

# Simulated microgravity decreases apoptosis in fetal fibroblasts

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**Abstract.** Space travel is a major challenge for human beings. Especially, the mechanisms through which space conditions might alter animal development have been questioned for a long time. The two major physical stress factors that are of relevance in this context are space radiation and weightlessness. While it has been extensively shown that high doses of ionizing radiation induce deleterious effects on embryonic development, so far, little is known about the potential harmful effects of radiation in combination with microgravity on the developing organism. In the present study, we investigated the effects of simulated microgravity on irradiated STO mouse fetal fibroblast cells using a random positioning machine (RPM). Radiation-induced cell cycle changes were not affected when cells were subjected to simulated microgravity for 24 h. Moreover, no morphological differences were observed in irradiated samples exposed to simulated microgravity compared to cells that were exclusively irradiated. However, microgravity simulation significantly decreased the level of apoptosis at all doses as measured by caspase-3 activity and it prevented cells from undergoing radiation-induced size increase up to 1 Gy.

## Introduction

Almost simultaneously with the first space flight, the potential hazard of the space environment became of particular interest. Especially in the context of long-term space flight and extra-terrestrial habitation, it is imperative to characterize the potential impact of space conditions, most prominently ionizing radiation and microgravity, on development. Performing animal experiments in space is non-trivial and developmental biology studies in space are even more complicated as mating and sampling at set time points are difficult to achieve. On the

other hand, space conditions are very difficult to reproduce on Earth: space radiation is too complex and variable in terms of composition and energy (1), and there is no present way to cancel gravity on Earth.

Animal studies have shown that ionizing radiation has a detrimental impact on embryonic development (2). While lethality is the main effect of irradiation during the pre-implantation period, embryos may show a variety of congenital anomalies after irradiation during the organogenesis period which follows. Those could be caused by the arrest of the development of a structure at an early stage of a specific organ development (2). Albeit being less radiosensitive than in younger embryos, the fetal period is affected by radiation, especially at the level of the central nervous system, the hematopoietic system and tissue formation (2). The origin of these developmental defects at the molecular level is assumed to lie in the generation of DNA double strand breaks (DSBs) by ionizing radiation (3-7). If not repaired properly, these lesions can lead to cell death, proliferation arrest or mutations which increase the risk of genetic diseases or cancer.

In-flight experiments have shown an impact of microgravity during critical periods of mammalian development, especially on bones, muscles and brain (8). However, most of the observed effects were recovered upon return to Earth. Despite extensive research, risk assessment of development in space will depend on the understanding of how space conditions affect organs and tissues at the molecular and cellular levels. To this end, robust models are required that allow for simulating space conditions on Earth. For microgravity simulation, various devices are currently used such as rotating wall vessel (RWV) or the random positioning machine (RPM) (9,10). The latter randomizes in three dimensions the direction component of the gravity vector in order to obtain, on average, a net force close to zero as the sample is taken as a reference. In this way, the RPM has effects comparable to real microgravity if the changes in direction are faster than the response time of the sample and it is therefore considered as a valuable microgravity simulation model (9-13).

We investigated the impact of simulated microgravity on the cellular response to ionizing radiation by incubating cells for 24 h in a RPM after exposure to increasing doses of X-rays. Fetal skin fibroblasts were used as an *in vitro* model. While simulated microgravity had no impact on radiation-induced morphological or cell cycle alterations, it induced a significant decrease in apoptosis.

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## Materials and methods

**Cell culture, X-irradiation and simulated microgravity.** The STO [Sandos inbred mice (SIM)] thioguanine- and ouabain-resistant cell line is derived from a continuous line of SIM skin fetal fibroblasts (14,15). Two milliliters of cells at a density of  $2.5 \times 10^5$  cells/ml were seeded in 12.5-cm<sup>2</sup> flasks and cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all were from Invitrogen, Merelbeke, Belgium).

