ARTICLE
Journal of Cellular Biochemistry 107:723–731 (2009)

Journal of Cellular Biochemistry

Simulated Microgravity Decreases DNA Repair Capacity and Induces DNA Damage in Human Lymphocytes

Ragini Kumari, Kamaleshwar P. Singh, and James W. DuMond Jr.*

Department of Biology, Texas Southern University, Houston, Texas

ABSTRACT

The effect of simulated microgravity on DNA damage and apoptosis is still controversial. The objective of this study was to test whether simulated microgravity conditions affect the expression of genes for DNA repair and apoptosis. To achieve this objective, human lymphocyte cells were grown in a NASA-developed rotating wall vessel (RWV) bioreactor that simulates microgravity. The same cell line was grown in parallel under normal gravitational conditions in culture flasks. The effect of microgravity on the expression of genes was measured by quantitative real-time PCR while DNA damage was examined by comet assay. The result of this study revealed that exposure to simulated microgravity condition decreases the expression of DNA repair genes. Mismatch repair (MMR) class of DNA repair pathway were more susceptible to microgravity condition-induced gene expression changes than base excision repair (BER) and nucleotide excision repair (NER) class of DNA repair genes. Downregulation of genes involved in cell proliferation (*CyclinD1* and *PCNA*) and apoptosis (*Bax*) was also observed. Microgravity-induced changes in the expression of some of these genes were further verified at the protein level by Western blot analysis. The findings of this study suggest that microgravity may induce alterations in the expression of these DNA repair genes resulting in accumulation of DNA damage. Reduced expression of cell-cycle genes suggests that microgravity may cause a reduction in cell growth. Downregulation of pro-apoptotic genes further suggests that extended exposure to microgravity may result in a reduction in the cells' ability to undergo apoptosis. Any resistance to apoptosis seen in cells with damaged DNA may eventually lead to malignant transformation of those cells. J. Cell. Biochem. 107: 723–731, 2009.

KEY WORDS: MICROGRAVITY; DNA REPAIR; DNA DAMAGE

Evidence from studies utilizing ground-based simulated microgravity conditions as compared to real space flight suggest that microgravity conditions result in several physiological changes that mimic those seen in space flight. Those changes include decline in cellular immune function [Leach et al., 1990; Wu et al., 1993], cardiovascular changes [Baevsky et al., 2007], bone loss [Vico et al., 2000], muscular atrophy [Hayes et al., 1992], and renal stones [Pietrzyk et al., 2007]. The physiological effects of microgravity may increase the risk for human diseases including cancer [Barr et al., 2007]. However, the molecular mechanisms which produce the adverse effects of microgravity on living cells have not been elucidated.

Recent studies have shown that exposure to microgravity conditions induce change in the expression of genes that control important cellular functions [Ward et al., 2006; Clement et al., 2007]. The aberrant expression of DNA repair genes resulting in genomic instability is characteristic of various human diseases. Previous reports suggest that both real space flight and simulated microgravity conditions result in gene expression changes and genomic instability at both the chromosome and DNA level. For example, chromosomal aberrations have been observed in the lymphocytes of space crews after space flight [Obe et al., 1997; Yang et al., 1997; George et al., 2001]. A high frequency of mutations was detected in*Drosophila melanogaster* after space flight as compared to the ground control group [Ikenaga et al., 1997]. Changes in the expression of several genes associated with cell proliferation and growth factor cascades have been reported in male rats after 17 days on the NASA-STS-90 Neurolab space flight [Taylor et al., 2002]. In this study, gene expression analysis at mRNAs level was performed by using DNA microarray and the result revealed 12 genes were upregulated by over twofold, and 38 were downregulated in rats group from 17 days space flight as compared to control group of rats. Cell-cycle genes and signal transduction proteins, such as *p21 Cip1*, *retinoblastoma* (*Rb*), *Cyclins G1/S*, *-E*, and *-D3*, *MAP kinase 3*, *MAD3*, and ras-related protein *RAB2* were among the genes that were inhibited in the muscle of the rat from space flight as compared

Grant sponsor: NIH; Grant number: RR03045; Grant sponsor: NASA-URC; Grant number: NCC9-165.
*Correspondence to: Dr. James W. DuMond, Jr., Department of Biology, Texas Southern University, Houston, TX 77004. E-mail: dumond_jw@tsu.edu
Received 17 December 2008; Accepted 12 March 2009 • DOI 10.1002/jcb.22171 • © 2009 Wiley-Liss, Inc.
Published online 4 May 2009 in Wiley InterScience (www.interscience.wiley.com).

to the control group of rat on ground [Taylor et al., 2002]. Changes in mitochondrial genes have also been reported in space-flown rats [Nikawa et al., 2004]. DNA damage as detected by comet assay has been reported in mice exposed to irradiation and simulating high-altitude flight conditions [Sirota et al., 2007]. These studies clearly indicate that exposure to microgravity causes gene expression changes and genetic aberrations. However, the effect of microgravity on the expression of DNA repair genes is not well understood. Therefore, one of our objectives in this study was to determine whether prolonged exposure to simulated microgravity affects the cellular DNA repair capacity that may lead to DNA damage and genetic aberrations and consequently the risk of patho-physiological changes including neoplastic transformation of cells.

The published reports suggest that base excision repair (BER), nucleotide excision repair (NER), and post-replicative mismatch repair (MMR) are three important DNA repair pathways in mammalian cells and each pathway involves many DNA repair genes [Kunkel and Erie, 2005; Denver et al., 2006]. In addition to these DNA repair genes, P53 and proliferating cell nuclear antigen (PCNA) are other crucial genes that are involved in the important cellular function associated with DNA repair process [Xu and Morris, 1993]. The P53 pathway has been shown to mediate cellular stress response and can initiate DNA repair [Vazquez et al., 2008]. In vitro assay for DNA repair has shown that PCNA participates in both NER and MMR [Johnson et al., 1996]. It has also been reported that loss of expression of genes involved in these DNA repair pathways results in oncogenesis [Buermeyer et al., 1999; Cleaver, 2005]. To gain an insight about the effect of prolonged exposure to simulated microgravity on DNA repair capacity that may explain the space flight associated cancer risk, we measured the expression of representative genes from BER, NER, and MMR as well as genes such as *P53*, *Gadd 45 gamma*, and *PCNA* involved in DNA repair process.

