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Assessment of Global Ocular Structure Following Spaceflight Using a Micro-Computed Tomography (Micro-CT) Imaging Method

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Abstract

Reports show that prolonged exposure to a spaceflight environment produces morphologic and functional ophthalmic changes in astronauts during and after an International Space Station (ISS) mission. However, the underlying mechanisms of these spaceflight-induced changes are currently unknown. The purpose of the present study was to determine the impact of the spaceflight environment on ocular structures by evaluating the thickness of the mouse retina, the retinal pigment epithelium (RPE), the choroid and the sclera layer using micro-CT imaging. Ten-week-old C57BL/6 male mice were housed aboard the ISS for a 35-day mission and then returned to Earth alive for tissue analysis. For comparison, ground control (GC) mice on Earth were maintained in identical environmental conditions and hardware. Ocular tissue samples were collected for micro-CT analysis within 38(±4) hours after splashdown. The images of the cross-section of the retina, the RPE, the choroid, and the sclera layer of the fixed eye was recorded in an axial and sagittal view using a micro-CT imaging acquisition method. The micro-CT analysis showed that the cross-section areas of the retina, RPE, and choroid layer thickness were changed in spaceflight samples compared to GC, with spaceflight samples showing significantly thinner cross-sections and layers compared to controls. The findings from this study indicate that micro-CT evaluation is a sensitive and reliable method to characterize ocular structure changes. These results are expected to improve the understanding of the impact of environmental stress on global ocular structures.

Introduction

In the microgravity environment of spaceflight, increased intracranial pressure (ICP) caused by fluid shift may have contributed to spaceflight-associated neuro-ocular syndrome (SANS)[1,2,3,4,5]. Indeed, over 40% of astronauts have

experienced SANS during and after an International Space Station (ISS) mission[6], including the spaceflight subject of the NASA Twins Study[7]. The current pathophysiology of SANS includes physiological changes such as optic disc edema, globe flattening, choroidal and retinal folds, hyperopic refractive error shifts, and nerve fiber layer infarcts (i.e., cotton wool spots) and are well documented[5,8]. However, the underlying mechanisms of the changes and factors contributing to the development of damage are unclear. In order to have a better understanding of SANS, animal models are available for characterizing the spaceflight-associated changes in retinal structure and function.

In a previous investigation on the same animals, we reported the impact of 35 days of spaceflight on the mouse retina. The results elucidate that spaceflight induces significant damage in the retina and retinal vasculature, and some proteins/pathways associated with cell death, inflammation and metabolic stress were significantly altered following spaceflight[9].

Currently, there are a variety of noninvasive imaging techniques established to monitor disease development and progression, as well as physiological responses to various environmental stressors, which are also widely used in small rodent models. One of these techniques is micro-CT, which evaluates anatomical structures and pathological processes, and has successfully been used on organisms as small as mice[10].

Micro-CT can achieve a microsized resolution, and it can provide high contrast for volumetric analysis of soft tissues with the addition of the appropriate contrast agent[10,11,12,13,14]. Micro-CT technology is advantageous compared to traditional methods such as gross anatomy, light microscopy, and histology examination, as it minimizes physical damage to the geometric profile of the specimens and does not alter the spatial relationship among structures. In addition, three-dimensional (3D) models of structures can be reconstructed from micro-CT images[12,14]. To date, despite evidence showing vision impairment following exposure to the space environment, few data in animal models are available for a better understanding of the spaceflight-associated changes in retinal structure and function. In the current study, mice were flown on a 35-day mission aboard the ISS to determine the impact of the spaceflight environment on ocular tissue structures by quantifying the microstructure of the retina, the RPE, and the choroid layers using micro-CT.

Protocol

The study followed the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and was approved by both the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University (LLU) and The National Aeronautics and Space Administration (NASA). More detailed information regarding this flight experiment can be found elsewhere[9,15].

1. Flight and control conditions

NOTE: A 12th Commercial Resupply Service (CRS-12) payload was launched By SpaceX at the Kennedy Space Center (KSC) on a 35-day mission in August 2017 which included onboard 10-week-old male C57BL/6 mice (n = 20) for NASA's ninth Rodent Research experiment (RR-9).

1. Prior to returning to Earth via SpaceX's Dragon capsule, have the mice live in NASA's Rodent Habitats (RH) aboard ISS for 35 days at an ambient temperature of 26–28 °C with a 12-hour light/dark cycle throughout the flight.

