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Examination of Molecular Mechanisms on Vascular Formation and Stress Response in Zebrafish by Different Microgravity Environments

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Abstract

It has been proved that the presence of humans in space requires meticulous mission design and a critical understanding of physiological parameters. Space is a hostile environment that has caused numerous health hazards in astronauts, including alterations in the vascularization system and high rates of muscle atrophy. Therefore, understanding the molecular pathways mediating space-induced alterations on human physiology is a necessity in making future missions a success. The goal of this study was to use zebrafish (*Danio rerio)* embryos as a unique model to study molecular mechanisms of simulated and real microgravity effect on vascularization system and stress response. To simulate microgravity, we exposed zebrafish embryos to a two-dimensional clinorotation device starting 1-day postembryonic fertilization (dpf) and lasting for a maximum of four days. Changes of multiple genes expression were measured by qRT-PCR. Thus, we used the KDRL-BSY zebrafish strain with the blue fluorescently labelled vascular system allowing to image vascularization development using confocal microscopy. Our preliminary results indicate that only a small proportion of genes are affected by clinorotation. Our next goal was to confirm our findings by exposing zebrafish embryos (days 2 and 3 dpf) to microgravity during the suborbital flight aboard Blue Origin's New Sheppard vehicle in the spring of 2019. Our project entitled Muscular characterization in Microgravity Universal Spacelab (McXIMUS) is a joint research collaboration between the Embry- Riddle Aeronautical University (ERAU) and the University of Texas Health Science Center in San Antonio (UTHSCSA). To ensure the safe environment for zebrafish embryos during the suborbital flight, we designed a NanoLab to guarantee stable thermal conditions inside the payload. Our team has established proper procedures and validation checks to maximize the outcome of this novel scientific experiment. Our data indicate that in contrast to clinorotation, zebrafish embryos exposed to suborbital flight had the up-regulated expression of multiple genes families, with the most profound effect observed in vascular endothelial growth factors and heat shock proteins. To the best of our knowledge, this is the first time when *Danio rerio* were flown on the suborbital flight mission to assess microgravity induced alterations on vascularization system and stress. Here, we present only the preliminary results of our ongoing gene expression analysis, as we are further elucidating the possible mechanisms of action. Findings from this experiment give insights into molecular pathways mediating vascular system and stress response and will assist in mapping out the strategies aimed to minimize the antagonizing effect of space travel in humans.

Keywords: Suborbital flight; zebrafish; cardiovascular; muscle atrophy; gene expression; simulated microgravity;

Nomenclature

NanoLab (10 cm × 10 cm × 10 cm)

Acronyms/Abbreviations

AAS: Applied Aviation Sciences; CC: Crew Capsule, ERAU: Embry-Riddle Aeronautical University; dpf: days post fertilization; Muscular characterization eXperiment In Microgravity Universal Spacelab: McXIMUS, PPF: Payload Processing Facility; UTHSCSA: University of Texas Health Science Center San Antonio.

1. Introduction

Over the past 50 years, since the Apollo flights (1968-1973), but most evidently during Skylab human spaceflight programs (1973-1974), loss of muscle mass and function was clear. No evidence of muscular disorganization and locomotor impairment was obtained during earlier flights in 1959-1963 with the Mercury and Gemini suborbital flights since these were short flights and there was no biomedical data (pre-flight, inflight, post-flight) to compare it with. It is well-known that astronauts can experience up to a 20% loss

of muscle mass on spaceflights that lasts up to 11 days. Longer duration space missions are clearly a concern and it is critical that we understand microgravity effects on the musculoskeletal system. Having the astronauts conduct physical exercise about 2-3 hours per day is critical to prevent further muscle volume loss and degradation of bone mineral density. Maintaining muscle function is critical so astronauts can perform safety activities and strenuous emergency procedures upon re-entry.

The musculoskeletal system is altered by microgravity environment. Recent studies [1] have suggested the use of non-mammalian systems (eg. Zebrafish) to refine present models that could be compared with other rodent models to better elucidate the effects of microgravity on bone and muscle. Previous research studies [2] have been conducted aboard the International Space Station (ISS) with regards to zebrafish in order to examine the gravity-dependence of skeletal muscles by monitoring a transgenic zebrafish line, to investigate the effect of microgravity on muscle mass and fiber morphology analysing the gene expression of mRNAs, and to examine the growth factors that are more sensitive to gravity. This type of research could lead to potential new drugs or treatments for weakened muscles. Zebrafish genes present generally about 80% similarity with their human homologs [8], and therefore it serves as a very promising model to tackle some of the challenging aspects of human space travel. However, little is known about the effect of microgravity on gene expression in zebrafish embryonic development. In present study, we exposed zebrafish embryos (day 2 and day 3 post-fertilization) to microgravity during the suborbital flight. Thus, prior to suborbital flight, we exposed zebrafish embryos to a slow rotating clinostat aiming to identify potential genes sensitive to simulated gravity. We evaluated the expression of multiple genes by conducting qRT-PCR experiments and identified specific genes that might be more susceptible to microgravity. Here, we present preliminary results derived from the comparative analysis of several genes affected by different types of microgravity platforms: suborbital space flight and clinorotation experiments. Our ongoing research efforts are aimed at further elucidating and refining the mechanisms of action.