Twenty-four hours after plating, cells were X-irradiated with the following doses: 0, 0.5, 1 or 4 Gy (250 keV, 15 mA, 1-mm Cu). The dose rate was  $0.273 \pm 0.006$  Gy/min and the accuracy of the given dose was higher than 99.99%.

Immediately after irradiation, the flasks were filled up with culture medium and half of them were submitted to simulated microgravity for 24 h while the others were placed in the same incubator and served as gravity-controls. The medium was placed in the same incubation conditions as during the experiment (37°C, 5% CO<sub>2</sub>) for 24 h prior to the experiment in order to be equilibrated in terms of temperature and gas. The desktop random positioning machine (RPM) (Dutch Space, Leiden, The Netherlands) was used for microgravity simulation at a rotational velocity between 55°/sec and 65°/sec. Direction, speed and interval were set as random. Directly after RPM treatment and for all measurements, all cells (adherent and floating) were washed with phosphate-buffered saline (PBS) (Invitrogen) and collected after exposure to 0.05% trypsin-EDTA (Invitrogen).

**Cellular morphology and cell area measurement.** After trypsinization, cells were washed twice with PBS supplemented with 10% FBS and resuspended in 1 ml of PBS supplemented with 1% FBS. Next, cells were centrifuged onto a slide by cytospinning at 500 rpm for 5 min. The slides were stained with May-Grünwald Giemsa and were analyzed by eye with light microscopy. Cells (n=100) from the 3 biological replicates prepared for each of the doses were scored for normality, vacuoles and morphological signs of apoptosis (cell blebs).

Cell area was measured on the slides prepared for cellular morphology assessment, with ImageJ freeware, 1.45h [W.S. Rasband, ImageJ; US National Institutes of Health, Bethesda, Maryland, USA; <http://rsb.info.nih.gov/ij/> (1997-2010)]. In brief, color images were converted to 8-bit grayscale and binarized using a fixed threshold. After watershed-based segmentation, the projected area was derived for the cellular regions of interest. Incorrectly segmented cells were manually removed by visual inspection. Fig. 1 shows an example of a 4 Gy irradiated sample before and after segmentation.

**Flow cytometry.** Cell cycle analysis and caspase-3 activity were measured using a Beckman-Coulter EPICS XL flow cytometer. For all assays and each condition, 20,000 cells were analyzed in triplicates.

**Cell cycle analysis.** After trypsinization, cells were fixed in cold ethanol (80%) for 1 h, after which they were washed in PBS and suspended in a solution of propidium iodide (PI) red (Sigma-Aldrich, Belgium) containing RNase (10 mM Tris,

5 mM MgCl<sub>2</sub>, 10 µg/ml RNase, 40 µg/ml PI) before being incubated at 37°C for 1 h. Estimation of G1, S and G2 fractions was performed by flow cytometry after discrimination of doublets and aggregates using red fluorescence pulse width analysis. In addition to gating away doublets and aggregates, signals from cells with less than half the DNA content of the G1 peak were excluded to avoid micronuclei and apoptotic bodies. The mitotic index (MI) was calculated as follows:  $MI = [(1 \times G1) + (1.5 \times S) + (2 \times G2)] / 100$ .

**Apoptosis (caspase-3 activity and sub-G1 peak).** Caspase-3 activity assessment was performed following the manufacturer's instructions using the CaspGlow™ kit (Medical and Biological Laboratories Co., Ltd., Woburn, MA, USA).

While performing cell cycle analysis, cells that showed a red fluorescence corresponding to a DNA content situated between half the mean channel of G1 phase and the G1 phase mean were referred to as sub-G1 cells.

**Statistics.** Graphics were performed using GraphPad Prism version 5.00 (GraphPad Software Inc., USA), while statistics were performed with SPSS version 17.0 (IBM Corporation, USA). When data were parametric (Kolmogorov-Smirnov test) one-way or two-way ANOVA was performed with Sidak *post hoc* test to analyze differences between treatments. All data were tested for homogeneity of variance before ANOVA analysis. If not parametric, 2 sample tests were performed by Kolmogorov-Smirnov and k sample tests were performed by Kruskal-Wallis. *Post hoc* test for Kruskal-Wallis tests were performed according to Chan and Walmsley (16). Differences between means were considered as significant when P-values <0.05.