2. Place Ground Control (GC) mice into the same housing hardware used in flight and match environmental parameters such as temperature and carbon dioxide (CO_2) levels as closely as possible based on telemetry data.
3. Feed GC mice the same NASA food bar diet as their space-based counterparts. Provide both the spaceflight and GC mice with same ad libitum access to water and food.

2. Post-flight evaluation of the mice

1. Within 28 hours of splashdown on Earth, transport the mice to Loma Linda University (LLU). Once there, remove the mice from the animal enclosure hardware and assess for survival and health.
   NOTE: Upon observation, inspecting personnel reported that all the mice had survived the 35-day space mission and were in good condition, i.e. no noticeable deficiencies/abnormalities.

3. Dissecting and preservation of mouse eyes after spaceflight

1. Within 38(±4) hours of splashdown (n=20/group), euthanize the mice in 100% CO_2 and collect their eyes.
2. Dissect the right eye retinas and place individually in sterile cryovials, snap-freeze in liquid nitrogen, and keep at −80 °C prior to use.
3. Fix the whole left eyes in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and then rinse with phosphate-buffered saline (PBS) for micro-CT assays.

4. Sample preparation for micro-CT scanning

1. After fixation, dehydrate the mice's eyes in ethanol. To forestall any further or abrupt shrinkage of the fixed sample, use a graded series of ethanol solutions: beginning with 50% ethanol for 1 hour and then increasing the concentrations of the ethanol solutions as follows for 1 hour each: 70, 80, 90, 96 and 100%.
   NOTE: The mice's eyes must be handled in a hood chamber.
2. Phosphomolybdic acid (PMA) staining
   CAUTION: On account of PMA being corrosive, a carcinogenic, and toxic to organs, appropriate protective personal equipment is necessary, including the use of a fume hood.
   1. Prepare the staining solution: 10 mg of PMA in 100 mL of absolute ethanol.
   2. Stain the mice's eyes (10 wt. % phosphomolybdic acid - PMA dissolved in absolute ethanol) for 6 days.
   3. Prior to scanning, first wash the eye samples in absolute ethanol and then place each eye in individual 2 mL plastic containers that are filled with 100% absolute ethanol. Add a cotton pad to stabilized samples during scan.

5. Micro-CT scanning and analysis

NOTE: The SkyScan 1272 scanner, a desktop X-ray Micro-CT system, was used for evaluation of retinal damage in the mice's eyes

1. Mount the soft-tissue sample to an appropriate sample holder. To prevent any movement during the X-ray CT measurements, ensure a tight fit of the sample on its holder (**Figure 1**).

2. Upon meticulous alignment of each sample, individually scan the sample via X-rays.
    1. After opening the software, center the sample in the frame. In the protocol, use no filter and set the matrix to increase the pixel at 4 µm. Use micro-positioning to keep the sample center on the frame.
    2. After that, check the parameter to maximize the contrast agent. To perform the calibration, remove the sample and check that the flat-field correction is greater than 80%.
    3. After calibration, reinsert the sample into the scanning chamber. For scanning, use a rotation step of 0.400, a frame averaging of 4, a random movement of 30, and rotate the samples 180°.
3. Use a positioning jig for repeated measurements. Due to phase-contrast enhancement performed as described, object details as small as 4 µm can be detected from x-rays generated by a sealed micro-focus X-ray tube (tungsten anode) at 50 keV and 80 mA with an integration time of 90 minutes.
   NOTE: The acquisition parameters laid out in this section for selection to produce overview CT scans with the highest image quality.
4. After scanning, use software (e.g., NRecon) to reconstruct the data.
    1. Adjust the histogram and use the same range (0 – 0.24) for all the samples. Reconstruct region of interest was a circle, and no scales or labels were used.
    2. To reduce artifacts during the scanning, use a beam-hardening correction of 20, a smoothing correction of 1, a ring artifact reduction of 6, and perform no alteration in the misalignment compensation. After reconstruction, it was confirmed that the sample was within the region of interest.
    3. Reposition images using a plane parallel to the optic nerve and lens of the eyes.
5. After scanning, use software (e.g., DataViewer) to visualize the reconstructed images in all three views.
   NOTE: If necessary, with this software, the images can be re-positioned using a plane parallel to the optic nerve and lens of the eyes to perform a standardized analysis.
6. Descriptive analysis
    1. Measure the structures using a measuring tool in the software (e.g., CTAn). Use the optical nerve to delimit the region of interest for analysis. By calculation, the protocol used the middle slice to perform the measurements. This evaluation was performed by descriptive analysis (**Figure 2** and **Figure 3**).
    2. Perform measurements of the retina, retinal pigment epithelium (RPE), choroid, and sclera layer in the sagittal (**Figure 2**) and axial view (**Figure 3**). Take three measurements of each structure in order to calculate an average.