Although the literature about microgravity effect on gene expression in zebrafish embryos is still limited, previous studies in which researchers exposed zebrafish to simulated microgravity give insights into gene expression in the heart, notochord, eyes, somites, and rohon beard neurons [4]. They observed approximately 30% increase in gene expression in the heart development for 32 hours post fertilization (hpf) (1.3 days pf) and 56 hpf (2.3 dpf), while the other parts showed periods of susceptibility between 24 hpf (1dpf) and 72 hpf (3dpf). Additional studies [5] identified 315 genes for early zebrafish development, 1dpf, 2dpf, and 5dpf, and their genetic screen revealed that complex molecular pathways of development play an important role in vertebrates. Other studies [6] findings revealed that early zebrafish embryonic vasculature development undergoes vast growth and remodelling, and blood vessels can be formed by various complex processes, and the genetic and molecular pathways that initiate and control these processes is not known or well understood. Thus, past research findings [1] showed that heart rate of zebrafish under simulated microgravity was about 60 bpm for 24 hpf or 25% lower than control; and 130 bpm for 36 hpf or about 7% lower than its controls. This explains why older embryos adapt to simulated microgravity better than younger embryos. Previous microgravity studies utilizing zebrafish [3] have revealed that there are unique mechanisms of tissue differentiation, which are distinct from the research findings obtained from ground-based simulations. Therefore, our study will build upon current knowledge and will aid in leveraging some of the unknowns about the effect of microgravity on gene expression [4].

1.1 Relevance to astronauts during space flight

Our study used zebrafish embryos 2-3 dpf, in which we observed and quantified the gene expression using various simulated gravity methods (suborbital vehicle, their corresponding ground controls, and slow rotating device). We identified several genes affected by the simulated gravity forces with the most profound effect observed in stress and vascularization related genes. We cannot neglect the possibility that some gene expression changes may be caused by the shear forces [4] when zebrafish were exposed to simulated microgravity. If this is the case, microgravity may have an impact on astronauts' heart and blood vessels' gene expression during space flight, suggesting that astronauts' cardiovascular system may be challenged. Reductions in physical activity and exposure to slightly elevated CO2 levels [7] are thought to impair the blood vessels of the brain to respond to changes in arterial blood pressure and CO2. Thus, it is unclear, how these changes occur in astronauts and their relationship with complications with astronauts' vision. Therefore, the significance of our work lies in the ongoing analysis of gene expression which may point out specific mechanisms accounting for microgravity altered molecular changes.

1.1.1 Sub-subsection headings

2. Material and methods

2.1 Animals

Zebrafish (*Danio rerio, strain: KDRL-BSY*) were maintained under standard conditions (14 h light, 10 h dark, fed twice daily) in the Greehey Children's Cancer

Research Institute (Greehey CCRI) zebrafish facility. The day before breeding, adult male and female zebrafish were set up in several breeding tanks, separated by a clear plastic separator. After the light was turned on the next morning, separators were removed, eggs were generated by natural mating and collected from 30 minutes to 2 hours after spawning. After sorting, clean eggs were moved to Petri dishes and incubated at 28°C in E3 medium (5 mM Na Cl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00001% Methylene Blue). For clinorotation studies, embryos 1-5 days post fertilization (dpf) were used, while for suborbital space experiment, embryos 2-3 dpf were used. All animal studies were approved by the UT Health San Antonio IACUC committee under the protocol #20170101AR.

2.2 Clinorotation parameters

For simulated gravity, we used the clinostat device (benchtop 2D clinostat, Eisco cat #BIO244) which allowed parallel positioning of max fifteen tubes rotating at a precisely 360 degrees every 20 minutes to provide a simulated weightless environment of 2.33×10^{-7} g. Zebrafish embryos (1-2 dpf) were placed in 5 ml tubes for continuous rotation for various time-points not exceeding 5 dpf. In each tube, five embryos were placed into 5 ml of E3 medium. The clinostat was placed in the zebrafish facility and covered by an aluminium foil to keep the embryos in the dark, isolated from possible visual clues concerning the rotation. E3 medium was changed every day. Control tubes (not exposed to rotation) were prepared exactly the same way but placed on the bench. After the study, embryos were euthanized by using the MS-222, were placed in RNAlater and kept at -80°C or imaged.

2.3 Transportation and housing at West Texas Launch Facility

Zebrafish embryos were transported to West Texas 2 days prior to the launch. Zebrafish embryos 0 and 1 dpf were placed in 50 ml conical tubes filled up to 90% with E3 medium. Tubes then were placed in a Styrofoam box and packaged using packaging peanuts to prevent moving during transport. Embryos were maintained at 27-32°C. It took approximately 6 hours to arrive at West Texas Launching Site in Van Horn, Texas. Upon arrival, fish were transferred into Petri dishes and placed onto a 30°C heating block to ensure the optimum temperature. E3 medium was changed to fresh daily. Zebrafish were live imaged under the inverted Nikon Microscope with the DS-L3 Control Unit. Some zebrafish embryos were left at the zebrafish facility, placed at the incubator and referred to as incubator controls.

