Original Article

MnSOD downregulation induced by extremely low 0.1 mGy single and fractionated X-rays and microgravity treatment in human neuroblastoma cell line, NB-1

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A human neuroblastoma cell line, NB-1, was treated with 24 h of microgravity simulation by clinostat, or irradiated with extremely small X-ray doses of 0.1 or 1.0 mGy using single and 10 times fractionation regimes with 1 and 2 h time-intervals. A quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) examination was performed for apoptosis related factors (*BAX*, *CYTC*, *APAF1*, *VDAC1–3*, *CASP3*, *CASP8*, *CASP9 P53*, *AIF*, *ANT1* and *2*, *BCL2*, *MnSOD*, autophagy related *BECN* and necrosis related *CYP-40*. The qRT-PCR results revealed that microgravity did not result in significant changes except for a upregulation of pro-apoptotic *VDAC2*, and downregulations of proapoptotic *CASP9* and antiapoptotic *MnSOD*. After 0.1 mGy fractionation irradiation, there was increased expression of proapoptotic *APAF1* and downregulation of proapoptotic *CYTC*, *VDAC2*, *VDAC3*, *CASP8*, *AIF*, *ANT1*, and *ANT2*, as well as an increase in expression of anti-apoptotic *BCL2*. There was also a decrease in *MnSOD* expression with 0.1 mGy fractionation irradiation. These results suggest that microgravity and low-dose radiation may decrease apoptosis but may potentially increase oxidative stress.

***Key Words*:** **space radiation, microgravity, extremely small dose, neuron, NB-1**

To ensure the long-term safety and normal expected life span of astronauts exposed to space-related environmental stresses and to help clarify the risks associated with long-term manned space missions, basic data regarding biological responses to such stressors need to be collected. Studies of these biological responses may contribute to a better understanding of the longer-term effects (e.g., carcinogenicity, neurotoxicity, lifespan changes) of exposure to conditions in outer space. Recently accumulated data have revealed that astronauts living on the International Space Station (ISS) are exposed to 0.2–1.0 mSv of space radiation on a daily basis.[1] Thus, several days spent in space will expose an individual to more radiation exposure than one year spent exposed to natural space radiation on Earth (~1.0 mSv/year). The biological effects of long-term, low-dose exposure to space radiation, including intracellular DNA and mitochondrial damage, may increase the risk of developing a number of disorders.[2,3] Such radiation effects are important factors to be considered in planning extended manned space missions.[3–5] Similarly, microgravity may influence cellular metabolism and function, including gene expression and signal transduction, and may result in cell death.[6]

Biological experiments involving radiation are aimed at examining dose-effect relationships in which a minimum dose yields a significant irradiation effect. The minimum dose is dependent on study endpoints, and the smaller the minimum dose, the more sensitive the endpoint. Studies of chromosomal aberration and micronucleus formation,[7–12] as well as mutation and transformation assays,[13,14] are sensitive approaches that utilize small doses of approximately 0.1 Gy (100 mGy). However, it remains controversial whether or not the dose-effect relationship is linear at lower doses, according to linear non-threshold (LNT), or non-LNT theory.[15] It is likely that the dose-effect relationship (whether LNT or non-LNT) is partially dependent on the endpoints used in a particular study. To verify the LNT dose-effect relationship, it would be necessary to conduct thousands of experiments.[15] Moreover, examinations at doses smaller than 0.1 Gy (100 mGy) would be difficult due to the lack of a model and/or endpoints. Transcriptomics is an effective tool for examining grouped signaling pathways such as apoptosis, and for investigating changes in the expression patterns of mitochondrial or protein-synthesis genes.

In the present study, we performed a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis in order to determine radiation and microgravity-related effects on gene expression in the human neuroblastoma cell line NB-1. The cells were exposed to single, low doses of 0.1 or

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1.0 mGy, or 10 times fractionated 0.1 or 1.0 mGy X-ray irradiation at 1 or 2 h time intervals. In addition, the cells were separately exposed to clinostat microgravity simulation conditions for 24 h.

Materials and Methods

Cells. The human neuroblastoma cell line NB-1 (RBC1953),[16] purchased from the Riken Bioresource Center Cell Bank (Ibaraki, Japan), was used in this study. The *p53* gene of these cells has a G215C polymorphism, a common polymorphism substituting an arginine for a proline at codon 72.[17] Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Inc., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C in humidified air containing 5% CO_2.