There are several studies conducted in space and in simulated microgravity on ground that suggest that human lymphocytes in culture are sensitive to gravity changes as evidenced by profound alterations of several cellular function [Cogoli et al., 1984, 1993; Cogoli-Greuter et al., 1995; Schmitt et al., 1996; Cooper and Pellis, 1998; Walther et al., 1998; Cooper et al., 2001; Risin and Pellis, 2001]. Moreover, these cells are easy to grow in suspension culture. These reports suggest that human lymphocyte cell lines are very sensitive and responsive to microgravity condition and therefore in this study we used the human lymphocytes as an in vitro cell model to determine the effects of microgravity on gene expression changes and cell survival.

Space flight conditions include both microgravity and exposure to space radiation. Previously reported genetic aberrations seen under space flight conditions have not been specifically attributed to microgravity or to space radiation. Moreover, previously reported studies of gene expression changes induced by ground-based simulated microgravity were conducted for shorter durations (in hours) of simulated microgravity exposure. The purpose of this study was to determine the effect of short-term (4 and 72 h) as well as extended-duration exposure (7 days) to simulated microgravity on the expression of DNA repair genes and genes involved in cell growth in human lymphocytes. The effect of an extended period of exposure to simulated microgravity on DNA damage was also investigated.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Trizol® reagent for total RNA isolation was purchased from Invitrogen, Inc. (Carlsbad, CA). One-step RT-PCR kit for real-time PCR was obtained from BioRad Laboratories, Inc. (Hercules, CA). Antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The comet assay kit was purchased from Trevigen (Gaithersburg, MD).

CELL CULTURE AND SIMULATION OF MICROGRAVITY

Human B-lymphocytes (Cat # GM037978, Coriell Cell Repository) and T-lymphocytes, (Cat # GM04155, Coriell Cell Repository) cell cultures were maintained in DMEM F-12 medium supplemented with 10% fetal bovine serum. For ground-based simulation of microgravity, a rotary cell culture system (RCCS), also known as a Clinostat, developed by NASA was used. Rotation in the Clinostat mimics the microgravity environment by nullifying the gravitational vector through continuous averaging. To investigate the effect of microgravity, actively growing lymphocyte cells were maintained in the Clinostat for 4 h, 72 h, and 7 days. In parallel, cells at similar cell densities were maintained in culture flasks for similar time points in normal gravity condition on ground as controls. Both groups of cells were kept under sterile conditions in a cell culture incubator maintained at 5% CO_2 and 37°C.

TRYPAN BLUE EXCLUSION METHOD FOR CELL VIABILITY

Cell viability was measured using the trypan blue exclusion method. Cells were collected by centrifugation at 1,500 rpm for 5 min. The cell pellets were washed twice with PBS and stained with 0.025% trypan blue in PBS for 3 min. Blue cells, having incorporated the dye, were scored as being dead. Cell counts were performed using a light microscope and a hemocytometer. The number of dead cells and live cells relative to the total number of cells present was expressed as a percentage of the total number of cells counted.

RNA ISOLATION

Total RNA from lymphocytes grown in the Clinostat was isolated using Trizol reagent (Invitrogen, Inc.). Total RNA from the ground condition control cells was isolated similarly. RNA was quantified spectrophotometrically, and the purity and integrity was checked by ethidium bromide staining after resolution on a 1.0% agarose gel.

QUANTITATIVE REAL-TIME PCR

Gene expression was measured by quantitative real-time PCR (QPCR) method and by using the one-step RT-PCR kit with SYBR green (BioRad Laboratories, Inc.). Single-step RT-PCR amplifications starting with total RNA (200 ng) were performed in 96-well optical reaction plates using an iCycler (BioRad Laboratories, Inc.) programmed for reverse transcription at 50°C for 15 min, denaturation and RT enzyme inactivation at 95°C for 5 min, followed by 40 cycles of 10 s denaturation at 95°C and 30 s primer annealing and strand extension at 60°C. The oligonucleotide primers

for real-time PCR were designed by using OligoPerfect™ Designer, a free software from Invitrogen, Inc. The specificity of PCR products was verified by melting curve analysis between 55 and 95°C at 0.5°C temperature increments, and the size specificity of the PCR product was further confirmed on agarose gel. The single peak in melt curve analysis and expected size of PCR product on agarose gel confirmed the specificity of the primers. Threshold cycle number (C_t value) was analyzed using iCycler IQ optical system software (BioRad Laboratories, Inc., version 3.0a). QPCR results were normalized to the C_t value of *GAPDH* from the same sample and the fold change in the expression of each gene was calculated by using the $\Delta\Delta C_t$ method [Livak and Schmittgen, 2001]. Amplification reactions for each sample were performed in triplicate. A non-template control was included in each experiment. The primer sequences used for the quantitative PCR experiments are given in Table I.

WESTERN BLOT ANALYSIS

Control and bioreactor grown cells were harvested and total cellular protein was extracted by lysis in extraction buffer (50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride). The samples were then centrifuged and the protein content of the supernatant was determined by Bradford assay [Bradford, 1976]. Thirty micrograms of total cell lysate from each group sample was electrophoretically separated on a 10% SDS–PAGE gel, followed by protein transfer onto nitrocellulose. The membranes were blocked with 5% non-fat dried milk in 1× PBS containing 0.05% Tween-20, overnight at 4°C. The blots were then reacted with a 1:100 dilution of primary antibody against *GADD 45 gamma*, *P53*, *PCNA*, *XPC*, or *GAPDH* (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After three washes of 5 min each with PBST, the membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) at a dilution of 1:1,000 for 1 h at room temperature. Membranes were again given three washes of 5 min each with PBST. The specific protein bands were then visualized by using an enhanced chemiluminescence system according to the manufacturer's instructions (Amersham, Piscataway, NJ).

COMET ASSAY

The comet assay was performed (comet assay kit; Trevigen) under alkaline conditions following the method as originally described earlier [Singh et al., 1988] with slight modifications. Briefly, harvested lymphocytes were centrifuged at 700*g*, and the cell pellets were washed twice in 1× PBS. The cell pellet was resuspended in 1× PBS at 10^5 cells/ml. An aliquot of 50 μl (5,000 cells) of cell suspension was mixed with 500 μl of 1% (w/v) low melting point (LMP) agarose dissolved in 1× PBS. Seventy-five microliters of the cell suspension in agarose was then quickly pipetted into each of three wells of a comet slide (Trevigen) and allowed to set at 4°C for 10 min in the dark. The slides were then immersed in prechilled lysis solution (2.5 M NaCl, 100 mM sodium-EDTA, 10 mM Tris, pH 10) for 60 min at 4°C to remove cellular proteins. Following lysis, the slides were placed in a horizontal gel electrophoresis unit, incubated in freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) then electrophoresed (1 V/cm tank length) for 45 min at room temperature. Following electrophoresis, the slides were immersed in neutralization buffer (0.4 M Tris–HCl, pH 7.5) and then gently washed three times for 5 min at 4°C to remove alkalis and detergents. Finally, the slides were fixed in 70% ethanol for 5 min and stored in the dark to dry completely. Immediately before image analysis, the gels on each slide were stained with 50 μl of diluted SYBR green (1:10,000 dilutions in Tris–EDTA buffer) in the dark for 5 min at 4°C. A coverslip was placed over the moist gel, and the slides were examined using 100× and 200× magnifications on an epifluorescence microscope (Nikon) at 460 nm. Images were captured using a digital camera and saved as TIFF/JPEG files to compact disk. To minimize the DNA damage from ambient ultraviolet radiation, all steps were performed with reduced light conditions.