2.4 Zebrafish Housing in the crew capsule (CC) and Ground Controls

On launch day, live 2 & 3 dpf zebrafish embryos (~100 from each batch) were transferred into 2 Oz round leak-proof bottles (Nalgene, cat # 562089-0001) each filled up to 90% with fish water to minimize sloshing and physical harm to the zebrafish. They were placed in the NanoLab (20cm x 10cm x 10cm) designed by the ERAU followed by the Nanorack team which put the NanoLab in the Nano Feather Frame Payload Locker 8-9 hours before launch. The NanoLab mass was about 500 g. The exact 2 Oz round leak-proof bottles containing 2 & 3 dpf zebrafish embryos were placed in the NanoLab kept at the West Texas Launch Payload Process Facility and were referred to as ground controls.

2.5 Mission Profile at Van Horn, Texas

Microgravity environment lasted about 4 minutes. During this time, embryos were assumed to have negligible shear and turbulence since the other mission phases such as launch, drogue parachute deployment, and landing, where sustain peak acceleration and vibrations can occur, do not last for more than a few seconds (see Fig. 2b).

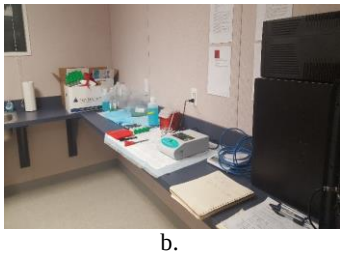
2.6 Post-flight Processing and Transporting to the UTHSCSA

The payload was retrieved and returned to our team after 6 hours. Imaging and video recording revealed that zebrafish embryos were alive and very active. After initial inspection and imaging/video recording zebrafish embryos were euthanized using Tricaine and then either placed in 4% Paraformaldehyde solution (for imaging experiments) or RNAlater solution (for qRT-PCR). These were stored on ice during transportation to the UTHSCSA facilities which occurred around midnight (same day of launch) for immediate analysis.

2.7 Confocal Imaging

The next day, 4% Paraformaldehyde fixed zebrafish embryos were collected from 4°C, washed three times with 1X PBS for 5 min each and placed in the 20 % sucrose solution for further imaging using Olympus FV3000 Confocal Microscope with 10X lenses and Midoriishi-Cyan blue channel.

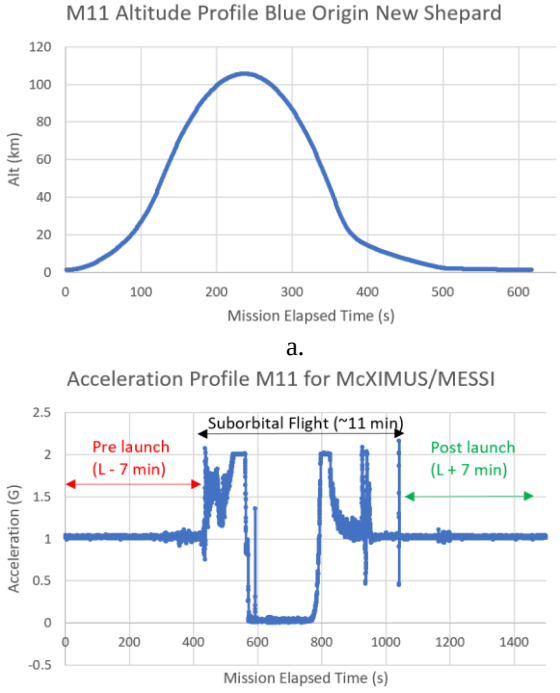
a.



b.

c d.

Fig. 1. Ground Control and Flight samples being prepared and secured. a. El Capitan Hotel, Van Horn, Texas. b. Separating control and flight samples at PPF. c. preparing NanoLab for flight samples at PPF. d. Second NanoLab containing control samples at PPF. White, red and non-lit 1.5 ml Eppendorf tubes to the right of the 2 zebrafish bottles were part of an adjacent experiment that did not involve zebrafish embryos.



a.

b.

Fig. 2. Flight profile of New Shepard's M11 mission. a. Altitude profile. b. Acceleration profile.

2.8 RNA extraction and reverse transcription

Total RNA was extracted by homogenizing embryos using pestles, followed by the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The concentration of total RNA was evaluated and measured at 260/280nm by spectrophotometer (NanoVue Plus, GE Healthcare). Synthesis of cDNA was performed from 0.5 µg of total RNA, which was reverse transcribed using (iScript cDNA Synthesis Kit, Bio-Rad) according to the manufacturer's instructions.