X-irradiation. Each flask of cells was irradiated with a dose of 0.1 or 1.0 mGy at room temperature using a Hitachi MBR-1505R X-ray generator (Hitachi, Tokyo, Japan). The machine was operated at 120 kVp and at either 0.2 mA with a filter of Al 1.0 mm + Cu 1.7 mm to achieve a dose of 0.1 mGy, or at 0.2 mA with a filter of Al 1.0 mm + Cu 0.2 mm to achieve a dose of 1.0 mGy. The dose rates were 0.59 mGy/min (9.84 nGy/s) and 3.69 mGy/min (61.5 nGy/s) for doses of 0.1 and 1.0 mGy, respectively, at a focus-surface distance of 60 cm in all cases. The doses were measures by Radiation Monitor (10x5-60, 9015; Radical Corporation, Monrovia, CA).

Clinostat treatment. Each flask of cells was subjected to microgravity treatment using a clinostat (Portable Microgravity Simulator PMS-1; Advanced Engineering Services Co., Ltd.). The rotation rates around the X- and Y-axes were 4.8 and 7.2 rpm, respectively. The machine operates to give theoretically 0 g to cells for 24 h.

Isolation of the total RNA. Total RNA was isolated using ISOGEN from cultured cells as recommended by the manufacturer (Nippon gene Toyama, Japan). Briefly, after washing cells by PBS 3 times, 1 ml of ISOGEN was added to cells and collected into 1.5 ml tubes. Two hundred µl of chloroform was added to the samples and vortexed. After centrifugation at 12,000 *g* for 15 min at 4°C, the aqueous phase was transferred to new 1.5 ml tubes and 0.5 ml of 2-propanol was added. After incubation for 5 min at R.T., centrifugation was performed at 12,000 *g* for 10 min at 4°C to precipitate RNA. The pellets were washed with 70% of EtOH and after centrifugation 7,500 *g* for 5 min at 4°C, pellets were dried briefly and dissolved in TE. The RNA quality was checked by measuring 260 nm absorbance and electrophoresis. All cDNAs were prepared by reverse transcription of 1 µg total RNA using oligo dT(20) primer (0.4 µM/50 µl final volume), and ReverTra Ace (TOYOBO) as recommended by the manufacturer. An equivalent volume of 0.1 µl of cDNA solution was used for quantification of specific cDNA by qRT-PCR.

SYBR Green-based qRT-PCR. Total RNA was isolated from NB-1 cells and cDNA was synthesized as described above. The sequences of primers were compared to those from the available human genome and the EST database (http://blast.genome.jp/) in order to select primers that would produce a single amplification product. The forward and reverse primers are listed in Table 1. The qRT-PCR assays were performed on an ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). Four sets of PCR profiles were used, depending on the primer sets (conditions A–D, Table 1). The experimental conditions were those recommended by the manufacturer, except for the annealing temperature, annealing time, elongation temperature, and elongation time; specifically, the differences were as follows: condition A, annealing and elongation 60°C 1 min; condition B, annealing 55°C 30 s and elongation 72°C 40 s; condition C, annealing 62°C 5 s and elongation 72°C 30 s; and condition D, annealing 55°C 5 s and elongation 72°C 30 s. All PCR assays were performed for 40 cycles. The size of single amplification products was further verified by gel electrophoresis. All data were normalized to an internal standard (glyceraldehyde-3-phosphate dehydrogenase; GAPDH). Three triplicate samples were used in an assay of qRT-PCR and repeated for three times. The average values were calculated and the bar was expressed as SD.

The NB-1 cells were irradiated with X-rays or were subjected to microgravity for 24 h prior to qRT-PCR gene expression analysis of cell death-related genes. The list of the genes is shown in Table 2. To examine the activation of apoptosis, the following genes were targeted: Bax (*BAX*); cytochrome *c* (*CYTC*); apoptotic protease activating factor 1 (*APAF1*); voltage-dependent anion channels (*VDACs*) 1, 2 and 3 (*VDAC1, 2 and 3*); caspases 3, 8 and 9, (*CASP3, 8 and 9*); p53 (*P53*); apoptosis-inducing factor (*AIF*); and adenine nucleotide translocators (*ANT*) 1 and 2 (*ANT1 and 2*). In addition, three more gene groups were targeted to examine the following. Apoptosis suppression: B-cell CLL/lymphoma 2 (*BCL2*) and manganese superoxide dismutase (*MnSOD*), autophagy related gene, Beclin1 (*BECN1*), and necrosis related gene, cyclophilin 40 (*CYP-40*).

Cells were exposed to single doses of radiation of 0.1 and 1 mGy, and analyses were performed 30 min and 2 h after irradiation. To evaluate the effects of long-term, low-dose exposure, cells were irradiated with doses of 0.1 and 1 mGy 10 times in 1 h-intervals (×10 [1 h]) and analyses were performed immediately, 30 min, and 2 h after irradiation. Cells were also irradiated at 2 h-intervals (×10 [2 h]) and analyses were performed immediately, 30 min, and 2 h after irradiation. The effects of microgravity, which was applied over a 24 h period using the clinostat, were also evaluated immediately after the procedure. Analyses of cell death-related genes were subsequently conducted.