## Results

**Cell morphology.** In order to assess the effect of simulated microgravity in combination to irradiation, STO cells were irradiated and subsequently exposed to RPM for 24 h. After RPM treatment, a portion of the cells appeared to have detached from the flasks and formed globular cell aggregates (data not shown). The occurrence of these aggregates was radiation-independent and found at all doses, non-irradiated cells included. For morphological assessment, the cells were mounted by cytospinning and May-Grünwald-Giemsa stained (Fig. 2A). Subsequent visual inspection of stained slides showed an increase in the number of vacuolated and blebbing cells with radiation dose but no significant differences in the number of normal and apoptotic cells (blebs) between RPM-treated cells and gravity-controls, irrespective of the dose (Fig. 2B).

Using automated cell size analysis, a significant increase was detected in gravity-controls for all doses (Fig. 3). However, this increase was not observed for RPM treatment exposed to 0.5 Gy and 1 Gy of X-rays, but only at 4 Gy, where it was comparable to the gravity-controls irradiated with the same dose.

**Cell cycle.** The observations of increased cell area after exposure to X-rays indicated a possible shift in cell cycle distribution towards the G2 phase. To verify this, a cell cycle analysis was performed by flow cytometry (see Fig. 4A for representative examples of cell cycle distributions). Irradiation of gravity-

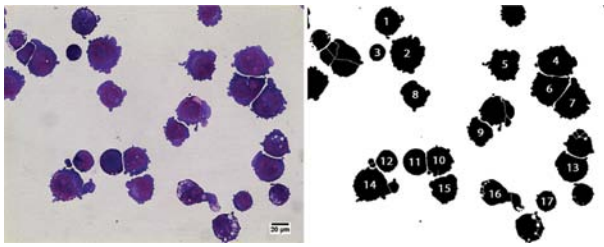


Figure 1. Example of cell segmentation performed with ImageJ version 1.45h for cell area measurement. Each cell is isolated from the background, and defined as a separate object with an identification number if its size, shape and circularity are fitting the criteria defining cells. The software measures the area of every identified object.