2.7 Real Time-PCR

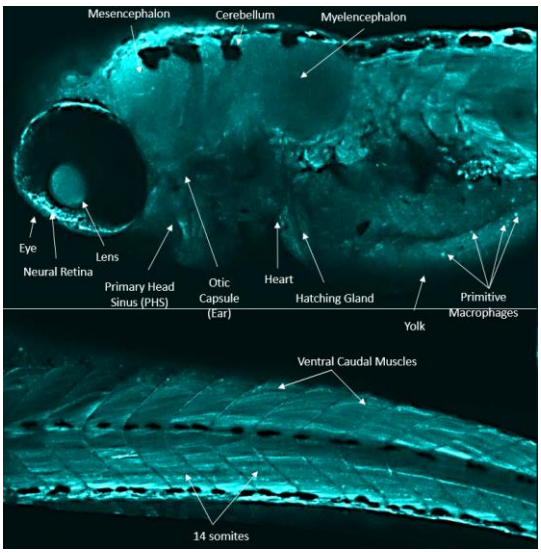
Gene-specific oligonucleotide primers were designed using Primer3 software to span exon-exon junctions (see Appendix A). qRT-PCR was done using SsoAdvanced Universal SYBR Green Supermix and SFX96 Touch real time PCR detection system (Bio-Rad, T100 Thermal Cycler). The cycling parameters (need to double check) were as follows: initial denaturation 95°C, 2 min; denaturation 95°C, 5 s; annealing/extension 60°C, 30 s; number of cycles 40; melt curve 65°-95°C (0.5°C increments). The comparative CT (2-ΔΔCT) method was used for all quantification. Values were normalized to the 18S housekeeping gene. Data from biological replicates were averaged and shown as mean normalized gene expression ± SEM. Data derived from at least two independent experiments.

2.8 Characterization of Fluorescence Intensity from Confocal Microscopy

Pictures for each condition (incubator, clinostat, control, ground 2dpf and 3dpf at PPF, and flown samples for 2dpf and 3dpf) were taken. ImageJ software was used to quantify the maxima of fluorescence intensity obtained from each picture. To find the maxima each picture was processed by excluding the edge maxima, and provided the count of selected points with these maxima. These counts were stored in an array and plotted for comparison (see Fig. 5).

2.9 Statistical Analysis

Data were graphically displayed using Prism 8 software. Differences in gene expression across different conditions were determined and compared using unpaired t-tests with a two-sided alpha of 0.05/0.005. Data are represented as mean ± standard error mean (SEM).



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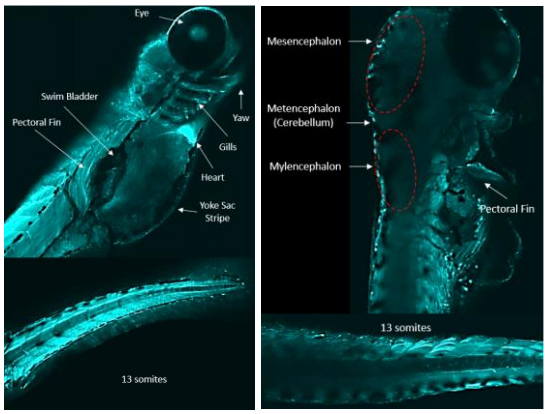

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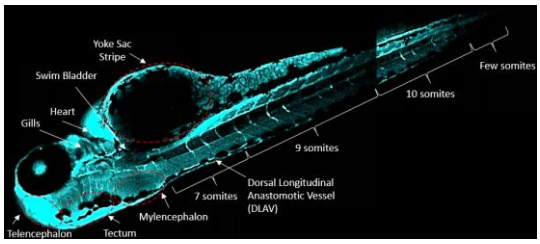

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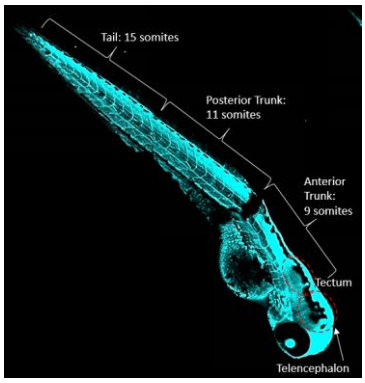


e.

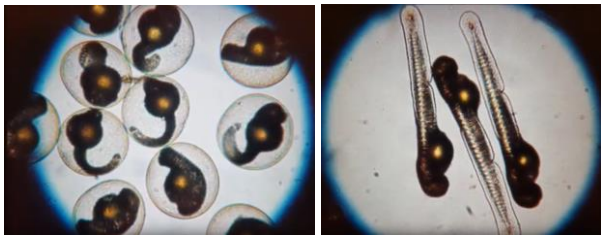
Fig. 3. Examples of zebrafish embryos at 2 dpf. a. Clinostat. b. Control incubator in the lab. c. Control in the lab. d. Flight. e. Ground at PPF. Images were taken with Olympus FV3000 Confocal Microscope with 10X lenses and Midoriishi-Cyan blue channel.

4. Results

This section addresses research findings of 2dpf and 3dpf zebrafish behaviour before and after the suborbital flight (Figures 6, 7), and the effects of clinorotation (Fig. 8) for 5dpf zebrafish during for ten genes. Flight condition was compared with ground for both 2dpf and 3dpf. Clinostat condition was compared with control.