Statistical analysis. Statistical analyses were performed using Student's *t* test. All *p* values less than 0.05 were considered to be statistically significant. Data are presented as the mean ± SD. Calculations were carried out using Microsoft Excel (Microsoft Corporation, Redmond, WA) on a MacBook Air (Apple Inc. Cupertino, CA).

Results

NB-1 cells were either subjected to microgravity for 24 h or irradiated with extremely low 0.1 and 1.0 mGy single or 0.1 mGy 10 times fractionated X-ray irradiation. The expression levels of cell death-related genes were then analyzed by qRT-PCR assay (Table 2). As shown in Table 3, microgravity treatment resulted in increased expression of *VDAC2* and decreased expression of *MnSOD*, while *CASP9* was also downregulated. Other genes investigated did not change significantly after a 24 h period of exposure of the cells to microgravity. On the other hand, 0.1 mGy single irradiation significantly decreased the expression of *CYTC*, *APAF1*, *VDAC1–3*, *CASP3*, *P53*, *AIF*, *ANT1,2*, *BCL2*, *MnSOD*, autophagy related *BECN1* and necrosis related *CYP-40*. 1.0 mGy single irradiation significantly downregulated *BAX*, *VDAC2*, *ANT1 and 2*, *BECN1* and *CYP-40*. 0.1 mGy fractionated irradiation increased the expression of *APAF1* and *BCL2* at both 1 and 2 h intervals. The expression of other genes (*CYTC*, *VDAC2 and 3*, *AIF*, *ANT1*, *MnSOD*, and *CYP-40*) was significantly downregulated at both 1 and 2 h intervals. For three of the genes (*CASP8*, *ANT2* and *BECN1*) expression was decreased at only the 1 h intervals, with no changes observed in the 2 h interval regime. A significantly downregulation of *BAX* was shown at the 2 h interval regime.

Discussion

The effects of 0.1 and 1.0 mGy irradiation and 24 h microgravity treatment on gene expression were examined using a human neuroblastoma cell line, NB-1, with active neuronal func-

Table 1. Primer sequences for genes examined in this study

Gene Group	Primer name	Primer sequence	PCR conditions
Apoptosis activation	*BAX*-F	5'-TTGGGTGAGACTCCTCAAGCCTCC-3'	C
	BAX-R	5'-TCTGAAGATGGGGAGAGGGCACCA-3'	
	CYTC-F	5'-GGAGAGGATACACTGATGGAGTATTTGGA-3'	A
	CYTC-R	5'-GAGATAAGCTATTAAGTCTGCCCTTTCTTC-3'	
	APAF1-F	5'-ATATTAAGTGGTGGAACGTTGTCACTGGG-3'	A
	APAF1-R	5'-AGTCACATATGTTTTGAAGTCAGGGGACAC-3'	
	VDAC1-F	5'-GGATACACTCAGACTCTTAAAGCCAGG-3'	A
	VDAC1-R	5'-ATGCTTGAAATTCCAGTTCCTAGACCAAGC-3'	
	VDAC2-F	5'-TTGGATCCCACTGCTTCCATTTCTGCAAA-3'	D
	VDAC2-R	5'-ATTAATGCTCTTCCCATCTACCAGAGCAGA-3'	
	VDAC3-F	5'-AAATAATGCCAGCCTGATTGGACTGGGTT-3'	A
	VDAC3-R	5'-TTCAAATCCCAAGCCAACCTTGTGACCT-3'	
	CASP3-F	5'-CAGAGGGGATCGTTGTAGAAGTCT-3'	B
	CASP3-R	5'-CGGCCTCCACTGGTATTTTATGAC-3'	
	CASP8-F	5'-AGAGCGATGTCCTCGAGGCGATGATATT-3'	A
	CASP8-R	5'-AAGTAGGCTGAGGCATCTGTTTCCCCAT-3'	
	CASP9-F	5'-CAAGAGTGGCTCCTGGTACGTTGAGA-3'	A
	CASP9-R	5'-CTGTTTATAAATCCCTTTCACCGAAACAGC-3'	
	P53-F	5'-TCAGTCTGAGTCAGGCCCTTCTGT-3'	B
	P53-R	5'-AGGCCTTGGAACTCAAGGATGCCC-3'	
	AIF-F	5'-CAAAGGTGTCATCTTCTACCTCAGGGAC-3'	A
	AIF-R	5'-CTCACCGTCCTTAATGATCTTCCTTGCT-3'	
	ANT1-F	5'-TTCTTAGGGGGTGTGGATCGGCATAA-3'	A
	ANT1-R	5'-CACATCAGCAGCCAACCTGGTCCTA-3'	
	ANT2-F	5'-ACTGTCACTGCTGTTGCCGGGTTGA-3'	A
	ANT2-R	5'-AAAAGCTTTGCCTCCTTCATCACGAGCA-3'	
Apoptosis suppression	*BCL2*-F	5'-GGAGGCTGGGATGCCTTTGTGGAA-3'	C
	BCL2-R	5'-TAGGCACCCAGGGTGATGCAAGCT-3'	
	MnSOD-F	5'-TTCTGGACAAACCTCAGCCCTAACGGT-3'	A
	MnSOD-R	5'-AACAGATGCAGCCGTCAGCTTCTCCTTAAA-3'	
Autophgay	*BECN1*-F	5'-AGTGGCGGCTCCTATTCCATCAAAAC-3'	A
	BECN1-R	5'-AAGACCCCACTTAAGATTCGTCAGCATGAA-3'	
Necrosis	*CYP-40*-F	5'-CAGAGCTCAAGGGATGGCAAGGATTAAAAG-3'	A
	CYP-40-R	5'-GACTTTCAGCAATTCTGCCTGGATAGCTTT-3'	
Internal control	*GAPDH*-F	5'-GGGCAAGGTCATCCCTGAGCTGAA-3'	A, B, C, D
	GAPDH-R	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	