Representative Results

The mean thickness of the retina, RPE, choroid, and sclera layer was recorded using the micro-CT scans after following the protocol above (**Figure 1**). The technique showed a multiplanar reconstruction of the eyes in three different views. During the analysis, the observer was able to scroll through the whole sample to standardize the analysis right in the middle of the sample.

The micro-CT analysis showed the cross-section areas of the eyes in the sagittal and axial view (**Figure 2** and **Figure**

3) in which were performed the linear measurements. RPE and choroid layer were significantly or trend lower in the spaceflight group when compared to the GC group (**Figure 3**).

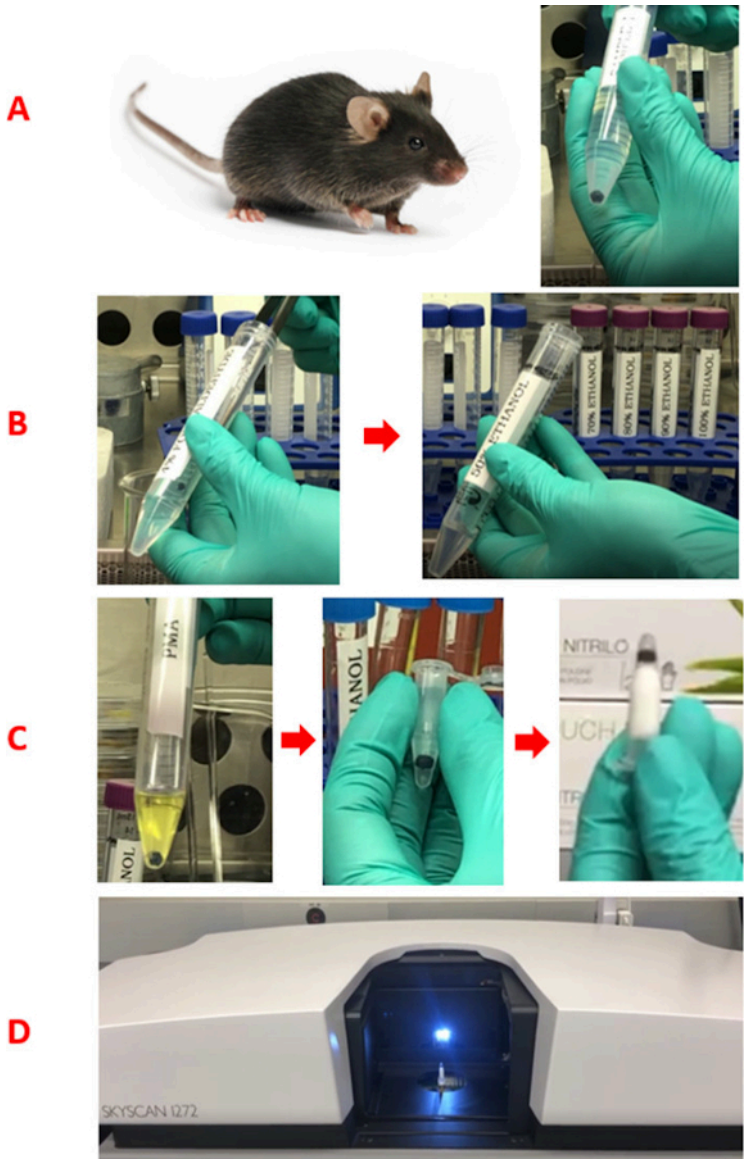

Figure 1: Micro-CT procedure of soft tissue. (**A**) Soft tissue sample (mouse eye). (**B**) Samples were fixed in 4% formaldehyde in phosphate buffer solution (PBS). After fixation, the mice's eyes were dehydrated in ethanol. To prevent a further and abrupt shrinkage of the fixed sample, a graded series of ethanolic solutions were used, beginning with 50% ethanol for 1 h and the following ethanol solutions in the concentrations listed, for 1 hour each: 70, 80, 90, 96 and 100%. (**C**) The mice's eyes were stained in phosphomolybdic acid (PMA) for 6 days, washed in absolute ethanol and then placed in individual 2 mL plastic containers filled with absolute ethanol. (**D**) A desktop X-ray micro-CT system scanner was used to evaluate the retinal injury in mice eyes. Please click here to view a larger version of this figure.