Assessment of zebrafish behaviour pre- and post-suborbital flight

We assessed the behaviour of the zebrafish embryos prior and post suborbital flight to ensure their survivability of the transportation and suborbital space exposure by inspecting their movements. Figure 4a displayed 28 moves for 11 embryos (L-36h) where every embryo moved at least once during 30 seconds time span. The right of Figure 4a depicts some of these embryos eight hours later (L-28h) –these showed no motion for even tens of minutes at a time, and provided a clear visualization of their heart pumping, with a heartbeat of about 168 bpm. Figure 4b depicts the embryos placed on the Petri dish. These showed no major moves over hours. Figure 4c illustrates several snapshots of the bottles with active embryos at L-18 hours before the flight. Figure 4d shows the same embryos about 6 hours post-flight. 2dpf embryos did not show much mobility while 3dpf embryos revealed to be very active suggested by their rapid swimming moves.



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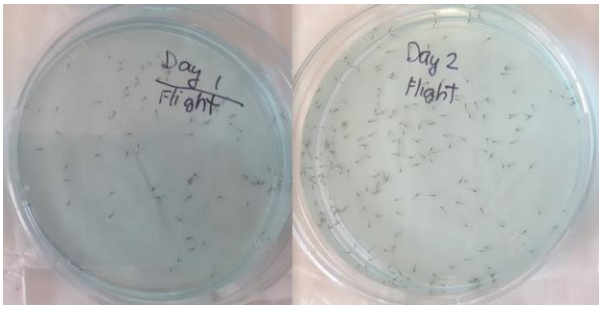

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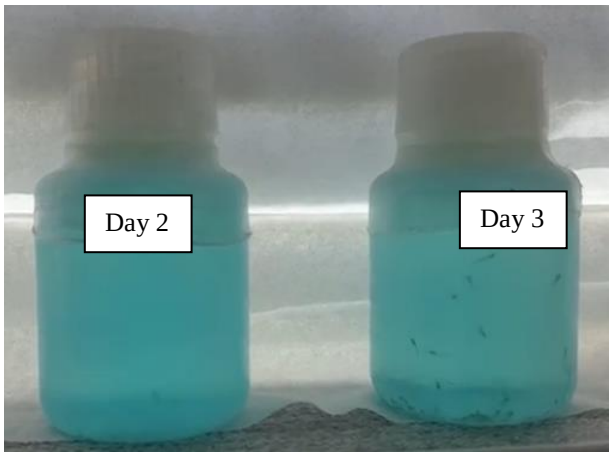

d.

Fig. 4. Visual examination of zebrafish embryos. a. Embryos being examined under the microscope at hotel El Capitan, Van Horn. Left: L-36h. Right: L-28h. b. Embryos placed in Petri dish plates at PPF for easy access collection prior to placing them in the bottles. c. Snapshots of actively moving embryos at PPF. d. Bottles recovered 6 hours post-flight showing day-2 embryos with low activity (left) and day-3 embryos being very active (right).

Effects of suborbital flight and clinorotation on the expression of the KDRL-BSY fluorescently labelled vascular system by confocal microscopy

We next assessed the intensity of the fluorescently labelled vascular system in zebrafish embryos by confocal microscopy. We compared flown zebrafish embryos to the ground controls and the ones left at the incubator at the zebrafish facility which were not exposed to the transportation stressors. Thus, we extended our analysis by adding an additional comparison group which consisted of embryos exposed to clinorotation (5 days). Our results (see Fig. 5) suggest that the fluorescence levels processed by ImageJ software for flown zebrafish embryos (2dpf and 3dpf) are over 3 times higher than for the control incubator conditions. Clinorotation effects also show increasing levels of fluorescence intensity, almost twice as that one of the control incubator conditions. However, it is important to note that zebrafish in this condition were older (5dpf) than the other samples, therefore this could justify for the increased fluorescence intensity. Ground samples showed over 30% and 15% increase of fluorescence intensity for 2dpf and 3dpf, respectively. Very little difference for control condition was observed, only 7% increase with respect to control incubator conditions. Temperature of incubator was 28.5°C (constant), temperature inside the zebrafish facility was 30°C, thus control and clinostat samples were exposed at this temperature; temperature inside the PPF varied between 27.5°C and 29.5°C, temperature inside the NanoLab (ground control at PPF) also varied from 23°C to 26°C according to a thermal sensor next to the bottles. Bottles were enclosed by some foam as a fluid level of containment and to damp vibrations of launch. Thus, the temperature inside the bottles was estimated to be 3 degrees warmer (26°C to 29°C). Temperature inside NanoLab (flight) was between 18.5°C and 19°C. Temperature variations may have affected our results.

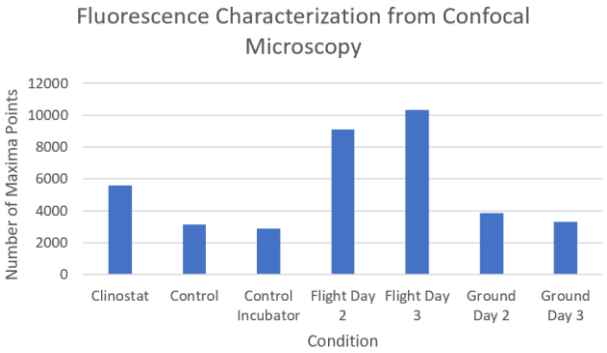

Fig. 5. Number of maxima points (based on fluorescence intensity) for each condition.