Condition A	Annealing and elongation: 60°C 1 min
Condition B	Annealing: 55°C 30 s, elongation: 72°C 40 s
Condition C	Annealing: 62°C 5 s, elongation: 72°C 30 s
Condition D	Annealing: 55°C 5 s, elongation: 72°C 30 s

tion.[16,17] The radiation doses used in this study (0.1 or 1.0 mGy) are considered extremely low-dose. The surviving fraction after 2 Gy (2,000 mGy), which is the daily dose in conventional radiation therapy, ranges from 0.35–0.62.[18,19] In conventional radiation therapy, patients receive 2 Gy × 30 times in 6 weeks, totally 60 Gy to treat cancer. Therefore, the endpoint to evaluate such extremely dose, 0.1 and 1.0 mGy is limited to molecular methods, such as qRT-PCR.

Experimental microgravity data. DNA microarray technology is a powerful tool for identifying "space genes" that play key roles in cellular responses to microgravity.[20–23] In animals, the hindlimb suspension model simulates space flight models and is well tolerated by the animals with minimal evidence of stress.[24] Frigeri and co-workers investigated the effects of microgravity on gene expression in the mouse brain by using 2-week-old hindlimb-unloaded mice.[23] They found that the TIC class of genes (i.e., the class responsible for the transport of small molecules and ions into cells) had the highest percentage of upregulation, including that of *VDAC1*, whereas the most commonly downregulated genes were those of the JAE class [cell junction, adhesion, and extracellular matrix (ECM)].[23] Schatten *et al.*[25] reported that space flight and clinorotation causes apoptosis. However, this study showed relatively no change of apoptosis elements. In this study, microgravity conditions increased expression of apoptotic *VDAC2*[26] and decreased expression of antiapoptotic *MnSOD*,[27,28] while *CASP9* was downregulated (Table 2 and 3), suggesting that these cells may be more susceptible to further oxidative stress.

Wang and Good[29] demonstrated the similarity between old age and prolonged exposure to microgravity (i.e., in terms of heart, muscle, and bone atrophy), and emphasized that determining the underlying signal transduction pathways (e.g., the control mechanisms for the processes of proliferation, differentiation, and apoptosis) remains important for creating therapeutic modalities to retard, reduce, and prevent tissue atrophy, both in space and on earth. Experiments examining the differentiation of neuron-like PC12 and SH-SY5Y cells revealed less neurite extension under conditions of microgravity created by a rotating wall bioreactor (RWB) than that observed in static cultures, suggesting that less-

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Table 2. Cell death-related genes analyzed by qRT-PCR

Gene group related to apoptosis activation	
Gene	Description
BAX	Bax
CYTC	Cytochrome *c*
APAF1	Apoptotic protease activating factor 1
VDAC1	Voltage-dependent anion channel 1
VDAC2	Voltage-dependent anion channel 2
VDAC3	Voltage-dependent anion channel 3
CASP3	Caspase 3
CASP8	Caspase 8
CASP9	Caspase 9
P53	p53
AIF	Apoptosis-inducing factor
ANT1	Adenine nucleotide translocator 1
ANT2	Adenine nucleotide translocator 2

Gene group related to apoptosis suppression	
Gene	Description
BCL2	B-cell CLL/lymphoma 2
MnSOD	Manganese superoxide dismutase (SOD2)