DNA damage was analyzed by visual scoring of comet tail morphology (intact nuclei with no tails indicate no DNA damage vs. nuclei with tails which indicate DNA damage), and comet tail length. The percentage of comet tail DNA was chosen as the parameter for expression of DNA damage because it has been shown to have minimum variation between experimental trials and has also been recommended for DNA damage analysis by comet assay [Halliwell, 1994; Sohal and Weindruch, 1996; Marlin et al., 2004].

TABLE I. A List of Genes and Their Forward and Reverse Primer Sequences Used for Gene Expression Analysis by Quantitative Real-Time PCR

Gene	Forward Primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (bp)
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT	116
CyclinD1	AACTACCTGGACCGCTTCCT	CCACTTGAGCTTGTTCACCA	204
PCNA	TGCGGCCGGGTTCAGGAGTCA	CAGGCAGGCGGGAAGGAGGAAAGT	348
P53	GGCCCACTTCACCGTACTAA	GTGGTTTCAAGGCCAGATGT	156
Bcl2	GGATGCCTTTGTGGAACTGT	AGCCTGCAGCTTTGTTTCAT	236
Bax	TTTGCTTCAGGGTTTCATCC	CAGTTGAAGTTGCCGTCAGA	246
ERCC1	CCCTGGGAATTTGGCGACGTAA	CTCCAGGTACCGCCCAGCTTCC	273
ERCC3	CCAGGAAGCGGCACTATGAGG	GGTCGTCCTTCAGCGGCATTT	171
ERCC5	AATCGAAGGCAGGCCCGTGGG	ATTCGGGAGCCCAGGTGCGTC	462
ERCC6	TTGAGCTGCAGGGTTTGGGTG	TGCATCCTCCTCCAGACTGGC	383
MSH2	GCCATTTTGGAGAAAGGACA	CTCACATGGCACAAAACACC	228
MSH4	TGGTGCAAGCTCATCTTCTG	TCACCTCTGGCAAGTCCTCT	204
MSH6	CCCCACCAGTTGTGACTTCT	CACGTTGCATTGCTCTCAGT	284
PMS2	CAGGACATGTCAGCCTCTCA	GGCTGCTTGATTTTCTCCAG	189
MLH1	TGGGACGAAGAAAAGGAATG	GATCAGGCAGGTTAGCAAGC	250
XPC	CCAGAGCAGGCGAAGACAAGA	AAGCGGGCTGGGATGATGGAC	215
OGG1	GCGTGCGCAAGTACTTCCAGC	GCATCACATGACCAATTACTG	584

STATISTICAL ANALYSIS

Two-tailed *t*-tests (paired samples for the means) with a hypothesized difference of 0 were used to analyze the cell counts and gene expression data. An ANOVA was completed on all data to confirm that the source of variation of the data was between and not within treatment groups. The criterion for significance [alpha (α)] was set at 0.05 for all statistical tests. Thus, data with a $P \leq 0.05$ were determined to be significantly different.

RESULTS

ABERRANT EXPRESSION OF GENES IN RESPONSE TO SIMULATED MICROGRAVITY CONDITIONS

DNA REPAIR GENES. The effect of both, the short durations (4 and 72 h) and an extended (7 days) period of exposures to a simulated microgravity environment on the expression of DNA repair genes in human lymphocytes was measured by quantitative real-time PCR. Twelve DNA repair genes tested were representatives from BER, NER, and MMR class of DNA repair pathways. Among the 12 DNA repair genes analyzed, three genes at 4 h and four genes at 72 h were significantly downregulated in lymphocytes grown in simulated microgravity condition as compared to the lymphocytes grown in normal gravity condition on ground as control (Fig. 1). *OGG1* was the only gene that was significantly upregulated at 72 h simulated microgravity condition. Exposure to simulated microgravity condition for 7 days resulted in significant downregulation of eight genes and significant upregulation of one gene (Fig. 1). As compared to BER and NER, the genes from MMR class of DNA repair pathway were more susceptible to microgravity condition. For example, two out of three genes downregulated at 4 h were from MMR. Similarly, three out of four genes at 72 h and four out of eight genes at 7 days that were significantly downregulated in microgravity condition were from MMR class of DNA repair pathway. In terms of fold change also the MMR genes had higher fold changes as compared to the BER and NER genes at all the three time points of simulated microgravity exposure (Fig. 1). A time-dependent decrease in the expression of MMR genes (*MSH2*, *MSH4*, *MSH6*, and *MLH1*) was observed in lymphocytes grown on simulated microgravity condition (Fig. 1).

CELL CYCLE AND APOPTOSIS-RELATED GENES

To determine the effects of extended exposure (7 days) to simulated microgravity on cell growth and apoptosis, the expression of cell-cycle genes, *CyclinD1*, *PCNA*, and *P53*, an anti-apoptotic gene, *Bcl-2*, and a pro-apoptotic gene, *Bax*, was measured by quantitative real-time PCR. Expression of *CyclinD1* and *PCNA* was decreased by 15- and 32-fold, respectively, in lymphocytes grown in simulated microgravity as compared to the ground-based control group (Fig. 2). The expression of *Bcl-2*, *Bax*, and *P53* was also decreased by 7-, 4-, and 5-fold, respectively (Fig. 2).

