Simulated Microgravity Induced Damage in Human Retinal Pigment Epithelial Cells

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Abstract

Purpose: The goal of this study was to determine the potential damage to the human retina that may occur from weightlessness during space flight using simulated microgravity.

Methods: Human retinal pigment epithelial (hRPE) cells were cultured for 24 hours in a NASA-designed rotating wall bioreactor vessel [RWV] to mimic the microgravity environment of space. Single-stranded breaks in hRPE DNA induced by simulated gravity were measured using the Comet Assay. In addition, the production of the inflammatory mediator prostaglandin E$_2_$ (PGE$_2_$) was measured in these cells 48 hours after recovery from simulated microgravity exposure**.**

Results: Simulated microgravity induced single-stranded breaks in the hRPE DNA that were not repaired within 48 hours. Furthermore, prostaglandin E$_2_$ production was dramatically increased 48 hours after the initial

microgravity-induced damage, indicating the induction of an inflammatory response. There was less DNA damage and no prostaglandin E$_2_$ release in hRPE cells pretreated with the anti-inflammatory agent cysteine during their exposure to microgravity**.**

Conclusions: We have demonstrated that the microgravity environment generated by a NASA-designed rotating wall bioreactor vessel induces an inflammatory response in hRPE cells. This system thus constitutes a new model system for the study of inflammation in the retina, a system that does not involve the introduction of an exogenous chemical agent or supplementary irradiation. This in vitro method may also be useful for testing novel therapeutic approaches for suppression of retinal inflammation. Furthermore, we suggest a safe prophylactic treatment for prevention of acute, transitory, or enhanced age-related permanent blindness in astronauts or flight personnel engaged in long-haul flights.

Introduction

Space travel subjects the human eye to the stress of solar and cosmic radiation, and at the same time to microgravity. It is well established that solar and cosmic radiation induce cataracts and retinal degeneration, (1-7) but the long term hazards of microgravity to ocular tissues have not been established. On earth, the human eye is constantly exposed to environmental hazards including smoke, environmental toxins and ambient radiation. Damage is averted in the young eye by the presence of a very efficient antioxidant system including, melanin (8), lutein and zeaxanthin (9), vitamins C (10)and E (11), and glutathione (12), superoxide dismutase, catalase, and the co-factors zinc and selenium (13). However, with age (above 40 years old), the levels of protective endogenous antioxidants decrease, and both the clarity of the lens and the function of the retina deteriorate (8, 14). Astronauts and flight personnel engaged on long-haul flights (15) are exposed to higher and more virulent environmental insults, and blinding disorders may appear decades after the original injury.

In the present work, we sought to examine the pathogenic mechanisms of potential damage to the retina induced by microgravity independent of radiation damage. Dutt et al. (16) have shown upregulation of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in the human retinal cell line 301-SV-40T(17) in a NASA Bioreactor. Barstable and Tombran-Tink have demonstrated photoreceptor rod outer segment damage in rats exposed to hypergravity and space travel. (18, 19) We present here an experiment which demonstrates that microgravity (simulated using a NASA designed RWV bioreactor) (20, 21) can induce an inflammatory response in human retinal pigment epithelial cells. Our results have also suggested a potential method to block this damage and avoid transient or permanent damage to astronauts on long space flights.

Methods

Cell culture

A human retinal pigment epithelial (RPE) cell derived from a human donor eye as described by Hu et al. (22) was used in these studies. The purity of the cell line was demonstrated by immunocytochemical methods: RPE cells display S-100 and cytokeratin, uveal melanocytes display S-100 antigen but not cytokeratin, and fibroblasts display neither of these proteins (23). The cells used in the present experiments were cultured using an F12 nutrient mixture supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 mu g/ml gentamicin (GIBCO, BRL Products, Rockville, MD). The cells used in these experiments were generally from passages two to three.

Simulated microgravity exposure.

Growing hRPE were detached with trypsin-EDTA solution (GIBCO) diluted 1:3. Detached cells were spun for 5 min at 1000 rpm at 4^0+^$ C and resuspended to 30 ml volume in RPMI-phenol red free media containing 10 % FCS, 2 mM glutamine and 50 mu g/ml gentamicin. The cells were counted with trypan blue and demonstrated 97-98% viability. Cells were reseeded and grown either in tissue culture vessels at unit gravity or in the simulated microgravity environment of the NASA-designed rotating wall vessel (RWV) bioreactor (Synthecon, Houston, TX) (20, 21)in the presence and absence of 1 mu M cysteine.

Prior to adding the cells, each 50 ml disposable, sterile Rotary cell culture filter (Synthecon, Houston, TX) was flooded with media. All bubbles were removed by gentle flushing. Sufficient hRPE cells were added into each filter for a final concentration of 250,000 cells per ml. Each loaded cell culture filter was then attached to the baseplate drive of the rotating wall vessel (RWV) bioreactor. The assembled bioreactor cultures were individually rotated at 8 rpm within a humidified incubator at 37^0+^$ C under 5% CO$_2_$ for 24 hours. Lewis et al. (24) have previously found that rotation of the RWV bioreactor at eight rpm was optimal for maintaining cells in suspension.