Gene group related to autophagy	
Gene	Description
BECN1	Beclin1

Gene group related to necrosis	
Gene	Description
CYP-40	Cyclophilin 40 (Cyclophilin D)

than-optimal differentiation occurs under conditions of simulated microgravity relative to normal gravity.[29]

Stein *et al.*[30] found that energy intake, as well as protein synthesis, decreased in a correlated manner during extended (>3 months) flights on the Russan space station, MIR. Post-flight energy intake and protein synthesis returned to, but did not exceed, the pre-flight levels. Stein and Leskiw further examined oxidative damage, urinary excretion of F2 isoprostane (isoprostane), 8-iso-prostaglandin F2a (PG), and 8-oxo-7,8-dihydro-2 deoxyguanosine (8-OHdG), during and after space flight.[31] Samples were obtained from MIR space flight participants between 88 and 186 days in orbit. They concluded that oxidative damage was decreased in-flight, and oxidative damage was increased post-flight.[31] While the putative increase in oxidative stress associated with space flight may persist after a return to normal gravity, this effect appears to be more pronounced after an extended period of time spent in space.[32] However, Hollander *et al.*[33] reported that space flight itself was associated with the downregulation of antioxidant defense systems in the rat liver. Space flight (STS-63 for 8 days, $n = 6$, male Sprague-Dawley rats) significantly reduced catalase, GSH reductase, and GSH sulfur-transferase activity in the liver. The relative levels of mRNA for Cu-Zn SOD and catalase were also significantly reduced by space flight. In addition, space flight dramatically reduced hepatic GSH, glutathionedisulfide, and total GSH contents, and these reductions were accompanied by reduced γ-glutamyl transpeptidase activity. Furthermore, space-flight rats exhibited an increase in the liver malondialdehyde concentration. Taken together, these results indicate that space flight can induce a downregulation of the antioxidant defense capacity and promote oxidative stress in the liver.[33] Degan *et al.*[34] reported that exposure to simulated microgravity reduced the intracellular ATP concentration in freshly drawn lymphocytes and lymphoblastoid cells (LB and COR3) by about 40–50% of the level found under normal growth conditions. Poly(ADP-ribose) polymerase (PARP) activity increased, indicating that cells exposed to reduced gravity

Table 3. Results of gene expression analysis by qRT-PCR

Apoptosis activation	*BAX*	*CYTC*	*APAF1*	*VDAC1*	*VDAC2*	*VDAC3*	*CASP3*
Microgravity	0.94 ± 0.31	1.22 ± 0.31	1.04 ± 0.27	1.03 ± 0.30	1.25 ± 0.10¶	1.02 ± 0.13	1.06 ± 0.16
0.1 mGy × 1–0.5 h	0.99 ± 0.15	0.70 ± 0.12§	0.63 ± 0.16§	0.57 ± 0.18§	0.58 ± 0.13§	0.51 ± 0.07§	0.79 ± 0.07§
0.1 mGy × 1–2 h	0.83 ± 0.33	0.66 ± 0.14§	0.67 ± 0.23§	0.63 ± 0.12§	0.45 ± 0.09§	0.50 ± 0.11§	0.89 ± 0.13
1 mGy × 1–0.5 h	0.65 ± 0.19	0.88 ± 0.26	0.95 ± 0.51	0.96 ± 0.23	0.20 ± 0.05§	1.03 ± 0.17	0.81 ± 0.14
1 mGy × 1–2 h	0.55 ± 0.12§	1.02 ± 0.28	0.78 ± 0.27	0.85 ± 0.16	0.76 ± 0.26	0.98 ± 0.21	0.85 ± 0.11
0.1 mGy-1 h × 10–0 h	0.93 ± 0.21	0.78 ± 0.15	1.35 ± 0.34¶	0.80 ± 0.17	0.42 ± 0.18§	0.37 ± 0.11§	0.82 ± 0.20
0.1 mGy-1 h × 10–0.5 h	0.91 ± 0.16	1.03 ± 0.24	1.49 ± 0.33¶	1.18 ± 0.30	0.33 ± 0.08§	0.53 ± 0.04§	0.87 ± 0.14
0.1 mGy-1 h × 10–2 h	1.04 ± 0.40	0.71 ± 0.19§	1.14 ± 0.19	0.87 ± 0.18	0.45 ± 0.22§	0.55 ± 0.06§	0.89 ± 0.18
0.1 mGy-2 h × 10–0 h	0.72 ± 0.27	1.00 ± 0.18	1.26 ± 0.26¶	1.11 ± 0.13	0.74 ± 0.18§	0.72 ± 0.09§	0.83 ± 0.13
0.1 mGy-2 h × 10–0.5 h	1.09 ± 0.25	0.94 ± 0.15	1.66 ± 0.41¶	0.94 ± 0.14	0.47 ± 0.21§	0.64 ± 0.06§	0.92 ± 0.21
0.1 mGy-2 h × 10–2 h	0.70 ± 0.16§	0.71 ± 0.13§	1.17 ± 0.29	0.93 ± 0.09	0.54 ± 0.34§	0.63 ± 0.06§	0.86 ± 0.13

