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Effects of Simulated Microgravity on Vascular Development in Zebrafish

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Abstract: Research in microgravity is of utmost importance for disclosing the impact of gravity on biological processes and organisms. With the development of space technology, scientists pay more attention to cardiovascular diseases associated with microgravity. However, up to date only sparse data exist on microgravity and cardiovascular development mechanisms. In this study, zebrafish was chosen as the model organism. Zebrafish embryos were exposed to microgravity using a ground-based simulation microgravity (SM) bioreactor. The effects of SM on the development of early embryonic vascular system were studied *in vivo* in real-time. Zebrafish embryos were selected and divided into two groups at 12 hpf. One group was cultured in the MG-IIA bioreactor whereas the control group was cultured under normal gravity conditions. SM did not affect the number of live zebrafish and there were nonspecific developmental phenotypes in two groups. The heart rate in SM zebrafish embryos was significantly decreased. Then the vascular development differences between two groups were analyzed by qPCR and whole mount *in situ* hybridization. The effect of SM on zebrafish vascular development was not evident at 12 hpf-24 hpf stage, but it had significant influences at 24 hpf-36 hpf stage.We also found that nos2b expression was up-regulated in the SM group both at 24 hpf and 36 hpf, interesting, all nos2b expression was observed in the hypothalamus at 24 hpf, it was no difference in the hypothalamus but significantly increased in the dorsal near the vascular at 36 hpf. These data suggested that the effect of SM on vasculogenesis stage is not obvious, but it has significant influences on angiogenesis, which maybe has relationship with the expression of nos2b.

Keywords: Simulated microgravity, gene expression, caudal vascular development, zebrafish.

1 Introduction

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Several studies on space flight and microgravity have demonstrated that physiological changes occur in humans during space flight, causing cardiovascular deconditioning[Aubert, Beckers and Verheyden (2005)],bone loss-[Hughes-Fulford (2001), Muller, Dalcq, Aceto et al. (2010)]muscular atrophy[Delp, Colleran, Wilkerson et al.(2000)]and others adverse health effects. The physiological changes caused by microgravity and space flights are not well understood. Several studies have used tail-suspended, parabolic flight[Wassersug and Izumi-Kurotani (1993)], or ground-based instrument simulated microgravity (SM)[Schwarz, Goodwin and Wolf (1992)]to look into mechanisms of post spaceflight diseases and on the impact on development of organisms. Such research may contribute to our understanding of how terrestrial animals can overcome diseases associated with microgravity, especially for astronauts.

Recent studies show that microgravity can influence the development oforganism. Though SM had no significant effect on the expression of B6D2 *in vitro* fertilization in mice, there is obvious adverse effect *in vitro* in the early embryonic development[Kojima, Sasaki, Kubota et al.(2000)]. Mouse embryonic development was restrained, which may be related to that mammalian early embryo development needs gravity vector. In the process of early embryonic development, the establishment of embryonic polarity is critical for the normal development of embryos [Ubbels and Brom (1984)]. Development speedof drosophila was significantly delayed in microgravity environment. The male drosophila life expectancy fell significantly compared with controls[Miller and Keller (1999)]. Zebrafish larvae were exposed to simulated microgravity environment had less inflated swimbladder at 96 hpf (hour post-fertilization)[Lindsey, Dumbarton, Moorman et al. (2011)], the number of otolith was reduced and the size became smaller [Moorman, Burress, Cordova et al. (1999)]. Microgravity also influenced the gene expression in zebrafish[Shimada and Moorman (2006); Shimada, Sokunbi and Moorman (2005)]. The studies referred to above show that microgravity influence the development of animals. However, studies on the effect of SM on vascular development seem to be lacking.