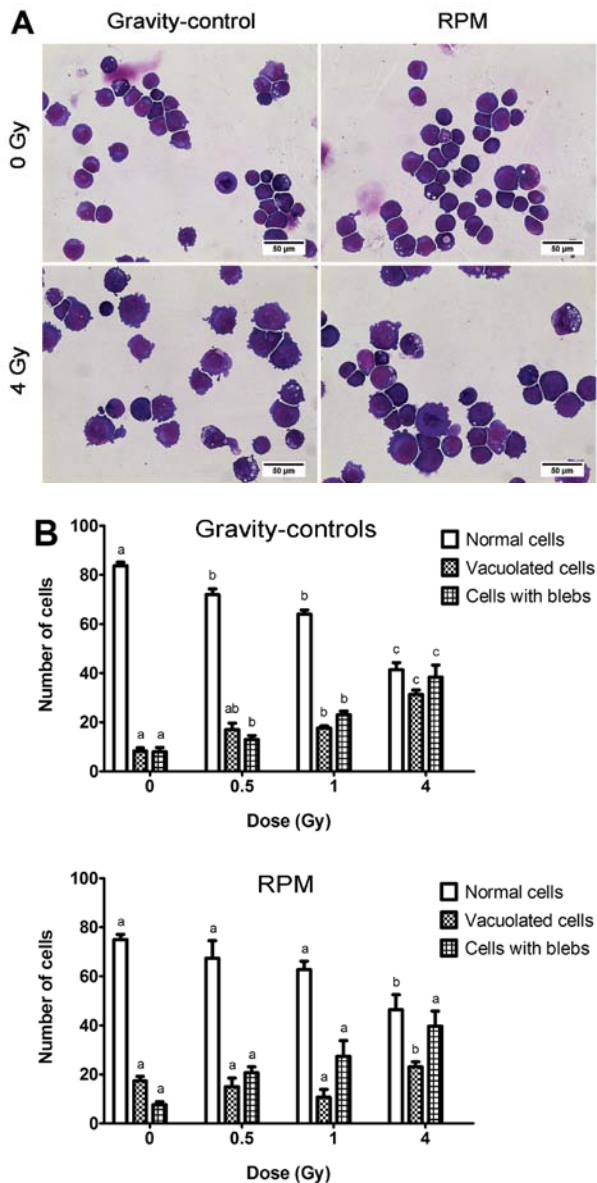


Figure 2. Morphological alterations of STG cells submitted to RPM for 24 h after X-irradiation. (A) Representative pictures of May-Grünwald Giemsa stained slides performed on cytopinned control cells (top left), submitted to RPM (top right) and 4 Gy irradiated (below) cells. (B) Histogram of the number of normal, vacuolated and apoptotic (blebs) cells, counted from May-Grünwald Giemsa stained slides (mean  $\pm$  SEM). Data were analyzed by one-way ANOVA and means separated per phenotype (a-d) by a Sidak *post hoc* test. Significantly more cells with vacuolization or blebs were observed from 1 Gy onwards compared to controls (top). However, no significant difference was detected between RPM treated samples (below) and their controls (top).

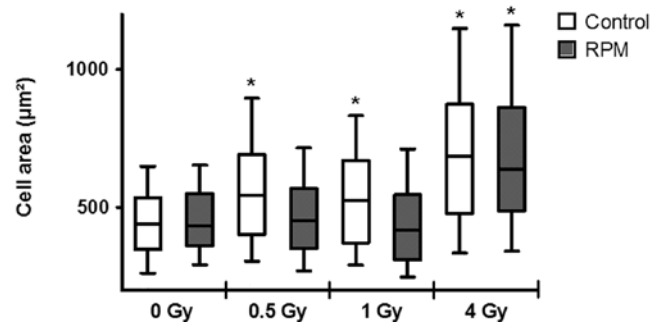


Figure 3. Box plots of the area of STG cells submitted to RPM for 24 h after X-irradiation. A significant increase was observed for all doses in cells that were not submitted to the RPM (Kruskal-Wallis non parametric test). In RPM treated cells, no cell area increase was detected at lower doses (0.5 and 1 Gy), indicating that RPM prevented the radiation-induced area increase.

control samples with 4 Gy significantly increased the number of cells in G2 phase and decreased the number of cells in G1 and S phases (Fig. 4B), which induced an increase of the mitotic index (Fig. 4C). RPM treatment had no significant effect on the number of cells in the G2 phase, irrespective of the X-ray dose (Fig. 4B). Likewise, no difference was observed in the mitotic index between RPM-treated and gravity-control cells.

**Apoptosis.** The sub-G1 peak measured by cell cycle analysis increased significantly after exposure of gravity-control cells to 4 Gy, but no significant effect of the RPM was detected on the sub-G1 peak, irrespective of the dose (Fig. 4B). However, the accuracy of this assay is limited by its sensitivity and the underestimation of cells encountering apoptosis while undergoing the S and G2 phases of the cell cycle. We therefore measured a second marker of apoptosis based on caspase-3 activity that we had previously described as a more suitable assay to measure radiation-induced apoptosis in STG cells (17). In the gravity-control cells, no increase in the caspase-3 activity was observed following X-ray doses of 0.5 or 1 Gy while a 2-fold increase in caspase-3 activity was observed after exposure of the cells to 4 Gy (Fig. 5). Cells from the samples submitted to simulated microgravity for 24 h exhibited a significantly lower caspase-3 activity at all doses compared to the gravity-controls. On the other hand, 4 Gy caused a relative increase in caspase-3 activity which was similar to the one observed for the gravity-control cells exposed to the same dose.