Apoptosis activation	*CASP8*	*CASP9*	*P53*	*AIF*	*ANT1*	*ANT2*
Microgravity	0.65 ± 0.20	0.73 ± 0.36§	0.94 ± 0.17	0.91 ± 0.22	1.10 ± 0.34	1.04 ± 0.14
0.1 mGy × 1–0.5 h	0.98 ± 0.37	0.91 ± 0.16	0.65 ± 0.11§	0.66 ± 0.14§	0.74 ± 0.18§	0.76 ± 0.18§
0.1 mGy × 1–2 h	1.10 ± 0.43	0.88 ± 0.19	0.71 ± 0.16§	0.69 ± 0.14§	0.79 ± 0.22§	0.68 ± 0.16§
1 mGy × 1–0.5 h	1.00 ± 0.40	1.00 ± 0.45	1.10 ± 0.19	0.97 ± 0.28	1.06 ± 0.31	0.81 ± 0.12§
1 mGy × 1–2 h	0.83 ± 0.14	1.03 ± 0.43	1.22 ± 0.42	0.92 ± 0.13	0.77 ± 0.16§	0.75 ± 0.28§
0.1 mGy-1 h × 10–0 h	0.76 ± 0.33§	0.84 ± 0.38	0.79 ± 0.21§	0.66 ± 0.14§	0.75 ± 0.17§	0.87 ± 0.10§
0.1 mGy-1 h × 10–0.5 h	0.85 ± 0.35§	0.79 ± 0.24	0.87 ± 0.17	0.77 ± 0.21§	0.78 ± 0.18§	0.79 ± 0.22§
0.1 mGy-1 h × 10–2 h	0.65 ± 0.13§	0.86 ± 0.45	0.92 ± 0.18	0.87 ± 0.28§	0.83 ± 0.23§	0.84 ± 0.17
0.1 mGy-2 h × 10–0 h	1.15 ± 0.16	0.87 ± 0.32	1.01 ± 0.32	0.71 ± 0.13§	0.82 ± 0.16§	0.99 ± 0.23
0.1 mGy-2 h × 10–0.5 h	0.93 ± 0.13	0.85 ± 0.25	1.13 ± 0.13	0.82 ± 0.12§	0.77 ± 0.10§	0.95 ± 0.19
0.1 mGy-2 h × 10–2 h	1.03 ± 0.11	0.86 ± 0.40	0.99 ± 0.15	0.95 ± 0.11	0.82 ± 0.19	0.86 ± 0.20

§Decreased significantly by *t* test ($p<0.05$). ¶Increased significantly by *t* test ($p<0.05$).

are stressed. Maillet *et al.*[35] reviewed microgravity in terms of an accelerated model of nutritional disturbances, and concluded that adopting an integrated approach will be essential for optimizing the health of astronauts. They also found that current dietary approaches for adults exposed to normal gravity are inadequate for preventing significant changes in the nutritional status of astronauts, and they suggested areas for further research in both fields of ground and space medicine. It is noted that since space experiments consist of microgravity and space radiation conditions, these findings might be caused by space radiation and not by microgravity.

Experimental data from studies of exposure to time spent in space and extremely low-dose radiation. In this study, 0.1 mGy single dose irradiation resulted in significant downregulation of *CYTC*, *APAF1*, *VDAC1–3*, *CASP3*, *P53*, *AIF*, *ANT1 and 2*, *BCL2*, *MnSOD*, autophagy related *BECN1* and necrosis related *CYP-40* (Table 3 and 4). However, 1.0 mGy single irradiation revealed significant downregulation in *BAX*, *VDAC2*, *ANT1 and 2*, autophagy related *BECN1*[36] and necrosis related *CYP-40*[37] (Table 3 and 4), suggesting the effects of 0.1 and 1.0 Gy single dose irradiation differentially affect the cells. In the extremely low 0.1 mGy dose fractionated irradiation group, proapoptotic *APAF1* significantly increased. On the other hand, antiapoptotic *BCL2*[38] significantly increased, and downregulation of proapoptotic *CYTC*, *VDAC2 and 3*, *CASP8*, *AIF*, *ANT1 and 2* was observed. These results demonstrate changes in the expression profiles of apoptosis-related genes in NB-1 cells subjected to low-dose radiation, with a shift towards an inhibition of apoptosis. Radiation-induced apoptosis is considered to be p53-dependent.[39–48] In this study, the level of *P53* did not show significant change. In addition, the levels of *CASP3* and *CASP9* did not show change. Taken together, these results suggest that long-term, low-dose radiation exposure may inhibit apoptosis, and that non-apoptotic death pathways may also not be affected by such exposure due to downregulation of *BECN1* and *CYP-40* (Table 4). However, the level of *MnSOD*, which suppresses apoptosis, was downregulated in the fractionated regimes, suggesting the possibility of apoptosis. These results may indicate extremely low dose 0.1 mGy X-irradiation fractionation regimes do not promote cell death, but may make cells susceptible to further oxidative stress due to MnSOD downregulation.