Treatments consisted of (1) cells exposed to microgravity for **24** hours, (2) cells exposed to microgravity for 24 hours and then incubated in media for **48** hours, (3) cells pretreated with 1 mu M cysteine, exposed to microgravity for 24 hours, and then incubated in media for **48** hours.

Comet assay

DNA damage (single stranded breaks) induced by microgravity was quantified using the comet assay, in which the amount of the genetic material that migrated from the nucleus to form the comet tail is measured. Images of nuclei and migrated material were digitized so that the comet tail moment (% of pixel intensity in the tail × distance from head to tail in microns) divided by the negative control value could be calculated. This value was then used as a quantitative index of DNA single-stranded breaks and presented as the median of the comet tail moment.

The comet assay was performed essentially as described by Singh (25). Briefly, 85 mu 1 of molten 1% normal agarose in PBS was dropped onto a pre-coated microscope slide, covered with an 18 x 18 mm No.1 glass coverslip, and left in ice to set. Once set, the coverslip was removed. The hRPE cells were then mixed with 85 mu 1 of 1% low melting point agar and immediately pipetted onto the layer of agarose on the slide. The coverslip was replaced and allowed to set on ice. The entrapped cells were then lysed in 150 ml of ice cold lysis buffer (2.5 M NaCl, 83 mM EDTA, 10 mM TRIS) and the pH was adjusted to 10 using sodium hydroxide. The lysis buffer was supplemented with 1% (v/v) Triton X-100 and 10% (v/v) DMSO prior to use. Lysis was performed at 4^0+^$ C for 60-90 min.

Following lysis the slides were incubated in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 20 min prior to electrophoresis. Electrophoresis

was performed at 20 V/32 mA for 24 min. The slides were washed three times in 100 mM TRIS, pH 7.5, and exposed briefly to chilled methanol.

Comet Assay Data Analysis

After electrophoresis, fluorescent images of the nuclei stained with ethidium bromide were captured with a video camera and digitized using the Matrox Meteor II interface (Matrox Image, Quebec, Canada) to a PC computer controlled by the Matrox Inspector program. A script written in BASIC was used to measure the comet tail moment (% of pixel intensity in the tail x distance from head to tail in microns) from at least 50 cells in each group. The parameter used to measure DNA damage in this study was the relative comet tail moment, i.e., the comet tail moment divided by the negative control value. Negative controls were determined using untreated cells; the negative control was employed to calculate the relative comet tail moment for each experiment.

A frequency distribution of the comet tail moment was determined for each test. The difference in distribution of comet tail moments was analyzed using the Mann-Whitney non-parametric statistical test (26) and presented as the median of the comet moment.

Enzyme immunoassay for PGE$_2_$

Supernatant of the conditioned medium was taken from control RPE cells or RPE cells treated with microgravity. The medium was frozen immediately in liquid nitrogen and then stored at -80^0+^$ C until analyzed. PGE$_2_$ was measured using an enzyme immunoassay R&D Systems kit (catalog no. DE0100).

Results

Single-stranded DNA breaks induced by simulated microgravity.

DNA damage to hRPE cells induced by microgravity was assessed by single cell gel electrophoresis (Comet Assay). In this assay, cleaved DNA migrates

away from the nucleus, forming a comet which is directly visualized as a cell with a round head and a tail (Figure 1); normal cells do not produce a tail (Figure 1A). The comet assay revealed that hRPE cells exposed to 24 hours of simulated microgravity (Figure 1B) suffered significant damage in the form of single-stranded DNA breaks when compared with control cells (Figure 1A). This damage was not repaired following 48 hours of post-exposure incubation in culture medium (Figure 1C). However when the hRPE cells were pretreated with 1 mu M cysteine followed by 48 hours of post-exposure recovery (Figure 1D), single stranded DNA breaks were diminished compared to Figure 1B-C.

The median of the relative comet tail moment for hRPE cells exposed to microgravity ([**Mg**], Figure 2) was substantially increased compared to non-exposed controls ([**control**], Figure 2; 44 vs. 9), indicating that microgravity alone induced DNA single-stranded breaks. This microgravity damage had not been repaired after 48 hours of post-exposure recovery. Less than 10% of these cells were attached ([**RMg.A**], Figure 2) to the plates. The DNA from the attached cells had no single-stranded breaks as indicated by the median of their relative comet tail moments. The remaining 90%, which appeared as floaters (**[RMg.F]**, Figure 2), had no change in the amount of DNA damage compared to cells measured immediately after microgravity exposure (44 vs. 44). When hRPE cells were pretreated with 1 mu M cysteine **[RMg.C**] and subjected to microgravity and post-exposure recovery, the single stranded DNA breaks were reduced by at least half from the levels of either hRPE cells exposed to 24 hours of simulated microgravity or those subjected to microgravity and allowed post-exposure recovery (22 vs. 44).