Cultured vascular smooth muscle cells *in vitro* have shown that microgravity influenced the cell cycle, suppressed cell proliferation and migration, enhanced cell apoptosis, stimulated nitric oxide (NO) release, and destroyed the organization of the cytoskeleton [Kang, Fan, Sun et al. (2013)]. Other research showed that SM can influence rat hind limb-unloaded (HU) structural remodeling. Functional adaption of the arterial microvasculature occurred in skeletal muscles, resulting in decreased cross-sectional area of hindlimb feed arteries4. However, the change ofarterial cross-sectional area caused by SMcould recover via cultured in normal gravitycondition 4-weeks [Zhang (2001)] although the above-mentioned studies suggest that SM affect the vascular development, the mechanism remains unclear.

Under normal conditions for endothelial cells (EC), NO is involved in maintaining normal physiological function of the vascular system. NO is synthesized by endothelial nitric oxide synthesis (eNOS)[Huang, Wei and Hung (2006)]. eNOS is involved in vascular development processes[Kubes, Suzuki and Granger (1991); Palmer (1993)]. NO influence the cardiovascular system during early development of zebrafish and it plays a very important role in the development of the vascular system [Pelster, Grillitsch and Schwerte (2005)]. At present we do not identify the homologous genes of eNOS in fish

but some studies found that nos2b in zebrafish contain a common eNOS special gene function areas of mammalian: N-terminal myristoylation sequence [Lepiller, Franche, Solary et al.(2009)]. Based on these studies we can assume that nos2b in zebrafish is similar to eNOS of mammals. It is necessary to understand whether nos2b participates in regulating vascular development.

To address these questions, we used transgenic zebrafish as the animal model, in which vascular ECs and blood cells could be simultaneously labeled by fluorescent proteins. Zebrafish is a powerful developmental biology model for studying vertebrate development. Zebrafish organ structure, physiology and molecular aspects were strikingly similar to humans. The whole body of zebrafish embryo is transparent, making it possible to observe vascular development in the entire zebrafish *in vivo*. This includes dynamic processes such as heart beat, blood circulation, i.e. the vasculature can be better studied than in cell or organ cultures[Chico, Ingham and Crossman (2008)]. ECs migrate to the specific locations where they assemble into the original blood vessels, forming the main arterial and venous blood vessels, cardiac primordia and original embryonic capillary network. This is the process of vasculogenesis[Yancopoulos, Davis, Gale et al.(2000)]which occurs from 12 to 24 hours post fertilization (hpf) in zebrafish. Cardiac conduction commences approximately at 20 hpf and blood circulation approximately at 24 hpf [Chico, Ingham and Crossman (2008)]. Angiogenesis occurs from 24 hpf in zebrafish. The immature vascular system still needs to develop vascularization branch and initiate circulation in the capillary network. This occurs by original blood vessels growth into new blood capillaries by budding or nested type growth[Risau and Flamme (1995)]. Hence, zebrafish is an ideal model for studying angiogenesis and mechanisms of cardiovascular diseases.

The present study aimed to explore the effects of microgravity on the development of vasculature. Our previous research showed that horizontal rotary cultivation can retard development of vascular plexus in the tails of zebrafish embryos [Sun, Xie, Zhang et al. (2013)]. In the present study, embryos were treated by simulated microgravity bioreactor from 12 hpf. At 24 hpf and 36 hpf, we examined the effect of SM on vascular development. Fluorescent protein labeling enabled us to observe vascular anatomy in the same embryo. In addition, we studied vascular marker flk1, arterial vascular marker ephrinB2, and venous vascular marker flt4 and gravity response factor gene nos2b, in order to evaluate effects of microgravity on blood vessels at molecular levels. This research can impact on our understanding of vascular remodeling during pregnancy, space flights and the mechanism of cardiovascular diseases after space flight.

2 Materials and methods

2.1 Zebrafish

The animal housing and surgical procedures were in accordance with the Guide for the Chinese Animal Care and Use Committee Standards, which conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were also performed in accordance with protocols approved by the Animal Ethics Committee of Chongqing

University. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed.