CONFIRMATION OF GENE EXPRESSION CHANGES WITH ADDITIONAL LYMPHOCYTE CELL LINE AND ANTI-ORTHOSTATIC (HIND-LIMB) SUSPENSION ANIMAL MICE MODEL

In addition to B-lymphocytes (Cat # GM037978, Coriell Cell Repository), gene expression analysis was also performed with human T-lymphocyte (Cat # GM04155, Coriell Cell Repository). In our previous study, we have also used testicular tissue from well-accepted anti-orthostatic (hind limb) suspension animal mice model for microgravity simulation [DuMond et al., 2006]. The findings of

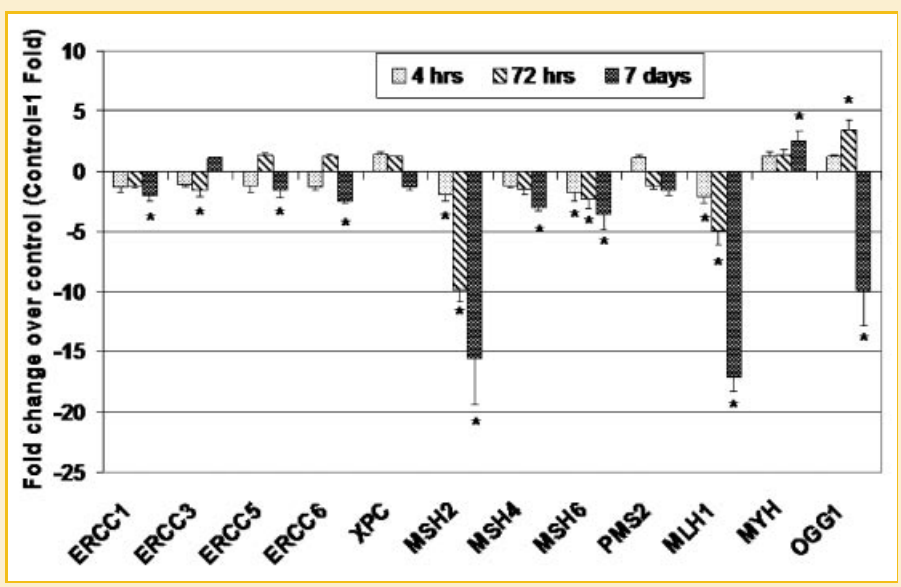

Fig. 1. Effect of modeled microgravity on the expression of DNA repair genes determined by quantitative real-time PCR (qRT-PCR). Lymphocytes were grown in a rotary cell culture system for 4 h, 72 h, and 7 days, and the expression of genes at transcript level was measured by qRT-PCR as mentioned in the Materials and Methods Section. Threshold cycle number (C_t value) for each gene obtained by qRT-PCR was normalized to the C_t value of *GAPDH* from the same sample and the fold change in expression for each gene was obtained by using the $\Delta\Delta C_t$ method. The graph shows the means of triplicate values. *Statistically significant differences relative to the control ($P < 0.05$).

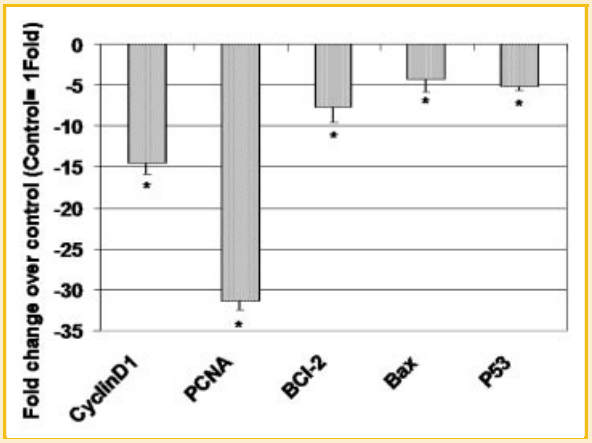

Fig. 2. Effect of modeled microgravity on the expression of genes for cell cycle and apoptosis determined by quantitative real-time PCR (qRT-PCR). Lymphocytes were grown in a rotary cell culture system for 7 days, and the expression of the gene transcripts was measured by qRT-PCR. Threshold cycle number (C_t value) for each gene obtained by qRT-PCR was normalized to the C_t value of *GAPDH* from the same sample and the fold change in expression of each gene was obtained by using the $\Delta\Delta C_t$ method. The graph shows the means of triplicate values. *Statistically significant differences relative to the control ($P < 0.05$).

gene expression changes were very similar in all the three independent experiments except some differences in the degree of fold changes. For example, at 7 days simulated microgravity exposure, the downregulation of *P53* was observed in both T- and B-lymphocyte cell lines as well as in mice model; however, the degree of fold changes was −5-fold in T-lymphocyte, −7.5-fold in B-lymphocyte and −3-fold in mice model of simulated microgravity. The similar pattern of downregulation of *ERCC1*, *ERCC5*, *ERCC6*, *XPC*, *OGG1*, *PCNA*, *CyclinD1*, and *Bax* was commonly observed in all the three independent studies with cell lines and animal model.

CONFIRMATION OF GENE EXPRESSION CHANGES AT THE PROTEIN LEVEL BY WESTERN BLOT ANALYSIS

Changes observed at the transcript level for *XPC*, *P53*, and *PCNA* were further confirmed at the protein level by Western blot analysis (Fig. 3). *GADD 45 gamma* was also included in gene expression analysis done by Western blot. The result of Western blot experiments largely confirm the changes in gene expression seen by quantitative real-time PCR and revealed reductions in the expression of *GADD45 gamma*, *XPC*, *P53*, and *PCNA* (Fig. 3). The expression of *GAPDH* (housekeeping gene) was similar in lymphocytes grown under simulated microgravity conditions and the time-matched control lymphocytes grown under ground-based conditions.

EXPOSURE TO SIMULATED MICROGRAVITY CONDITIONS CAUSES DNA DAMAGE IN HUMAN LYMPHOCYTES

The comet assay was performed to elucidate the effect of simulated microgravity on DNA damage. After electrophoresis, the damaged DNA migrates out of the nucleus and forms a comet tail-like

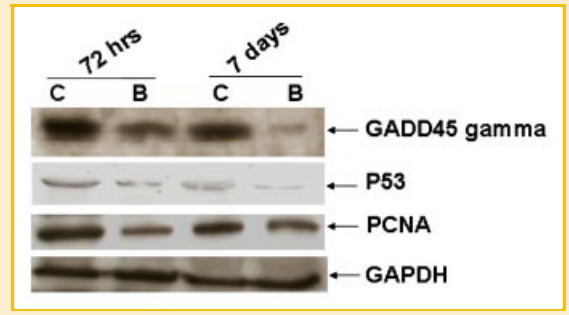

Fig. 3. Representative Western blots showing the effect of modeled microgravity on the expression of specific cell cycle and DNA repair genes. Lymphocytes were grown in a rotary cell culture system for 72 h and 7 days, and the expression of the genes at the protein level was measured in whole cell lysates by Western blot analysis. (C, control group grown under normal gravitational conditions; B, test group grown under simulated microgravity conditions in a bioreactor.)

structure. The tailed DNA in the comet assay is an indicator for DNA damage. The results of the comet assay revealed very few cells with tailed DNA in lymphocytes grown under ground-based conditions (Fig. 4A), whereas a higher number of cells with tailed DNA were observed in lymphocytes grown under simulated microgravity for 7 days (Fig. 4B). Visual scoring revealed that the length of tail DNA was greater in lymphocytes grown in simulated microgravity as compared to the control cells (Fig. 4).