Effects of suborbital flight on gene expression in day 2 and day 3 zebrafish embryos

We evaluated the expression of multiple genes in 2 and 3 dpf zebrafish embryos exposed to suborbital flight and compared their expression to the ground controls (see Figures 6, 7).

Given exposure to such flight might induce the stress response, initially, we assessed the expression of two well-known stress genes (heat shock proteins (HSPs) hsp70 and hsp47 which are induced in response to a variety of stress conditions [9]. Our data show the significant up-regulation of both genes in suborbital flight samples with a higher expression detected in the hsp47 gene than to hsp70 for both developmental stages of zebrafish embryos. Furthermore, the expression of hsp70 was higher in day 2 embryos, whereas the expression of hsp47 was higher in day 3 embryos. The significantly elevated expression of hsp47 could be more sensitive to stressors experienced by the zebrafish embryos as compared to hsp70. Other researchers [9] report that certain stressors, such as exposure to ethanol resulted in a significant upregulation of the hsp47 gene and only a slight increase of hsp70 gene in day 2 zebrafish embryos.

Next, we looked at the expression of the vascular endothelial growth factor (VEGF) family of genes including VEGF, FLK1 also known as vascular endothelial growth factor receptor 2 (VEGFR-2), and FLT4 (fms-related tyrosine kinase 4), vascular endothelial growth factor receptor 3 (VEGFR-3). VEGF signalling plays multiple roles during vascular development of the zebrafish, especially in embryonic vasculogenesis and angiogenesis (two distinct

mechanisms that implement the formation of the vascular network in the embryo).

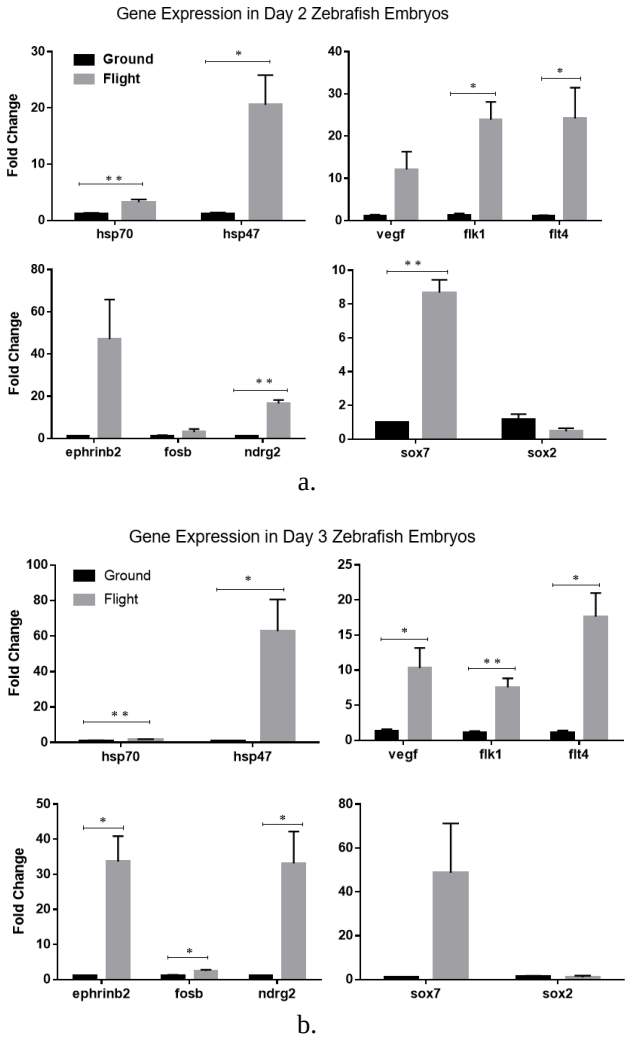

Fig. 6. Fold change expression (2^-(ΔΔCt) of genes affected by the exposure to suborbital flight. a. Day 2 zebrafish embryos. b. Day 3 zebrafish embryos. Values indicate mean ± SEM (derived from at least two independent experiments). * $p \leq 0.05$; ** $p \leq 0.01$. p shows the significance of gene expression between flight and ground sample.

Vasculogenesis is accountable for the formation of the heart and the first primitive vascular plexus inside the embryo and in its surrounding membranes, whereas angiogenesis expands this network [10]. During these processes, the Flk-1 is present in the endothelial cells. Our data show that both VEGF receptors FLK1 and FLT4 were significantly up-regulated in days 2-3 zebrafish embryos exposed to suborbital flight, followed by the increased expression of VEGF gene. Our data is in alignment with findings from another group [1] which demonstrated the up-regulation of the endothelial-specific marker FLK1 in 24-36 hpf stage zebrafish embryos exposed to simulated gravity. We further assessed the effect of vascular development by looking at the vein vascular marker FLT4 and the arterial vascular marker EPHRINB2 expressions which were both up-regulated in suborbital space samples. Thus, FOSB (activator protein 1) had no effect, whereas there was a significant up-regulation of N-myc downstream-regulated gene 2 (NDRG2) in zebrafish embryos. Our results suggest that exposure to suborbital flight may promote vascular angiogenesis and vasculogenesis in the zebrafish embryos.