This study used zebrafish Tg (Flk1: GFP) that express green fluorescent protein in the vasculature. The zebrafish were obtained from Tsinghua University developmental biology lab and then bred and maintained in the laboratory at Chongqing University. The fish were kept on a 14 h :10 h day-dark cycle in aquaria supplied with 28°C recirculating water. The zebrafish were fed salt water fairy shrimps daily. Embryos were collected within 3 h after fertilization and maintained at the same temperature until 12 hpf. Further incubation was in the SM bioreactor or at normal gravity condition (controls). Embryos were collected at 24 hpf and 36 hpf respectively. The embryos collected at 36 hpf were carried out in 0.003% 1-phenyl-2-thiourea (PTU) at 24 hpf to inhibit melanin pigment formation.

2.2 Zebrafish embryos were exposed to SM

We used a MG-IIA type of ground-based bioreactor provided by the institute of Mechanics at Chinese Academy of Sciences to simulate microgravity. [Zhe, Luo, Lin et al. (2015)]Embryos were placed in a special container for exposure to SM and an equivalent portion of embryos was placed in a stationary container as controls from 12 hpf. The container radius r was 2.5 cm and a maximum of 60 fertilized embryos were placed in the container for each experimental trial. Air bubbles were removed and the chamber was sealed and mounted to the bioreactor base. We chose zebrafish who have the same mother as the two groups: the SM group and the control group. All embryos were placed on the same table and received the same vibration and illumination time. Until 24 hpf and 36 hpf we observed the mortality, the fluorescence intensity, heart rate, and development of the vascular plexus of the zebrafish tail. Previously we found that horizontal rotary cultivation promoted apoptosis of zebrafish cells whereas it had no significant effect on proliferation, resulting in delayed development for embryo tail's vascular plexus during angiogenesis[Sun, Xie, Zhang et al. (2013)]. Hence, we chose 12 hpf zebrafish embryos for the experiments.

MG-IIA ground-based bioreactor is made up of four parts, including a rotating stent, tissue culture container, base stand, motor and transmission system (Figure1). There is no changing medium in this bioreactor. The bioreactor has a gas exchange membrane on both sides which provided oxygen for the bioreactor. In the horizontally rotating bioreactor, zebrafish are affected by gravity, buoyancy, centripetal force and fluid shear stress. The trajectories of zebrafish will be affected by particle radius, relative density, rotating speed as well as by other factors[Liu, Li, Sun et al. (2004)]. When the solution has the same density as the embryos, there is no relative movement when the forces offset each other. This is similar to the space microgravity by the MG-IIA type of ground-based bioreactor. The radius of the container was $0cm \leq r \leq 2.5cm$, the rotation speed was $5rpm \leq n \leq 50rpm$, and g0 was $981 cm/s^2$.

The angular velocity, $\omega = \frac{n \times 2\pi}{60}$

$K = \frac{\omega^2 r}{g_0}$ "K" is a characteristic parameter for describing microgravity environment.

Table 1. Zebrafish gravity environment inside the reactor critical value K. "K" is a characteristic parameter for describing microgravity environment. "Y" means "Yes".

n(rpm)	r(cm)	ù	K	microgravity
5	2.5	0.523	$0.07\ x10^{-2}$	Y
8	2.5	0.873	$0.18\ x10^{-2}$	Y
10	2.5	1.047	$0.27\ x10^{-2}$	Y
15	2.5	1.570	$0.63\ x10^{-2}$	Y
20	2.5	2.093	$1.12\ x10^{-2}$	Y
25	2.5	2.617	$1.74\ x10^{-2}$	Y
30	2.5	3.140	$2.51\ x10^{-2}$	Y
40	2.5	4.187	$4.47\ x10^{-2}$	Y
50	2.5	5.233	$6.98\ x10^{-2}$	Y