EFFECT OF SIMULATED MICROGRAVITY ON CELL VIABILITY

The effect of extended exposure to simulated microgravity on cell viability was determined by trypan blue staining. The cell viability data revealed no significant difference in the number of dead cells when a comparison was made between the bioreactor and ground conditions at day 7 (Fig. 5). An approximate 20% increase in non-viable cells was seen at day 7 as compared to 72 h (Fig. 5) under the control conditions and in cells grown under simulated microgravity.

DISCUSSION

The most novel and important finding of this study is that ground-based simulated microgravity alone, in the absence of space radiation, can impair DNA repair capacity and induce apoptotic resistance of cells with damaged DNA.

Genetic aberrations in space-flown animal models as well as in astronauts have been reported [George et al., 2001]. However, whether the observed DNA damage in these previously reported studies is due to microgravity or to space radiation is not clear. Decreased repair of radiation-induced DNA damage under microgravity conditions has also been reported [Sirota et al., 2007]. Moreover, whether microgravity alone can impair DNA repair capacity in a cellular system with normal, constitutively expressing DNA repair genes is not clear from these previous studies. It is in this context that the present study was designed to investigate whether simulated microgravity alone in the absence of space radiation could alter the expression of DNA repair genes and induce

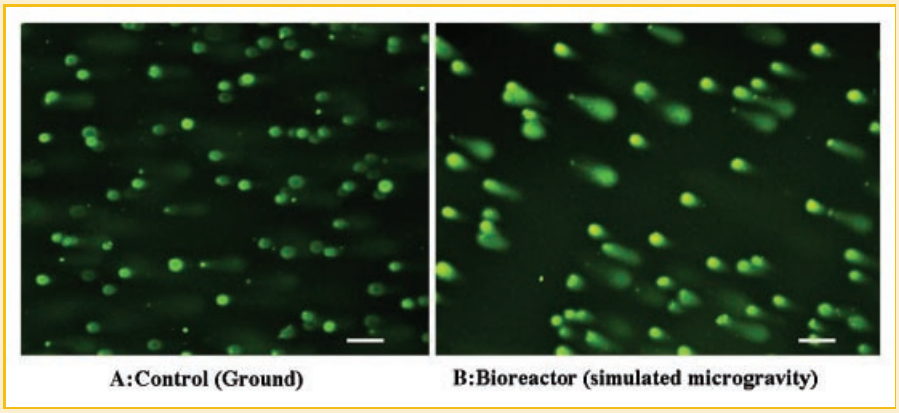

Fig. 4. Representative photographs of the comet assay results showing DNA damage in lymphocytes grown under ground conditions (A) and simulated microgravity conditions (B). Lymphocytes were grown in a rotary cell culture system for 7 days and the DNA damage in individual cells was measured by single cell gel electrophoresis (comet assay). Magnification scale bar (- - -) = 10×.

DNA damage. The results of this study show decreased expression of DNA repair genes and increased DNA damage under simulated microgravity, in the absence of space radiation, suggesting that microgravity alone can induce DNA damage.

Besides DNA repair genes, *P53* signaling pathways also mediate DNA repair via activation of *GADD 45* [Smith et al., 1996; Wang et al., 1999]. *P53* also controls DNA repair by modulating the levels of *PCNA* [Xu and Morris, 1993; Johnson et al., 1996]. The results of Western blot analysis revealed that genes involved in *P53*-mediated DNA repair pathways, such as, *P53*, *GADD 45 gamma*, and *PCNA*, are also downregulated under simulated microgravity conditions. Decreased expression of *GADD 45* [Ward et al., 2006] and *PCNA* [Hughes-Fulford, 2001] in microgravity has also been reported previously. Therefore, the previous report and the present findings showing decreased expression of *GADD45 gamma* and *PCNA* suggest that DNA damage under simulated microgravity conditions as observed in this study by comet assay could be the cumulative effect of loss of DNA repair genes and inhibition of a *P53*-mediated DNA repair pathway.

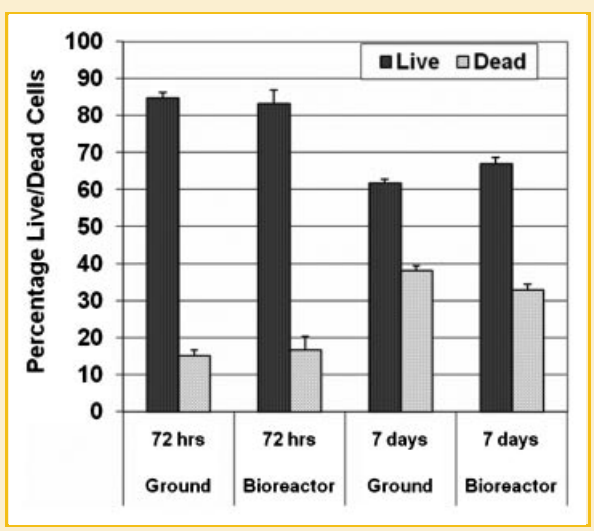

Fig. 5. Effect of simulated microgravity on the cell viability of lymphocytes. Lymphocytes were grown in a rotary cell culture system for 72 h and 7 days, followed by trypan blue staining to detect non-viable cells. Live and dead cells were counted after staining and cell count data was converted to percentage of dead/live cells. The data shown are the means of triplicate samples.

This study also determined the effect of exposure to microgravity for an extended period on cell survival or apoptotic capacity. The cell viability data revealed similar percentages of dead cells under both simulated microgravity conditions and ground conditions. This indicates that simulated microgravity has no effect on cell apoptosis. This is further supported by an observed decrease in expression of *P53* that may result in a decrease in cell-cycle arrest needed for apoptosis, and a decrease in the expression of pro-apoptotic gene *Bax* that would help the cells to adapt under simulated microgravity conditions. A recent report also suggests that microgravity does not induce apoptosis [Bucaro et al., 2007]. Yet another study suggests the inhibition of radiation- and activation-induced apoptosis in blood lymphocytes under simulated microgravity conditions [Risin and Pellis, 2001]. These previous reports and the findings of the present study together suggest that microgravity does not induce apoptosis and that prolonged exposure to microgravity results in acquisition of apoptotic resistance. This also suggests that the DNA damage seen in cells grown under simulated microgravity conditions as observed in this study through the comet assay is not due to increased apoptosis or cell senescence.