Effect of microgravity on PGE$_2_$ secretion

PGE$_2_$ secretion was measured (Figure 3) in the conditioned medium of untreated hRPE samples [**Control]**, hRPE cells subjected to 24 hours simulated microgravity [**Mg]**, hRPE cells subjected to 24 hours simulated microgravity and then incubated in medium for 48 hours [**RMg]**, and hRPE cells pretreated with 1muM cysteine subjected to 24 hours simulated microgravity and then incubated in medium for 48 hours [**RMg.C]**. Microgravity treatment did not cause secretion of PGE$_2_$ immediately after treatment. However, within 48 hours there was a significant secretion of PGE$_2_$, which was blocked by pretreatment of the hRPE cells with cysteine.

Discussion

Exposure to microgravity poses a unique hazard to astronauts. Our previous studies have shown that radiation exposure increases single-stranded breaks in DNA, as measured by the Comet Assay, and that this damage can be prevented by the antioxidant lutein (27). We have shown here using simulated microgravity that microgravity induced significant single stranded DNA breaks and that damage was not repaired within 48 hours.

Furthermore, based on the secretion of PGE$_2_$, the pathogenic mechanism of microgravity damage to retinal cells appears to be, at least in part, the induction of a delayed inflammatory response in the hRPE cells. Inflammation is a serious problem in the retina. The eye is immune privileged and except during a bacterial or viral infection, the blood ocular barriers prevent most immune cells from entering the retina and releasing reactive oxygen species (28). Should this barrier be broken and an immune response initiated, damage can occur that can lead to retinal degeneration and/or retinal detachment (29, 30) .It has been also shown that inflammation and PGE$_2_$ release in the retina are serious risk factors for the induction of proliferative vitreoretinopathy (PVR) (31) and macular degeneration (32) .The recent identification of a gene variant [common variant of the complement factor H (CFH) gene] found in half of the age-related macular degeneration cases in the United States further implicates inflammation in the etiology of macular degeneration. (33-35) We have shown here that an inflammatory response is induced in hRPE cells by simulated microgravity, without the introduction of an exogenous chemical agent or supplementary irradiation. This method may be useful for testing novel therapeutic approaches that suppress retinal inflammation.

We have succeeded in blocking much of the microgravity-induced DNA damage and all of the PGE$_2_$ release from hRPE cells with cysteine (Figure 1, 2). This protective effect is not surprising as we have seen similar protection against radiation and phototoxic induced inflammation using non-toxic sulhydryl quenchers (cysteine, N-acetyl cysteine and the radioprotector WR-77913) in clinical trials, (36) in vivo (37, 38) and in vitro. (39) Others have found these compounds useful against X-radiation. (40, 41) As these compounds are safe for human consumption, protecting against accumulated microgravity induced damage and reducing inflammation with cysteine or another non-toxic anti-inflammatory agent could be part of a treatment in preventing acute, transitory or enhanced age related permanent blindness in

astronauts after their return to earth. This treatment may also be applicable to flight personnel engaged on long-haul flights (15).

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Figure Legends

Figure 1. Images of single cell electrophoresis (Comet Assay) of hRPE cells exposed to microgravity.

A: Control cells. **B**: Cells treated for 24 h with simulated microgravity. These cells had single-stranded DNA breaks as indicated by the appearance of a comet (a cell with a round head and a tail). **C**: Cells treated for 24 h with simulated microgravity after 48 h recovery. Single-stranded DNA breaks have not been repaired. **D**: Cells pretreated with 1mu M cysteine, treated for 24 h with simulated microgravity after 48 h recovery. The number of damaged cells was diminished when compared with **B** or **C**.

Figure 2. Quantitative DNA damage from Comet Assay

[control] control cells; [Mg] Cells treated for 24 h with simulated microgravity; [RMg] Cells treated for 24 h with simulated microgravity after 48 h recovery; attached [RMg.A], floaters [RMg.F]; [RMg.C] Cells pretreated with 1mu M cysteine, treated for 24 h with simulated microgravity after 48 h recovery.

Figure 3. Effect of microgravity on PGE$_2_$ secretion

PGE$_2_$ secretion was measured in the medium of untreated hRPE samples [**Control**]; hRPE cells subjected to 24 hours simulated microgravity [**Mg]**; hRPE cells subjected to 24 hours simulated microgravity and then incubated in medium for 48 hours [**RMg**]; and hRPE cells pretreated with 1mu M cysteine, subjected to 24 hours simulated microgravity, and then incubated in medium for 48 hours [**RMg.C**].

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