The previous study [1] observed that simulated microgravity on zebrafish vascular development was not evident at 12 hpf - 24 hpf, but these effects were significant at 24 hpf - 36 hpf. These studies also revealed that FLK1, FOSB, and EPHRINB2 for 36 hpf zebrafish showed highly significant differences (26% higher, 20% lower and 38% higher, respectively) in the development of its cardiovascular system with respect to controls when using the simulated microgravity device.

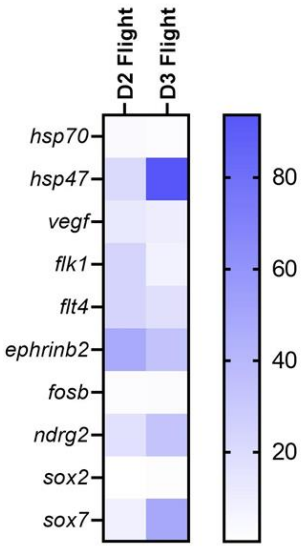

Fig. 7. Heat map of genes affected by the exposure to suborbital flight in days 2 & 3 zebrafish embryos. Graph was generated by plotting the relative expression (2^-(ΔΔCt) of each of these genes to the ground controls. Data derived from the qRT-PCR experiments. Colour intensity represents the level of expression.

Finally, we looked at the SOX (Sry-related HMG box containing) family of transcription factors genes SOX7 and SOX2. In zebrafish, little is known about the SOX genes and their role in the skeletal system. Our data show the up-regulation of SOX 7 in day 2 (significant) and day 3 (not significant) embryos, whereas SOX2 was downregulated in day 2 embryos and with no difference in day 3 embryos. In summary, our results suggest that exposure to suborbital flight might induce alterations in the vascular system of days 2-3 zebrafish embryos.

Effects of clinorotation in day 5 zebrafish embryos exposed to simulated rotation for 4 days

In preparation for suborbital flight, we first exposed zebrafish embryos to the clinorotation and sought to identify genes possibly affected by this simulated gravity platform. In contrast to exposure to suborbital flight, clinorotation did not significantly alter the expression of the majority of genes of which most were downregulated. The significant downregulation was observed in the following genes: VEGF, VEGFC, FLK1, FLT4 and NDRG2, whereas not significant up-regulation was observed in EPHRINB2. Other genes, HSP70, HSP47, and FOSB either had a very slight effect or were downregulated without significance. It is possible that this platform of simulated gravity might not induce changes in gene expression (see Fig. 8).

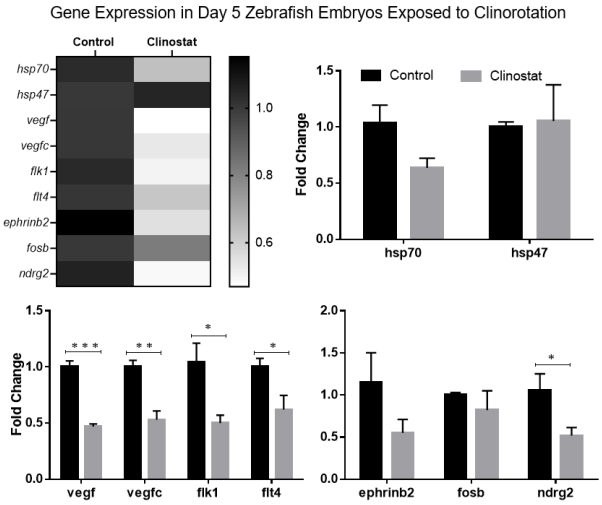

Fig. 8. Heat map of genes affected by the exposure to clinorotation in day 5 zebrafish embryos (top left). Bar graphs showing fold change expression (2^-(ΔΔCt) of the same genes. Data derived from the qRT-PCR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; values indicate mean ± SEM (from at least two independent experiments).

5. Discussion

To the best of our knowledge, there are no reports available on gene expression analysis conducted in zebrafish embryos exposed to suborbital flight. In this study, we presented preliminary data on the expression of several genes possibly affected by the suborbital flight. Our data show that the stress genes (HSP70 and HSP47) and genes involved in vascular system formation had the most profound up-regulated effect in zebrafish embryos exposed to suborbital flight. Given that a sudden adverse environmental change leads to the increased synthesis of stress proteins, we were not surprised about the elevated levels of HSP70 and HSP47. Thus, microgravity might alter the blood flow and, in such way, might increase the formation of the blood vessels and accelerate vascular system formation. Therefore, this might lead to the changes of the vascular system which we observed in zebrafish embryos exposed to suborbital flight. Our gene expression data also correlates with the fluorescence intensity measurements indicating that the vascular system might be truly affected by the brief exposure to microgravity. Interestingly, zebrafish embryos exposed to clinorotation had little or downregulated effect in most genes' expression. We hypothesize that the speed that the 2D-clinostat generates might not be sufficient to induce changes in the gene expression level.