In general, the *P53* is upregulated as a response to cellular DNA damage and the upregulation of *P53* is needed to arrest the cell-cycle progression either to repair the DNA damage [Vazquez et al., 2008], or to induce apoptosis [Polyak et al., 1997] if the damage cannot be fixed. In our study, the comet assay revealed that simulated microgravity induces DNA damage. In this condition the general expectation is that the *P53* expression should increase to arrest the cell-cycle progression or to induce apoptosis. Surprisingly, a downregulation of *P53* expression was observed in microgravity grown cells having damaged DNA. The similar number of live cells in simulated microgravity and ground conditions further suggests

that cells neither went under apoptosis nor the cell cycle was arrested in microgravity condition, as expected in cells with downregulated *P53*. Therefore, this study suggests that in microgravity condition the cells evade the apoptosis and cell-cycle arrest by downregulating *P53* and its downstream target gene *Gadd45 gamma*. It is possible that to adapt in microgravity condition the cells downregulate its *P53* by epigenetic mechanism of promoter DNA methylation as observed in many tumor cells [Kang et al., 2001]. However, it remains to determine the exact mechanism for downregulation of wild-type *P53* in microgravity condition.

Among the three different classes of DNA repair genes (MMR, NER, and BER) examined in this study, the influence of simulated microgravity on gene expression changes was relatively higher in MMR genes (downregulation of *MSH2* by 15.6-fold, and *MLH1* by 17.2-fold) and BER gene (downregulation by 10-fold) as compared to the NER genes (Fig. 1). This particular trend of higher impact on MMR and BER than NER systems by simulated microgravity may have some implications on cell survival and cell cycle as has been reported previously [Hawn et al., 1995; Meyers et al., 1997]. There are reports that suggest that MMR system influences the cell cycle. For example, *hMLH1* plays a regulatory role in G2-M arrest following exposure to ionizing radiation [Meyers et al., 1997]. An enhanced G2 arrest was observed after introduction of *hMLH1* in HCT116 cells exposed to 6-thioguanine, a DNA base analog that mimics a mismatch upon incorporation into DNA [Hawn et al., 1995; Berry et al., 1999]. Similarly, an attenuated G2 arrest after treatment with DNA alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in wild-type *hMSH2*-deficient human colon adenocarcinoma cell line LoVo, but not in wild-type *hMSH2* containing human colon adenocarcinoma cell line SW480 was observed [Carethers et al., 1996]. These previously reported studies suggest that MMR genes not only participate in the repair of damaged DNA but also may signal cell-cycle arrest in the presence of extensive DNA damage. Given this dual role of MMR and the finding of this study showing reduced expression of MMR in cells with damaged DNA in simulated microgravity suggest that reduced expression of MMR genes could be one of the pathways and mechanistic basis for evading the apoptosis and cell-cycle arrest in microgravity condition as observed in this study.

The PCNA is a multifunctional protein involved in the cell cycle and in DNA repair by interacting with DNA polymerase δ and cyclin/cyclin-dependent kinase complexes [Umar et al., 1996]. PCNA has also been found to interact with MMR proteins MSH2 and MLH1 [Umar et al., 1996]. The reduced expression of MMR genes and its interacting partner PCNA in cells grown in simulated microgravity condition further supports the view that downregulation of MMR pathway may be one of the mechanisms for cell survival in microgravity condition. MMR gene *MLH1* has P53-response elements within its first intron and has been suggested as a target gene of *P53* [Chen and Sadowski, 2005]. Therefore, it is possible that the reduced expression of *MLH1* may be because of the reduced expression of its regulator *P53* in microgravity environment, however, it needs to be experimentally verified. The downregulation of P53 pathway as observed in this study could be another mechanism for cells to evade apoptosis in microgravity condition. Taken together, our study suggests that multiple pathways may be involved in inhibition of apoptosis of cells with damaged DNA as a result of longer exposure to simulated microgravity. Further study with more representative DNA repair genes from each class of DNA repair pathway is needed for better understanding of survival of cells with damaged DNA in microgravity condition.

Interestingly, of the 18 genes analyzed in this study, all except two were downregulated. A similar trend whereby more downregulated genes are seen and fewer upregulated in simulated microgravity conditions has also been found in previous studies [Ward et al., 2006]. Thus, it appears that simulated microgravity exerts its effects largely through downregulation of critical genes. To investigate whether the pattern of gene expression changes is cell line-specific or microgravity condition-specific, the gene expression analysis was also performed with additional lymphocyte cell line. Furthermore, in our previous study, the gene expression analysis in microgravity condition has also been performed with testicular tissue from well-accepted anti-orthostatic (hind-limb) suspension animal mice model for microgravity simulation [DuMond et al., 2006]. Similar pattern of gene expression changes in three independent studies (two cell lines in the present study and our previous study with animal model) suggests that observed effects of simulated microgravity on gene expression changes are not cell line specific, rather it is simulated microgravity condition-specific changes.

In this study, we observed large reduction in the expression of BER and MMR genes as a result of exposure to simulated microgravity. The DNA MMR is an important repair mechanism and is essential for maintaining genomic stability. It plays key roles in the repair of base–base mismatches and insertion/deletion mispairs generated during DNA replication and recombination. MMR also prevents homologous recombination (HR) and in DNA damage signaling in eukaryotic cells [Kunkel and Erie, 2005; Jiricny, 2006; Modrich, 2006; Li, 2008]. *MSH2* and *MLH1* are two important genes among the MMR system. For example, germline mutations in *MSH2* and *MLH1* together account for nearly half of all hereditary non-polyposis colorectal cancer (HNPCC) patients [Raevaara et al., 2005]. *MSH2*-deficient cells may be more susceptible to DNA cross-link-inducing agents than normal, whereas *MLH1*-deficient cells have a greater potential to survive cross-linking treatment and that may lead to tumor initiation [Wu and Vasquez, 2008]. *OGG1* is another important gene that encodes the enzyme responsible for the excision of 8-oxoguanine, a mutagenic base byproduct which occurs as a result of exposure to reactive oxygen. Therefore, the deficiency in DNA repair enzyme OGG1 would have functional consequences, such as, compromising the ability of cells to repair DNA [Boiteux and Radicella, 2000], resulting in oxidative DNA damage. The published studies have shown the involvement of oxidative DNA damage in carcinogenesis, aging, and several age-related degenerative diseases [Fortini et al., 2003; Nishigori et al., 2004]. The large changes in BER and MMR observed in this study may explain the mechanistic basis for previously reported genomic instability and chromosomal aberrations in cells exposed to microgravity. Further study is needed to screen the genetic changes, particularly the point mutations that may have accumulated in these cells as a consequence of reduced expression of MMR and BER genes in simulated