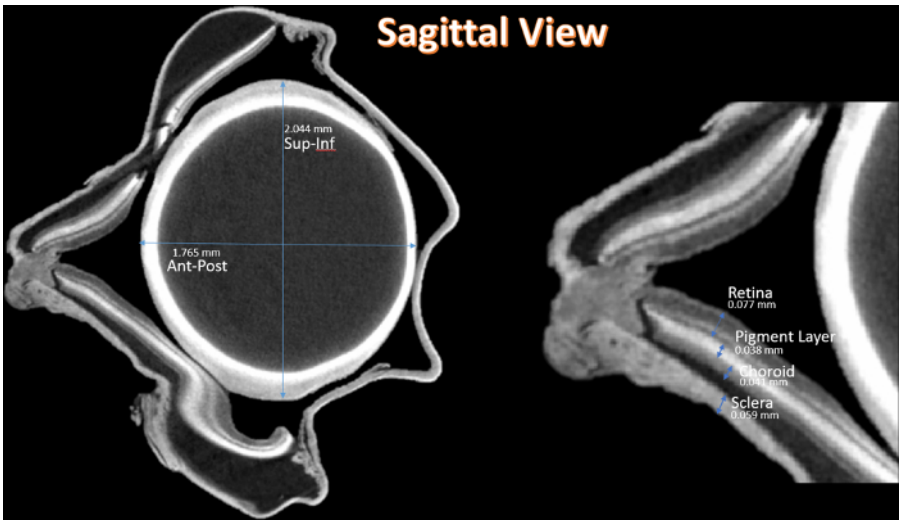

Figure 2: Sagittal view of a ground control mouse. Layers of the eye on the right side of the image are annotated, from top-to-bottom, retina (0.077 mm), retina pigment layer (RPE, 0.038 mm), choroid (0.041 mm), sclera (0.059 mm). This figure has been taken from Overbey et al.[15]. Please click here to view a larger version of this figure.

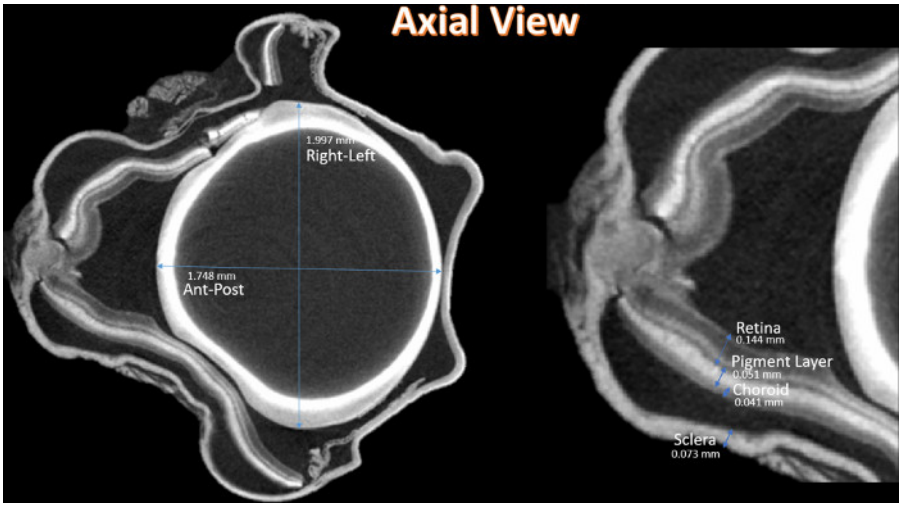

Figure 3: Axial view of a ground control mouse. Layers of the eye on the right side of the image are annotated, from top-to-bottom, retina (0.144 mm), retina pigment layer (RPE, 0.051 mm), choroid (0.041 mm), sclera (0.073 mm). This figure has been taken from Overbey et al.[15]. Please click here to view a larger version of this figure.

