whereas higher doses, from 8 to 80 Gy, reduced the ATP level. Thus, differences in effects between doses and cell lines existed.

In our study, once determined that 2 and 10 Gy doses were not cytotoxic and did not impair viability of HUVECs, we proposed to assess the effect of 2 Gy after 2 and 24 h on the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222* as they are involved in oxidative stress, apoptosis, and angiogenesis in atherosclerotic plaques, as well as in inflammation and endothelial dysfunction (16-19). In the present study, we observed that *miRNA-221* was most highly expressed after 2 h radiation, followed by *miRNA-222*, *miRNA-146a* and *miRNA-155*. In contrast, *miRNA-222* had

the highest expression at 24 h post-radiation, followed by *miRNA-155*, *miRNA-146a* and *miRNA-221*. Moreover, results showed that 2 Gy RT significantly increased the expression of *miRNA-221* and *miRNA-222* and significantly reduced *miRNA-155* expression at 2 h, whereas at 24 h, levels of *miRNA-221* and *miRNA-222* were restored and statistically decreased, respectively, and *miRNA-146a* and *miRNA-155* were significantly overexpressed. Similarly, in a previous study which also worked with HUVECs, it was observed that 1 Gy promoted an increase in the expression of *miRNA-221* and *miRNA-222* after 1 and 2 h, although their levels were restored at 4 and 8 h (35). In addition, other studies assessed the levels of some selected miRNAs in other endothelial cell lines. Kraemer *et al.* (36) found a similar response of *miRNA-146a* after RT, observing that 2.5 Gy up-regulated *miRNA-146a*, among others, at 4 h whereas its level was restored at 24 h in EA.hy926 endothelial cells. Furthermore, *miRNA-221* and *miRNA-222* were up-regulated after 6 h at 10 Gy, and *miRNA-146a* at both 6 and 24 h, in human coronary artery endothelial cells (37). Differences in RT-induced changes of miRNA expression between these studies and our results may be due to cell specificity, as well as RT dose and time-point variability. In fact, studies employing other cell types confirm this affirmation. For example, while the expression of *miRNA-146a*, *miRNA-221* and *miRNA-222* was not modulated in 2.5 Gy irradiated-lymphoblasts after 4 h or 6 days (38), Simone *et al.* (39) observed a decrease in *miRNA-222* level after 1 h irradiating at doses from 0.25 to 10 Gy on fibroblasts.

In addition, we wanted to study the mechanism by which these miRNAs might act by assessing inflammation and endothelial dysfunction at selected dose and time-points on HUVECs. We evaluated inflammatory response by measuring the release of several cytokines, as they promote local inflammation in the response of endothelial cells to radiation (8). IL8 is a pro-inflammatory cytokine that is involved in inflammatory response by contributing to the recruitment of leukocytes following radiation. Furthermore, it is involved in mitogenesis, angiogenesis, chemotaxis and neutrophil degranulation (40). It is highly expressed in atherosclerotic plaques whose main sources are endothelial cells as well as macrophages and smooth muscle cells. Therefore, IL8 has been intensively studied as a potential marker of atherosclerosis (41). Our results showed that the IL8 level of HUVECs was not affected by radiation at 2 Gy at 2 nor 24 h. Similarly, Meeren *et al.* (42) found no differences in IL8 release at the same dose after 24 h, despite it being enhanced after 18 h. Furthermore, other studies supported that 10 Gy increased IL8 until 6 h to 6 days in a time-dependent manner (25, 42). In our study, we also analyzed the release of other cytokines, as they form a complicated network by promoting specific cellular interactions in inflammation, endothelial dysfunction and in the course of atherosclerosis (41). We were not able to make valid assessment or quantification of these other cytokines because their levels were too low. In fact, many of these cytokines were not detectable from HUVECs after 10 days radiation at 1 to 20 Gy (42) or even when treated with progesterone (43) or proteases (44).

Moreover, we assessed sICAM1, sVCAM1 and sE-selectin levels as the release of these adhesion molecules have previously been measured in order to test endothelial dysfunction in HUVECs (45, 46), and it is described that their levels are enhanced in circulation of patients with CVD (11, 12). We found that levels of sICAM1 and sE-selectin were too low to analyze, whereas there was no difference in the release of sVCAM1 at the studied time-points. There are no previous data evaluating radiation effects on their release, but their cell surface expression was studied. It was described that 2 Gy enhanced their levels after 5 to 36 h (24, 47, 48). This was also observed when lower or higher doses were employed (*e.g.* 0.125, 0.250, 7, 8 and 10 Gy) at times up to 18 h (9, 23, 25, 49, 50). However, and similarly to our results, Gaugler *et al.* (24) and Ebrahimian *et al.* (51) found levels of VCAM1 to be unchanged at 2 Gy after 2 to 10 days and 1 to 10 weeks, respectively. It should be noted that these studies differed from ours as we measured soluble adhesion molecules, whereas they studied the expression at the cell surface. Moreover, while we employed ELISA, they performed other evaluation techniques such as flow cytometry, western blot, northern blot and mRNA extraction. Finally, we studied the acute response whereas their time-

points were from days to weeks, and there were also differences in the type of RT, the dose and the dose rate.

Conclusion

In conclusion, our results showed that single-dose RT at either 2 or 10 Gy under our experimental conditions did not affect cytotoxicity or viability from 1 to 48 h on HUVECs. Furthermore, the expression of *miRNA-146a*, *miRNA-155*, *miRNA-221* and *miRNA-222* was modulated by 2 Gy at 2 and 24 h in these cells. We observed that 2 Gy did not promote inflammation or endothelial dysfunction in the acute phase as a possible mechanism of action. Thus, future studies should be focused on assessing the chronic response of miRNA modulation, as well as inflammation and dysfunction of endothelium, after radiation exposure. Moreover, we suggest that RT induced-variation in selected miRNAs levels might be related to atherosclerosis progression and the subsequent development of future cardiovascular pathologies in patients who receive RT. In addition, further research is needed in order to confirm the role of miRNAs as biomarker or therapeutic agent in this pathology.

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