The critical value K of the zebrafish embryos was calculated, and its value keeps at 10^{-2}, which suggested that the whole volume of the containers was under microgravity conditions (Table 1). Embryos keep in a state of suspension, which avoids the larger shear force and bubble generation, three-dimensional culture system for the incubation of embryos created a good moderate mechanical environment. Rotating wall vessel was designed by American National Aeronautics and Space Agency (NASA) which provides a microgravity environment, and the rotating speed is limited in 8.5 rpm to 26.5 rpm are namely in microgravity condition[10], but the rotating speed 15 rpm to 20 rpm were commonly used in microgravity simulation experiment[Moorman, Cordova and Davies (2002)]. As the particle diameter becomes large to increase optimum speed, and higher the percentage of dissolved oxygen won, and besides zebrafish embryos diameter is about 1mm, so we choose 25 rpm to SM.

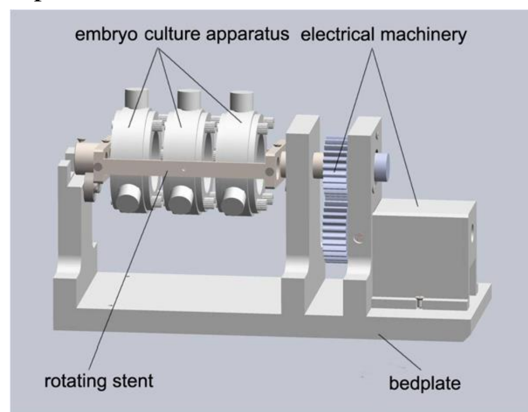

Figure 1. The MG-IIA type of ground-based simulated microgravity (SM) bioreactor was provided by the National Microgravity Laboratory, Institute of Mechanics, Chinese Academy of Sciences.

2.3 qPCR

Total RNA was extracted using trizol method. The concentration of total RNA was detected and reverse transcribed into cDNA. The gene sequences of flk1, flt4, ephrinB2, nos2b were found in National Center of Biotechnology Information (NCBI). Primers were designed by Primer 5 software (Table 2). We chose the fluorescence quantitative qPCR experiment SYBR Green kit (Bio-RAD). Total volume 25μL system: cDNA 1μL, Forward Primed 0.5μL, Reverse Primed 0.5μL, ddH2O 10.5μL, SYBR Green PCR Master Mix 12.5μL. The qPCR program: denaturation for 4min at 95°C, 39 cycles for 15s at 95°C, for 30s at 60°C, for 30s at 72°C follow by 15 s at 95°C and a melt curve for 30s at 55°C to 95°C.

Table 2. q-PCR primer sequences (5′→3′)

Genes	Primer sequences
flk1	F: 5'-GAGAACGGAACCAACAAGATCCACGAG-3'
	R: 5'-CCCTCCAGCAGAACTGACTCCTTAC-3'
flt4	F: 5'-CTGTCGGATTTGGATTGGGA-3'
	R: 5'-GGTGGACTCATAGAAAACCCATTC-3'
ephrin B2	F: 5'-CAAGGACAGCAAATCGAATG-3'
	R: 5'-TGAGCCAATGACTGATGAGG-3'
nos2b	F: 5'-CTTCAAGCCGACTTTCCTTG-3'
	R: 5'-GTCAGGAGAGGAGCTGATGG-3'
â-action	F: 5'-CTGTCTTCCCATCCATCGTGGGTC-3'
	R: 5'-CTCCATATCATCCCAGTTGGTGACA-3'

2.4 Thepreparation of in situ hybridization RNA probes

We designed specific primer sequences of flk1, flt4, ephrinB2, nos2b using Prime 5.0 software (Table 3) for hybridization *in situ*. The fragment was recovered, purified and connected to PMD-20T carrier. The plasmid was constructed and transformed into *E. coli*. The *E.coli* was expanded cultured to obtain a mass of recombinant plasmid. Sequence identification was carried out by the Invitrogen Company. Extinction enzymes Not I was used to cut the plasmid at specific places to get the linear plasmid. According to the digoxin RNA labeling kit (ROCHE), the digoxin-labeled probes were synthesized successfully from corresponding antisense RNA.