## Discussion

Microgravity is believed to induce various effects on many cellular processes. Among others, alterations in the cytoskeletal organization have been reported in many cell types, including lymphocytes (18), endothelial cells (19) and osteoblasts (20). It has been hypothesized that the cytoskeleton could act as a mechanosensitive element that would trigger cell signaling changes leading to differences in cell proliferation and cell death (18,20). A possible interaction between irradiation and simulated microgravity has already been investigated (21). Indeed, microgravity-induced alterations in DNA damage repair kinetics following irradiation could have dramatic consequences for cell survival, which is of

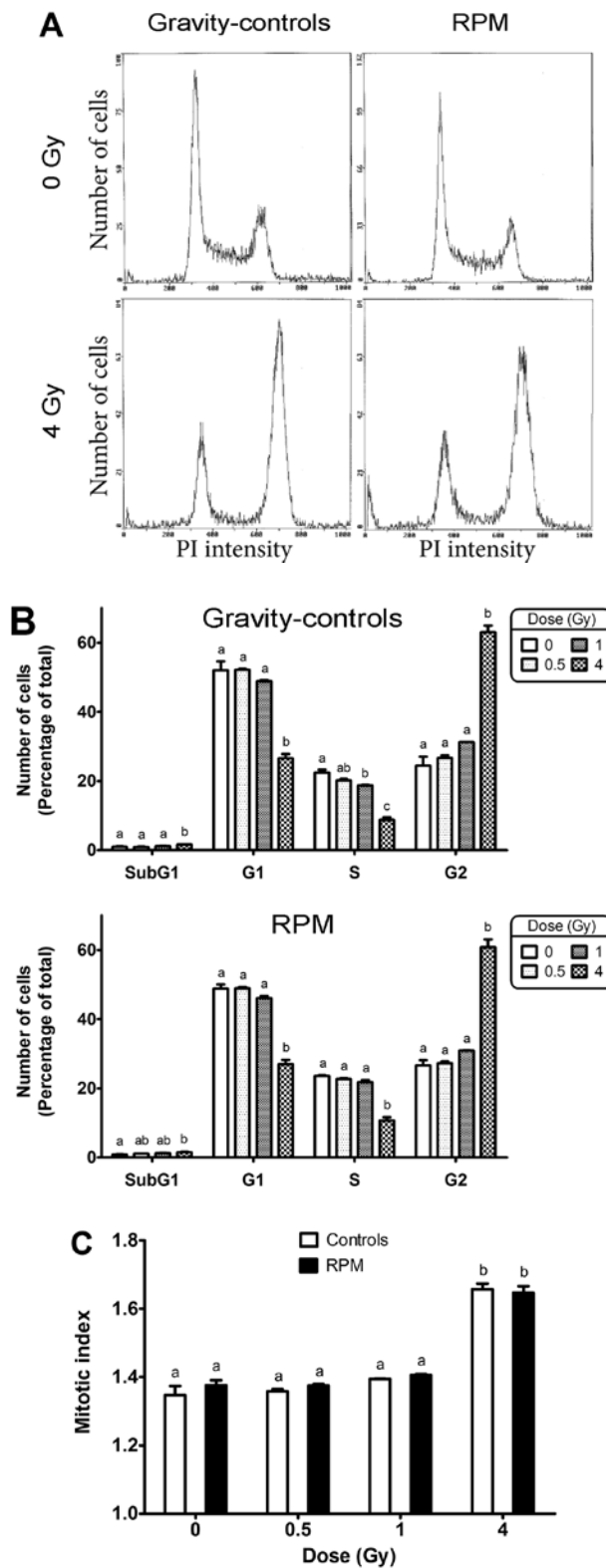


Figure 4. Cell division alterations of STO cells submitted to RPM for 24 h after X-irradiation. (A) Representative cell cycle distributions performed by flow cytometry analysis after propidium iodide (PI) staining of controls (top) and 4 Gy irradiated cells (below), RPM-treated cells (right) and their controls (left). The G2 cell cycle arrest observed in irradiation alone is also seen in RPM treated samples. No difference is observed between non-irradiated samples submitted to RPM compared to the gravity controls. (B) Quantitative analysis of the cell cycle measured by flow cytometry (mean  $\pm$  SEM). Almost no difference is observed between RPM-treated cells (below) and the gravity-controls (top). Data were analyzed by one-way ANOVA and means separated per cell cycle phase (a-d) by a Sidak *post hoc* test. (C) Mitotic indices calculated from the cell cycle (mean  $\pm$  SEM). No difference is observed between RPM-treated cells and the gravity-controls (two-way ANOVA).

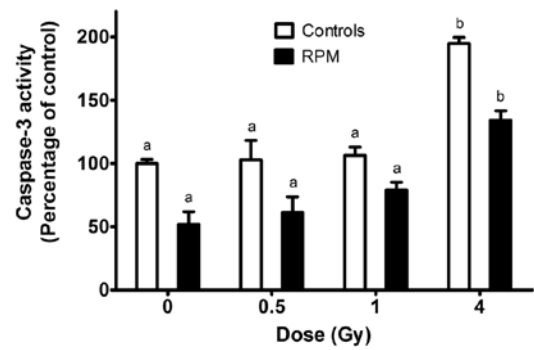


Figure 5. Histogram of caspase-3 activity measured by flow cytometry (mean  $\pm$  SEM). Significantly lower caspase-3 activity was measured in RPM-treated cells than in non-irradiated cells (50% of controls) as well as in irradiated cells (two-way ANOVA). However, a significant radiation-induced caspase-3 activity was measured in RPM-treated cells and the gravity-controls. This indicates that RPM reduced the basal level of caspase-3 activity, which protected cells from low dose radiation-induced apoptosis.