microgravity conditions. Taken together, the observations of loss of DNA repair capacity, DNA damage, and increased cell survival will provide the opportunity for accumulation of mutations. Some of these mutations may potentially lead to mutator phenotype that could eventually contribute in tumorigenesis. Therefore, this study has significant implications not only in understanding the molecular basis for space-flight-associated patho-physiological changes but also in its countermeasures.

In summary, the findings of this study suggest that simulated microgravity conditions can induce DNA damage and genomic instability directly by impairing DNA repair capacity, and indirectly by impairing *P53*-mediated cell-cycle control. The increased adaptability of those cells with genomic instability to grow further in a cellular environment that has reduced expression of genes for DNA repair and tumor suppressor, such as *P53*, may provide the opportunity to move unchecked on the path of oncogenesis in microgravity environment. The next logical question that remains to be addressed is how the expression of these genes is aberrantly regulated in microgravity condition. Recent reports suggest that in addition to gene mutations, the epigenetic changes of DNA methylation and histone modifications play important role in regulation of gene expression [Kang et al., 2001; Roh et al., 2006]. Whether these epigenetic changes have any role in gene expression changes in microgravity condition is another question that remains to be addressed. This study, therefore, provides the future directions for space life science research.

ACKNOWLEDGMENTS

This work was supported by grants from NIH (RR03045) and NASA-URC (NCC9-165).

REFERENCES

Baevsky RM, Baranov VM, Funtova II, Diedrich A, Pashenko AV, Chernikova AG, Drescher J, Jordan J, Tank J. 2007. Autonomic cardiovascular and respiratory control during prolonged spaceflights aboard the international space station. J Appl Physiol 103:156–161.

Barr YR, Bacal K, Jones JA, Hamilton DR. 2007. Breast cancer and spaceflight: Risk and management. Aviat Space Environ Med 78:26–37.

Berry SE, Garces C, Hwang H, Kunugi K, Meyers M, Davis TW, Boothman DA, Kinsella TJ. 1999. The mismatch repair protein, hMLH1, mediates 5-substituted halogenated thymidine analogue cytotoxicity, DNA incorporation, and radiosensitization in human colon cancer cells. Cancer Res 59:1840–1845.

Boiteux S, Radicella JP. 2000. The human OGG1 gene: Structure, functions, and its implication in the process of carcinogenesis. Arch Biochem Biophys 377:1–8.

Bradford MA. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–253.

Bucaro MA, Zahn AM, Risbud MV, Ayyaswamy PS, Mukundakrishnan K, Steinbeck MJ, Shapiro IM, Adams CS. 2007. The effect of simulated microgravity on osteoblasts is independent of the induction of apoptosis. J Cell Biochem 102:483–495.

Buermeyer A, Deschene SM, Baker SK, Liskay RM. 1999. Mammalian DNA mismatch repair. Annu Rev Genet 33:533–564.

Carethers JM, Hawn MT, Chauhan DP, Luce MC, Marra G, Koi M, Boland CR. 1996. Competency in mismatch repair prohibits clonal expansion of cancer cells treated with N-methyl-N′-nitro-N-nitrosoguanidine. J Clin Invest 98:199–206.

Chen J, Sadowski I. 2005. Identification of the mismatch repair genes PMS2 and MLH1 as P53 target genes by using serial analysis of binding elements. Proc Natl Acad Sci USA 102(13): 4813–4818.

Cleaver JE. 2005. Cancer in xeroderma pigmentosum and related disorders of DNA repair. Nat Rev Cancer 5:564–573.

Clement JQ, Lacy SM, Wilson BL. 2007. Genome-wide gene expression profiling of microgravity effect on human liver cells. J Gravit Physiol 14:121–122.

Cogoli A, Tschopp A, Fuchs-Bislin P. 1984. Cell sensitivity to gravity. Science 225:228–230.

Cogoli A, Bechler B, Cogoli-Greuter M, Criswell SB, Joller H, Joller P, Hunzinger E, Muller O. 1993. Mitogenic signal transduction in T lymphocytes in microgravity. J Leukoc Biol 53:569–575.

Cogoli-Greuter M, Sciola L, Spano A, Meloni MA, Pippia P, Cogoli A. 1995. Lymphocyte movements and interactions in microgravity. J Gravit Physiol 2:117–118.

Cooper D, Pellis NR. 1998. Suppressed PHA activation of T lymphocytes in simulated microgravity is restored by direct activation of protein kinase C. J Leukoc Biol 63:550–562.

Cooper D, Pride MW, Brown E, Risin D, Pellis NR. 2001. Suppression of antigen-specific lymphocyte activation in modeled microgravity. In Vitro Cell Dev Biol Anim 37:63–65.

Denver DR, Feinberg S, Steding C, Durbin M, Lynch M. 2006. The relative roles of three DNA repair pathways in preventing Caenorhabditis elegans mutation accumulation. Genetics 174:57–65.

DuMond JW, Singh KP, Ramesh GT. 2006. Increase of oxidative stress and loss in expression of DNA repair genes by modeled microgravity in the testis of mice. Proc Amer Assoc Cancer Res 47: Abstract #3558.

Fortini P, Pascucci B, Parlanti E, D'Errico M, Simonelli V, Dogliotti E. 2003. 8-Oxoguanine DNA damage: At the crossroad of alternative repair pathways. Mutat Res 531:127–139.

George K, Durante M, Wu H, Willingham V, Badhwar G, Cucinotta FA. 2001. Chromosome aberrations in the blood lymphocytes of astronauts after space flight. Radiat Res 156:731–738.

Halliwell B. 1994. Free radicals and antioxidants: A personal view. Nutr Rev 52:253–265.

Hawn MT, Umar A, Carethers JM, Marra G, Kunkel TA, Boland CR, Coi M. 1995. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Res 55:3721–3725.