It is important to note that due to the limited number of zebrafish we had, we isolated RNA from the whole zebrafish embryos, but it is possible that certain genes expression might be tissue-specific [11]. Therefore, for future studies, RNA should be isolated from different parts of the zebrafish embryos.

Here, we presented only the preliminary results of suborbital flight effect, as there is an ongoing investigation on more genes expression which will be presented in future manuscripts. Also, our further analysis will include incubator controls which might eliminate some of the confounders, such as the transportation variables, that could have affected our results.

6. Conclusions

In this study, we show that *Danio rerio* serves as a good model to investigate alterations in gene expression during the suborbital flight. We identified a group of genes with the most profound effect including stress and vascular formation genes. Appendix A depicts the summary of genes analysed in our study and their corresponding gene expression signatures. As described above, certain families of genes are more susceptible to suborbital flight exposure than others. Identifying their expression and studying their molecular mechanisms could lead to a design of gene therapy studies (such as targeting VEGF), so that we could tackle the most challenging aspects of human space travel.

Acknowledgements

This work was partially supported by the AAS department at Embry-Riddle Aeronautical University, the department of Molecular Medicine at UTHSCSA, and the Graduate School of Biomedical Sciences at UTHSCSA. Thanks to the Faculty Research Development Program (FRDP) that allowed the PI to travel to Texas in preparation for the suborbital payload during week launch. Special thanks to Dr. Myron Ignatius for allowing us to use the zebrafish facility at the Greehey Children's Cancer Research Institute. Also, thanks to Kunal Baxi and Nicole Hensch for their advice and help during early phases of the research. The

authors would like to thank the AAS for funding the travel expenses to present this research work.

Appendix A (Primer sequences for the quantitative PCR (qRT-PCR))

See end of paper.

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Appendix A: Primer sequences for the quantitative PCR (q-PCR) and suborbital flight effects on 2dpf, 3dpf, and clinorotation effects on 5dpf zebrafish embryos.

Name	Forward primer (5'->3')	Reverse primer (5'->3')
ndrg2	CCAATGATGGTTGGGGTTAG	AGGCTGATCTCACGAGGAAA
flk1	TGGTTCGTTACTCCACACCA	GCAGCCGACTGTACTCCTTC
flt4	GCGTCAGCGACTACTTCTCC	CCAGTGGCTGCTGAGAGAAT
ephrinb2	CAAGGACAGCAAATCGAATG	TGAGCCAATGACTGATGAGG
hsp70	CAAAGGCAAATCCTCAGAGC	CACAAAGTGGTTCACCATGC
hsp47	TGGGTCTGACTGAAGCTGTG	GTCGGCGTAGAAGAGTTTGG
vegf	AAGGATTGCCACTGGATCAC	GGAAAGCCGACGTTTAATGA
sox7	AGTCGAGCACCACACTCTCA	CATGTGCGCTGTCAAAACTT
sox2	AACCAGCGCATGGACAGTTA	GACTTGACCACCGAACCCAT
vegfc	GTCTAACCGCGACCAGAGAG	CCGTTTCCTTCTTCACAAGC
18S	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATCA

Name	Relevance	Effects on 2dpf (suborbital)	Effects on 3dpf (suborbital)	Effects on 5 dpf (clinorotation)
ndrg2	Cell proliferation, differentiation, cancer, cell stress	Up regulation (significant)	Up regulation (significant)	Down regulation (significant, 50%)
flk1	Blood vessel development, regulation heart contraction	Up regulation (significant)	Up regulation (significant)	Down regulation (significant, 55%)
flt4	Vein vascular marker, vasculature development	Up regulation (significant)	Up regulation (significant)	Down regulation (significant, 40%)
ephrinb2	Arterial vascular marker	Up regulation (not significant)	Up regulation (significant)	Down regulation (slight, 55%)
hsp70	Stress gene, response to heat, hemopoiesis	Up regulation (significant)	Up regulation (significant)	Down regulation (slight, 45%)
hsp47	Stress gene, fin regeneration	Up regulation (significant)	Up regulation (significant)	Down regulation (negligible)
vegf	Organ, circulatory system and vascular developments	Up regulation (not significant)	Up regulation (significant)	Down regulation (significant, ~50%)
sox7	Artery development and vasculogenesis	Up regulation (significant)	Up regulation (not significant)	Not studied
sox2	Organ development, cell population proliferation, fin regeneration	Down regulation (not significant)	Down regulation (not significant)	Not studied
vegfc	Positive regulation of endothelial cell proliferation, vasculature development	Not studied	Not studied	Down regulation (significant, ~50%)
fosb	Bone formation	Not studied	Not studied	Down regulation (not significant, 20%)
18S	Housekeeping	Comparison	Comparison	Comparison