particular interest for space mission risk assessment. Although Mognato *et al* (22) observed a slower re-joining of radiation-induced DSBs and a significant increase in apoptosis in human lymphocytes irradiated with an acute high dose of gamma-rays (5 Gy) and subsequently submitted to the rotating wall vessel (RWV) for 24 h (22), most studies showed no difference in repair kinetics or cell death upon simulated microgravity as reviewed by Manti (21). Our data showed that irradiated STO cells which had been exposed to simulated microgravity immediately after irradiation exhibited the same relative radiation-induced increase of apoptosis as those which had not been exposed to microgravity. On the other hand, we observed an overall decrease in the level of apoptosis, evidenced by a lower level of caspase-3 activity, in RPM-treated cells with respect to gravity-controls, irrespective of the irradiation dose. This indicates a lower background level of apoptosis, but no change in radiation-induced apoptosis. Apparently conflicting with this observation, no difference was detected in the percentage of sub-G1 cells between RPM and gravity-control conditions. However, sub-G1 peak assessment is known to have a low specificity to detect apoptotic cells (23).

In contrast to Canova *et al* (24), we observed no change in radiation-induced G2 phase arrest between RPM-treated cells and gravity-controls. Although a significant increase in cell area was measured at all doses in gravity-controls, the radiation-induced G2 phase arrest was significant only at 4 Gy. On the other hand, no increase in cell area was observed up to 1 Gy in RPM-treated cells. Based on these observations, we hypothesize that RPM treatment could protect STO cells from undergoing apoptosis after exposure to moderate doses of radiation, possibly by decreasing the basal levels of caspase-3 activity. Such decrease in apoptosis could have dramatic consequences at the organismal level, by allowing accumulation of cells with persistent DNA damage and/or mutations (25). Indeed, an increase in mutation frequency was observed in human lymphocytes gamma-irradiated (1-3 Gy) and subsequently submitted to the RWV for 24 h (26).

In conclusion, we showed that simulated microgravity (RPM) decreased the level of apoptosis in fetal skin fibroblasts. This could have important consequences for mutation

frequency in cells subjected to the combination of microgravity and irradiation.

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