Table 3. Primer sequence of the PCR for hybridization *in situ* probes

Genes	Primer sequences
flk1	F: 5’-TGCCCAGATTATGGTGATG-3’
	R: 5’-GATGCTATCCGACTGAACC-3’
flt4	F: 5’-TTCTGGCATCTCGTAAGTGTAT-3’
	R: 5’-ATGTTGTTGCTTATGCCAGTAG-3’
ephrin B2	F: 5’-AGGAAGTGAGTGGATACGACGAG-3’
	R: 5’-GAACAGGCAAACCCAGGAAAT-3’
nos2b	F: 5’-ATGGAGCACCCACAGTATGA-3’
	R: 5’-AGCCCAGGATGAGAACGATT-3’

2.5 Whole-Mount in situ hybridization

In our experiments, zebrafish was separately treated from 12 hpf to 24 hpf and 36 hpf. The shell of the zebrafish embryo was stripped and embryos were collected with EP tubes. Each tube contained 30 embryos that were fixed by 4% PFA/PBS (Paraformaldehyde/Phosphate Buffered Saline) and then kept in refrigerator -20°C.The *in situ* hybridization was performed according to the protocol from Hashimoto [Hashimoto, Maegawa, Nagai et al. (2004)]. An Olympus stereo microscope was used for making images.

2.6 Dataanalysis

The data obtained in this study are reported as means ± standard deviation. Data obtained from different treatment groups were statistically compared by SPSS 17.0. Differences between the control and experimental groups were compared using *t*-test. Differences were considered significant at $p<0.05$ and highly significant at $p<0.01$

3 Results

3.1 SM effects on zebrafish embryos vascular development

After zebrafish were treated by SM, we observed the effects of SM on the development of vascular in zebrafish across the Green Fluorescent Protein (GFP) fluorescence imagesusing stereo microscope (Olympus, Tokyo, Japan). The vascular development in the SM group was faster than in controls, i.e. the development of the caudal venous plexus was more complete at 24 hpf and 36 hpf (Figure 2A). But interesting, there was nonspecific developmental phenotypesbetween the two groups at 24 hpf and 36 hpf (Figure 2B). We also analyzed the number of live zebrafish at 24 hpf (Figure 2C) and 36 hpf (Figure 2D) (n=60), data shows that there was no significant difference between two groups. The heart rate of the SM group was significant different from the control group at 36 hpf (Figure 2F). At 24 hpf, there was a highly significant difference between the two groups (Figure 2E). Hence, SM affects the vascular development in zebrafish.