Hayes JC, McBrine JJ, Roper ML, Stricklin MD, Siconolfi SF, Greenisen MC. 1992. Effects of space flight on skeletal muscle performance. FASEB J 65:A1770.

Hughes-Fulford M. 2001. Reduction of anabolic signals and alteration of osteoblast nuclear morphology in microgravity. J Gravit Physiol 8: 1–4.

Ikenaga M, Yoshikawa I, Kojo M, Ayaki T, Ryo H, Ishizaki K, Kato T, Yamamoto H, Hara R. 1997. Mutations induced in Drosophila during space flight. Biol Sci Space 11:346–350.

Jiricny J. 2006. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol 7:335–346.

Johnson RE, Kovvali GK, Guzder SN, Amin NS, Holm C, Habraken Y, Sung P, Prakash L, Prakash S. 1996. Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair. J Biol Chem 271:27987–27990.

Kang JH, Kim SJ, Noh DY, Park IA, Choe KJ, Yoo OJ, Kang HS. 2001. Methylation in the P53 promoter is a supplementary route to breast carcino-

genesis: Correlation between CpG methylation in the P53 promoter and the mutation of the P53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma. Lab Invest 81(4): 573–579.

Kunkel TA, Erie DA. 2005. DNA mismatch repair. Annu Rev Biochem 74:681–710.

Leach CS, Dietlein LF, Pool SL, Nicogossian AE. 1990. Medical considerations for extending human presence in space. Acta Astronaut 21:659–666.

Li GM. 2008. Mechanisms and functions of DNA mismatch repair. Cell Res 18:85–98.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\text{delta delta Ct}}$. Methods 25:402–408.

Marlin DJ, Johnson L, Kingston DA, Smith NC, Deaton CM, Mann S, Heaton P, Vugt FV, Saunders K, Kydd J, Harris PA. 2004. Application of the comet assay for investigation of oxidative DNA damage in equine peripheral blood mononuclear cells. J Nutr 134:2133S–2140S.

Meyers M, Theodosiou M, Acharya S, Odegaard E, Wilson T, Lewis JE, Davis TW, Patten CW, Fishel R, Boothman DA. 1997. Cell cycle regulation of the human DNA mismatch repair genes hMSH2, hMLH1, and hPMS2. Cancer Res 57:206–208.

Modrich P. 2006. Mechanisms in eukaryotic mismatch repair. J Biol Chem 281:30305–30309.

Nikawa T, Ishidoh K, Hirasaka K, Ishihara I, Ikemoto M, Kano M, Kominami E, Nonaka I, Ogawa T, Adams GR, Baldwin KM, Yasui N, Kishi K, Takeda S. 2004. Skeletal muscle gene expression in space-flown rats. FASEB J 18:522–524.

Nishigori C, Hattori Y, Toyokuni S. 2004. Role of reactive oxygen species in skin carcinogenesis. Antioxid Redox Signal 3:561–570.

Obe G, Johannes I, Johanes C, Hallman K, Reitz G, Facius R. 1997. Chromosomal aberrations in blood lymphocytes of astronauts after long-term space flights. Int J Radiat Biol 72:727–734.

Pietrzyk RA, Jones JA, Sams CF, Whitson PA. 2007. Renal stone formation among astronauts. Aviat Space Environ Med 78:9–13.

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. 1997. A model for p53-induced apoptosis. Nature 389:300–305.

Raevaara TE, Korhonen MK, Lohi H, Hampel H, Lynch E. 2005. Functional significance and clinical phenotype of nontruncating mismatch repair variants of MLH1. Gastroenterology 129:537–549.

Risin D, Pellis NR. 2001. Modeled microgravity inhibits apoptosis in peripheral blood lymphocytes. In Vitro Cell Dev Biol Anim 37:66–72.

Roh TY, Cuddapah S, Cui K, Zhao K. 2006. The genomic landscape of histone modifications in human T cells. Proc Natl Acad Sci USA 103(43): 15782–15787.

Schmitt DA, Hatton JP, Emond C, Chaput D, Paris H, Levade T, Cazenave JP, Schaffar L. 1996. The distribution of protein kinase C in human leukocytes is altered in microgravity. FASEB J 14:1627–1634.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Biol 175:184–191.

Sirota NP, Kuznetsova EA, Peleshko VN. 2007. Development of genetic instability in mice in response to chronic irradiation simulating high-altitude flight conditions. Radiat Biol Radioecol 47:297–301.

Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Conner PM, Fornace AJ. 1996. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to U V-irradiation or cisplatin. Oncogene 13:2255–2263.

Sohal RS, Weindruch R. 1996. Oxidative stress, caloric restriction, and aging. Science 273:59–63.

Taylor WE, Bhasin S, Lalani R, Datta A, Gonzalez-Cadavid NF. 2002. Alteration of gene expression profiles in skeletal muscle of rats exposed to microgravity during a spaceflight. J Gravit Physiol 9:61–70.

Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Lisky RM, Kunkel TA. 1996. Requirement of PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87:65–73.

Vazquez A, Bond EE, Levine AJ, Bond GL. 2008. The genetics of the P53 pathway, apoptosis and cancer therapy. Nat Rev Drug Discov 12:979–987.

Vico L, Collet P, Guignandon A, Lafage-Proust M, Thomas T, Rehailia M, Alexandre C. 2000. Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355:1607–1611.

Walther I, Pippia P, Meloni MA, Turrini F, Mannu F, Cogoli A. 1998. Simulated microgravity inhibits the genetic expression of interleukin-2 and its receptor in mitogen-activated T lymphocytes. FEBS Lett 436:115–118.

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ, Harris CC. 1999. Gadd 45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci USA 96:3706–3711.

Ward NE, Pellis NR, Risin SA, Risin D. 2006. Gene expression alterations in activated human T-cells induced by simulated microgravity. J Cell Biochem 99:1187–1202.

Wu Q, Vasquez KM. 2008. Human MLH1 protein participates in genomic damage checkpoint signaling in response to DNA interstrand crosslinks, while MSH2 functions in DNA repair. PLoS Genet 4(9): e1000189.

Wu AH, Taylor GR, Graham GA, McKinley BA. 1993. The clinical chemistry and immunology of long-duration space missions. Clin Chem 39:22–36.

Xu J, Morris G. 1993. P53-mediated regulation of proliferating cell nuclear antigen expression in cells exposed to ionizing radiation. Mol Cell Biol 19:12–20.

Yang TC, George K, Johnson AS, Durante M, Fedorenko BS. 1997. Biodosimetry results from space flight Mir-18. Radiat Res 148:S17–S23.