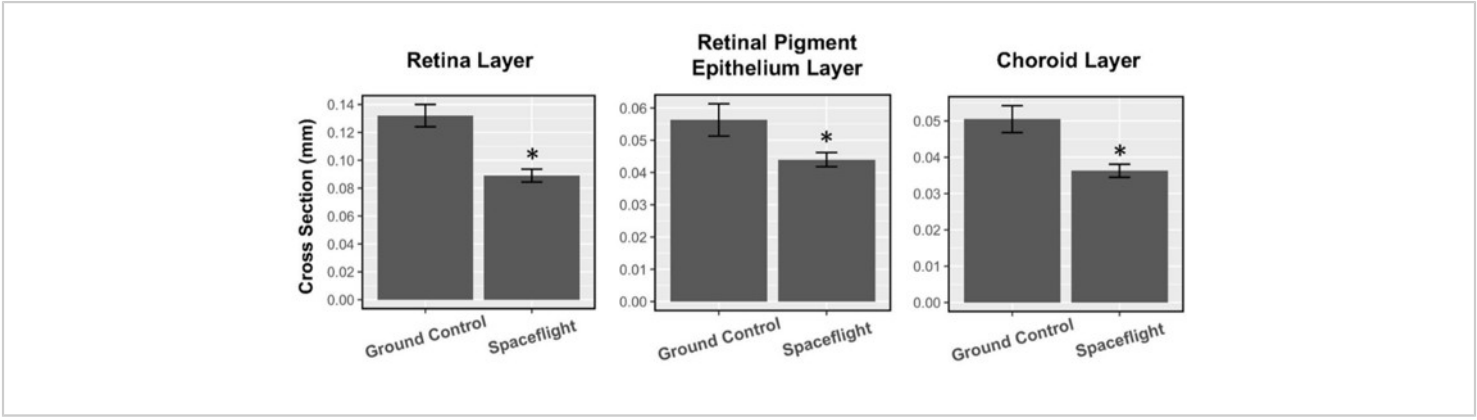

Figure 4: Average thickness of the retinal layer, RPE layer, and the choroid layer measured by micro-CT in the spaceflight and control groups. Counts were averaged across five retinas per group. Values were represented as mean thickness ± standard error (SEM). SEM of the mean is marked with error bars. Significantly lower in cross-section thickness in the spaceflight (FLT) group compared to the ground control (GC) group is denoted '*' ($p < 0.05$). This figure has been taken from Overbey et al.[15]. Please click here to view a larger version of this figure.

Discussion

The results of this study showed that there were structural changes in the spaceflight mouse eye using the micro-CT technique when compared to GC groups, particularly of the retina, the RPE, and the choroid layers of the eye, as evidenced by their decreased thickness. Micro-CT provides an efficient and non-destructive technique to characterize the changes with no need for manipulation. The use of PMA staining enhanced the quality of the micro-CT images to successfully obtain clear 3D tomographic images after reconstruction, foregoing any need to physically alter the structure of the specimen. An added benefit of these images is that they display the entire region of interest digitally, thereby increasing accessibility as well as reproducibility of the findings. Through the micro-CT images produced during this study, the targeted specimen showed differentiation of the multiple structures like the retina, RPE, choroid, and sclera layer for determination of the thickness of each layer.

A critical step within the protocol is the manipulation of the samples due to their size and texture. The handling of the specimen must be carefully done without putting pressure on the specimen during the preparation. The micro-CT has some limitations: resolution and the lack of standardized values for the parameters. During the scanning, the different micro-CT scanners may have diverse image processing algorithms; yet calibration for a grayscale might be pursued to overcome any problem. After scanning, reconstruction of the images should be based on the tissue and the analysis that will be performed. It can be critical since image quality depends on the tomographic system, the settings, the specimen size as well as the preparation methods[16,17].

Due to its successful application in studying several types of normal and pathologic tissues, micro-CT imaging capabilities should be used in future research to compile volumetric data for other analyses. Thus, based on the present study's purpose, it was acceptable to use bi-dimensional

measurements, but segmentation of the gross 3D structure can also be beneficial to provide a precise outline of the entire specimen. Even with all the advantages of a non-destructive technique, micro-CT will not replace other methods such as immunohistochemistry, but will supplement and allow subsequent histology analyses if desired.

A prolonged spaceflight condition produces a series of structural and functional ocular changes in the astronauts during and after the space mission defined as SANS. The findings include hyperopic shifts, globe flattening, choroidal/ retinal folds, and cotton wool spots[19]. In contrast to astronauts' optical coherence tomography (OCT) finding of retinal nerve fiber layer thickening, thinning of retina and choroidal layer was documented in this animal micro-CT study. These results were unexpected. This discrepancy may be due to confounding factors. Mice have limited cephalad fluid shift compared to human. This lack of fluid shift may have evoked different responses to gravitational changes. Secondly, mice were dissected within 38 hours after splashdown, and an acute response for re-adaptation may also contribute to morphological changes in the retina and choroid. Confirmation of this possibility requires further measurements during spaceflight and long term after the mission.

The results of this study indicate that space flight conditions, especially gravitational changes, may induce an acute and short-term response in the eye. Further investigation is needed to determine the consequences of the acute changes on ocular on retinal function and mechanism of spaceflight–induced structure changes.

Disclosures

All authors have nothing to disclose.

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