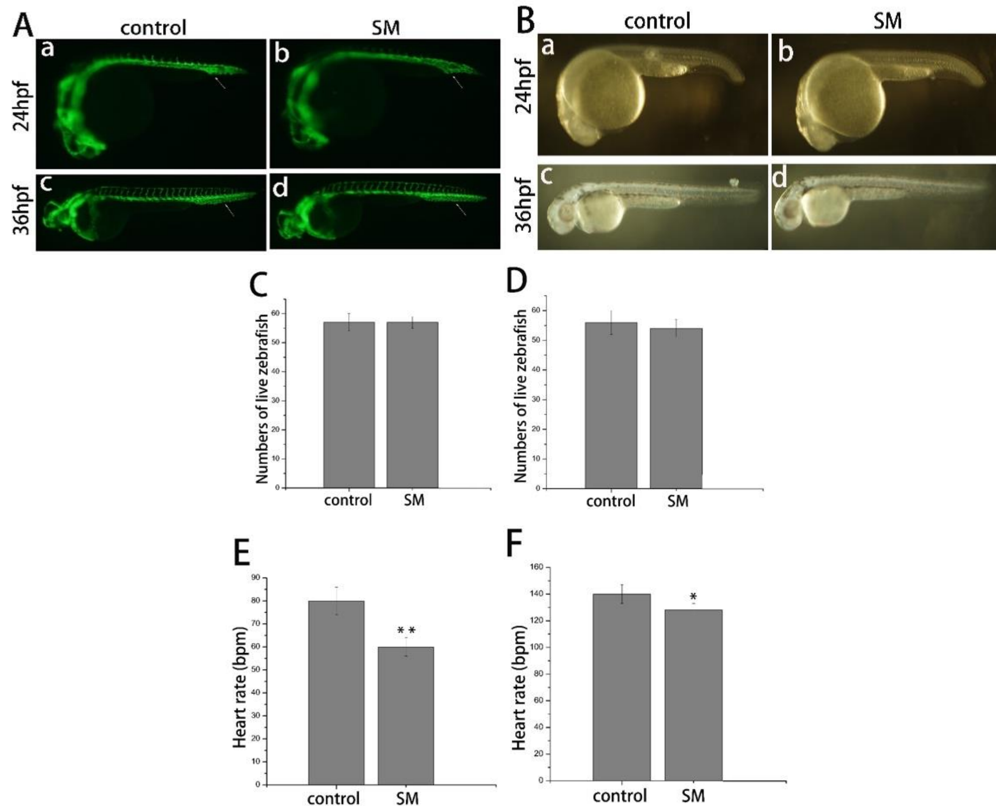

Figure 2. Effect of SM on the development of cardiovascular system. A) Zebrafish GFP fluorescence images using an Olympus stereo microscope. The arrows point at the embryos caudal venous plexus. B) White light pictures of zebrafish were imaged at 24 hpf and 36 hpf using an Olympus stereo microscope. C) The number of live zebrafish at 24 hpf (n=60). D) The number of live zebrafish at 36 hpf (n=60). E) The influence of SM on 24 hpf zebrafish heart rate (n=30). *t*-test: *indicates significant difference (p<0.05), **indicates highly significant difference (p<0.01). F) The heart rate of 36 hpf for SM embryos and in normal controls (n=30). *t*-test: *indicates significant difference (p<0.05), **indicates highly significant difference (p<0.01).

3.2SM regulated the vascular development in zebrafish

qPCR and *in situ* hybridization experiments were used to observe endothelial-specific marker flk1 gene expression and distribution in the whole vascular system. The flk1 expression was increased (Figure 3B) and the caudal venous plexus was more completely developed (Figure 3A) in the SM group at 24 hpf. The flk1 expression was significantly higher compared to the control group at 36 hpf (Figure 3C). The caudal venous plexus in SM group were more full and perfect than the control embryos at 36 hpf (Figure 3A). The results showed that the effect of SM on zebrafish vascular development was not obvious at 12 hpf-24 hpf, but it had significant influences at 24 hpf-36 hpf stage.