Few studies have investigated the effects of extremely low-dose irradiation. Suzuki and coworkers found that extremely low-dose X-ray irradiation (20–50 mGy) stimulated the proliferation of human cultured cells and the phosphorylation of extracellular signal-regulated kinase.[49] Ding and coworkers applied cDNA microarray analyses to G1-arrested normal human skin fibroblasts subjected to X-ray irradiation (low dose, 20 mGy; high dose, 4 Gy).[50] The predominant cell functions affected by low-dose radiation were those involved in cell-cell signaling, signal transduction, cell development, and DNA damage responses. At high-doses, the responding genes were involved in apoptosis and cell proliferation. Interestingly, several genes (i.e., the cytoskeleton components anillin (*ANLN*; actin binding protein), and keratin 15 (*KRT15*) and the cell-cell signaling genes Growth factor receptor-bound protein 2 (GRB2)-related adapter protein 2 (*GRAP2*) and G-protein-coupled receptor 51 (*GPR51*) responded to low-dose radiation, but not to high-dose radiation. Pathways that are specifically activated by low-dose radiation were also evident. The researchers concluded that these quantitative and qualitative differences in gene expression may help explain the non-linear correlation of biological effects of ionizing radiation ranging from low doses to high doses.[50] Moreover, Ogura and coworkers found that the mutation frequency in a 500 μGy-irradiated group of Drosophila melanogaster was significantly lower than that of the control group, whereas in the 10 Gy-irradiation group, the mutation frequency was significantly higher than that of the control group.[51] They suggested the apparent upregulation of a positive regulator of apoptosis immediately after irradiation with 500 μGy, furthermore suggesting that the linear non-threshold (LNT) model for stochastic effects of ionizing radiation was not applicable. Thus, their results will be relevant to further discussions of the LNT model.

Kiefer[52] addressed some pertinent questions related to the assessment of radiation risk to humans in space. The effects remain to be clarified for low-dose rates of γ-rays, protons, and heavy particles, which dominate in the case of space-acquired radiation. It will be necessary to conduct experiments in space in order to determine the combined effects of space radiation and microgravity. Ohnishi and coworkers[53] discussed cellular risk as well as molecular events following exposure to space radiation, and they have advocated for prioritizing space-radiation research. Gridley *et al.*[54] conducted a RT-PCR assay to investigate the effects of pre-exposure ("Pre") of low-dose/low-dose-rate photons (^{57}Co, total of 0.049 Gy at 0.24 mGy/h) on gene expression in the 2 Gy whole-body irradiated C57BL/6 mouse liver. There were various genes downregulated in all groups studied (13 in the "2 Gy-alone", 16 in the "Pre", and 16 in the "Pre + 2 Gy" groups). These results suggest that exposure to only 0.049 Gy at 0.24 mGy/h can alter gene expression.

Space radiation may increase oxidative stress by reducing levels of antioxidants. Manda *et al.*[55] tested the effects of high-linear energy transfer (LET) ^{56}Fe beams (500 MeV/nucleon, 1.5 Gy) on memory impairment and apoptosis using male C57BL mice. High-LET radiation substantially impaired the reference memory of mice 30 days after irradiation, whereas no significant effect was observed on the motor activities of the mice. The memory dysfunction caused by irradiation was attenuated by α-lipoic acid.

Table 4. Results of gene expression analysis by qRT-PCR (continued)