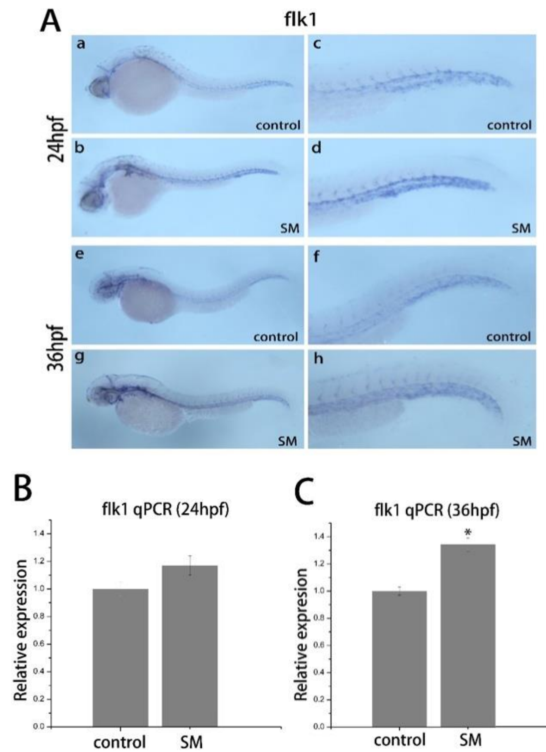

Figure 3. Zebrafish endothelial-specific marker flk1 was increased in vascular development under SM conditions. A) Whole-mount in situ hybridization images of flk1. The right picture shows the tail enlarged. B) qPCR results of zebrafish flk1 relative expression at 24 hpf (no significant difference). C) qPCR results of zebrafish flk1 relative expression at 36 hpf. *t*-test: *indicates significant difference ($p<0.05$), **indicates highly significant difference ($p<0.01$).

3.3 SM had different effects on the arteries and veins

To further confirm the effects of SM on blood vessels, we examined the vein vascular marker flt4 and the arterial vascular marker ephrinB2 expressions. The results showed that flt4 expression was no difference at 24 hpf (Figure 4C) but was significantly low at 36 hpf (Figure 4D) in SM group. The tail vasculature appeared intermittent and the posterior cardinal vein development showed obvious deformity and incomplete in SM group (Figure 4A, B). EphrinB2 expression did not differ between groups at 24 hpf (Figure 5C) but it was significantly increased in the SM group at 36 hpf (Figure 5D). Whole-mount *in situ* hybridization of ephrinB2 showed that the lumen diameter was largest in the SM group (Figure 5A, B), particularly at 36 hpf (Figure 5B). The above results demonstrated that SM inhibits vein vascular development and promote arterial vascular development in zebrafish angiogenesis.

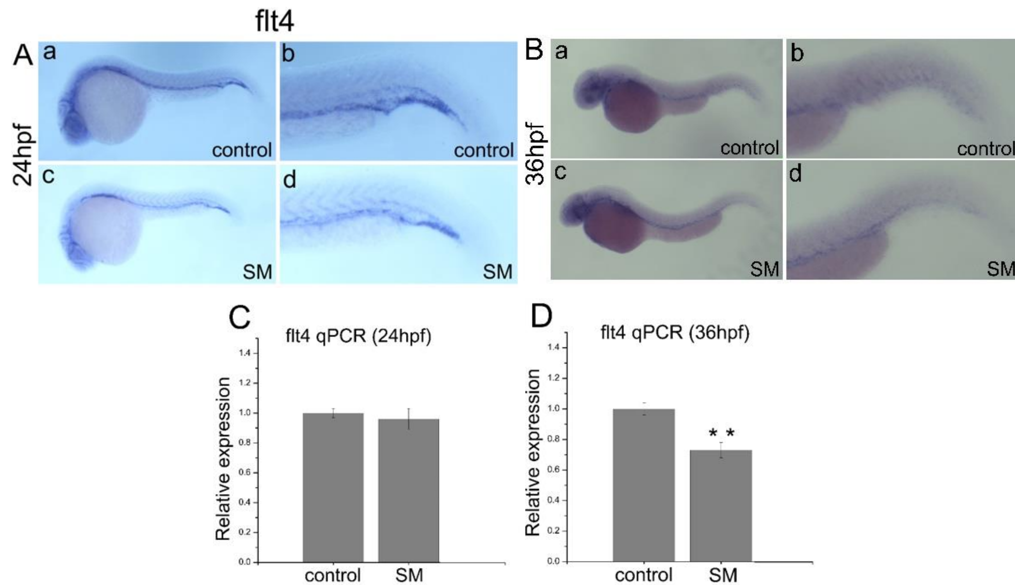

Figure 4. Zebrafish vein vascular marker flt4 was decreased in vascular development under SM conditions. A) Whole-mount *in situ* hybridization images of flt4. The right picture shows the tail enlarged. B) qPCR results of zebrafish flt4 relative expression at 24 hpf (no significant difference). C) qPCR results of zebrafish flt4 relative expression at 36 hpf. *t*-test: *indicates significant difference ($p<0.05$), **indicates highly significant difference ($p<0.01$).