Apoptosis suppression	*BCL2*	*MnSOD*	Autophagy	*BECN1*	Necrosis	*CYP-40*
Microgravity	1.07 ± 0.17	0.67 ± 0.18§	Microgravity	0.87 ± 0.11	Microgravity	0.88 ± 0.14
0.1 mGy × 1–0.5 h	0.75 ± 0.19§	0.61 ± 0.07§	0.1 mGy × 1–0.5 h	0.83 ± 0.19§	0.1 mGy × 1–0.5 h	0.74 ± 0.10§
0.1 mGy × 1–2 h	1.04 ± 0.32	0.64 ± 0.18§	0.1 mGy × 1–2 h	0.80 ± 0.20§	0.1 mGy × 1–2 h	0.72 ± 0.11§
1 mGy × 1–0.5 h	1.04 ± 0.23	0.94 ± 0.25	1 mGy × 1–0.5 h	0.73 ± 0.16§	1 mGy × 1–0.5 h	0.86 ± 0.10§
1 mGy × 1–2 h	0.85 ± 0.29	1.06 ± 0.33	1 mGy × 1–2 h	0.84 ± 0.12§	1 mGy × 1–2 h	0.81 ± 0.09§
0.1 mGy-1 h × 10–0 h	1.37 ± 0.19¶	0.50 ± 0.13§	0.1 mGy-1 h × 10–0 h	0.82 ± 0.11§	0.1 mGy-1 h × 10–0 h	0.74 ± 0.13§
0.1 mGy-1 h × 10–0.5 h	2.38 ± 0.39¶	0.65 ± 0.10§	0.1 mGy-1 h × 10–0.5 h	0.62 ± 0.09§	0.1 mGy-1 h × 10–0.5 h	0.78 ± 0.16
0.1 mGy-1 h × 10–2 h	1.33 ± 0.21¶	0.79 ± 0.15§	0.1 mGy-1 h × 10–2 h	0.83 ± 0.15§	0.1 mGy-1 h × 10–2 h	0.56 ± 0.17§
0.1 mGy-2 h × 10–0 h	1.56 ± 0.36¶	0.75 ± 0.14§	0.1 mGy-2 h × 10–0 h	0.96 ± 0.08	0.1 mGy-2 h × 10–0 h	0.73 ± 0.34§
0.1 mGy-2 h × 10–0.5 h	1.72 ± 0.49¶	0.80 ± 0.13§	0.1 mGy-2 h × 10–0.5 h	0.97 ± 0.06	0.1 mGy-2 h × 10–0.5 h	0.71 ± 0.30§
0.1 mGy-2 h × 10–2 h	1.57 ± 0.18¶	0.82 ± 0.11	0.1 mGy-2 h × 10–2 h	1.07 ± 0.14	0.1 mGy-2 h × 10–2 h	0.75 ± 0.36§

§Decreased significantly by *t* test ($p<0.05$). ¶Increased significantly by *t* test ($p<0.05$).

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Radiation-induced apoptotic and necrotic cell death of granule and Purkinje cells was also significantly inhibited by pretreatment with α-lipoic acid.[55] The effects of single whole-body irradiation of 3 Gy by γ-rays, 3 Gy by 1,000 MeV protons, or 50 cGy (500 mGy) by 1 GeV/nucleon iron ions were examined in terms of the total antioxidant status (TAS) of male CBA mice, and the protective effects of diet supplementation with Bowman-Birk Inhibitor Concentrate (BBIC), L-selenomethionine (L-SeM), or a combination of *N*-acetyl cysteine, sodium ascorbate, coenzyme Q10 (CoQ10), α-lipoic acid, and vitamin E succinate were also examined.[56,57] Guan *et al.*[57] found that BBIC, L-SeM, and certain antioxidant combinations that include CoQ10 exhibit protective effects, suggesting that antioxidants act as protectors against adverse biological effects induced by space radiation. Our qRT-PCR results regarding changes in cell death-related gene expression revealed that single doses of 0.1 mGy, as well as fractionated dose regimes, downregulated apoptosis-related genes (*CYTC*, *VDAC2 and 3*, *AIF*, *ANT1 and 2*). However, downregulation of antioxidant gene (*MnSOD*) and increased apoptosis related gene (*APAF1*) expression may indicate increased risk by extremely low dose irradiation.

Conclusion

The qRT-PCR examination of cell death-related gene expression changes revealed that microgravity treatment increased apoptosis related gene *VDAC2*, but reduced the expression levels of apoptosis-related gene *CASP9* and the antioxidant gene *MnSOD* using a neurobastoma cell line NB-1. After 0.1 mGy single irradiation, most of the genes examined were downregulated, while 1.0 mGy single irradiation revealed significant downregulation in only six genes (*BAX*, *VDAC2*, *ANT1 and 2*, *BECN1* and *CYP-40*), suggesting 0.1 and 1.0 mGy radiation have different effects on cells. After 0.1 mGy fractionated irradiation, apoptotic *APAF1* and anti-apoptotic *BCL2* were upregulated, and apoptosis related *CYTC*, *VDAC2 and 3*, *CASP8*, *AIF*, *ANT1* genes were significantly downregulated. These results suggest decreased risk of apoptosis. However, downregulation of *MnSOD* was also observed. These results may indicate that space environment including microgravity and space radiation may increase oxidative stress by reducing MnSOD.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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