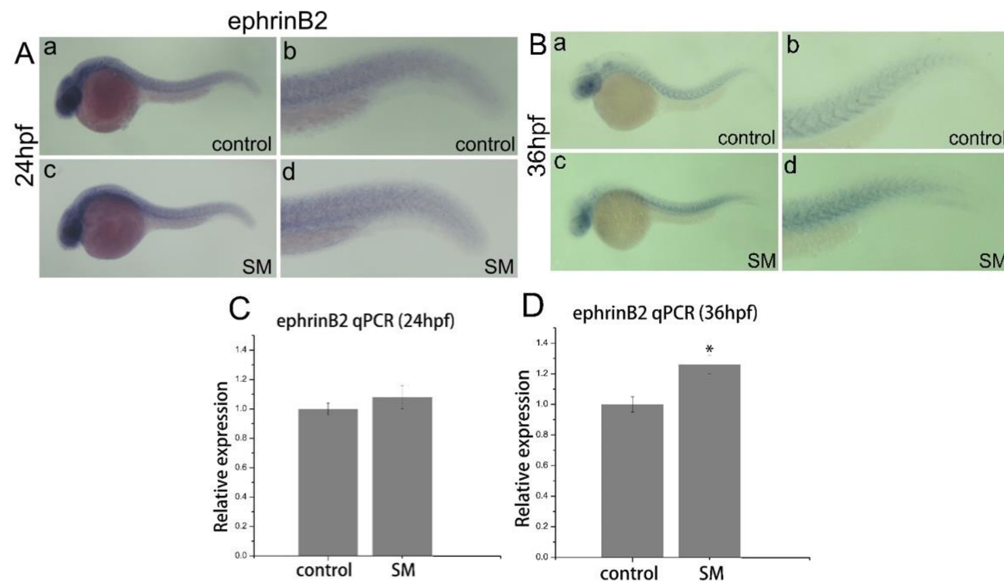

Figure 5. Zebrafish arterial vascular marker ephrinB2 was increased in vascular development under SM conditions. A) Whole-mount *in situ* hybridization images of ephrinB2. The right picture shows the tail enlarged. B) qPCR results of zebrafish

ephrinB2 relative expression at 24 hpf (no significant difference). C) qPCR results of zebrafish ephrinB2 relative expression at 36 hpf. *t*-test: *indicates significant difference ($p<0.05$), **indicates highly significant difference ($p<0.01$).

3.4 Nos2b may be involved in the process of angiogenesis by SM

NO can regulate early cardiovascular development of zebrafish embryos and play an important role in maintaining the vascular system during individual development. In order to explore whether nos2b is involved in microgravity regulated vascular development, we used whole-mount *in situ* hybridization and qPCR methods to examine the changes of expression and distribution of nos2b. The results showed that the expression of nos2b increased significantly in SM group at 24 hpf (Figure 6A, C). All nos2b expression was found in the hypothalamus posterior to the hypothesis and at the base of the primordium of the lower jaw ventral of the anterior hindbrain at 24 hpf (Figure 6A). The nos2b gene expression increased significantly at 36 hpf (Figure 6D). Interesting, nos2b gene expression was no difference in the hypothalamus between two groups (Figure 6B) but significantly increased at the dorsal near the vascular in SM groups at 36 hpf (Figure 6B, arrow). Therefore, before 24 hpf nos2b may not participate in the regulation of vascular development. With the change of nos2b expression location, we can infer thatnos2b is likely to involve in the process of microgravity regulated vascular development.

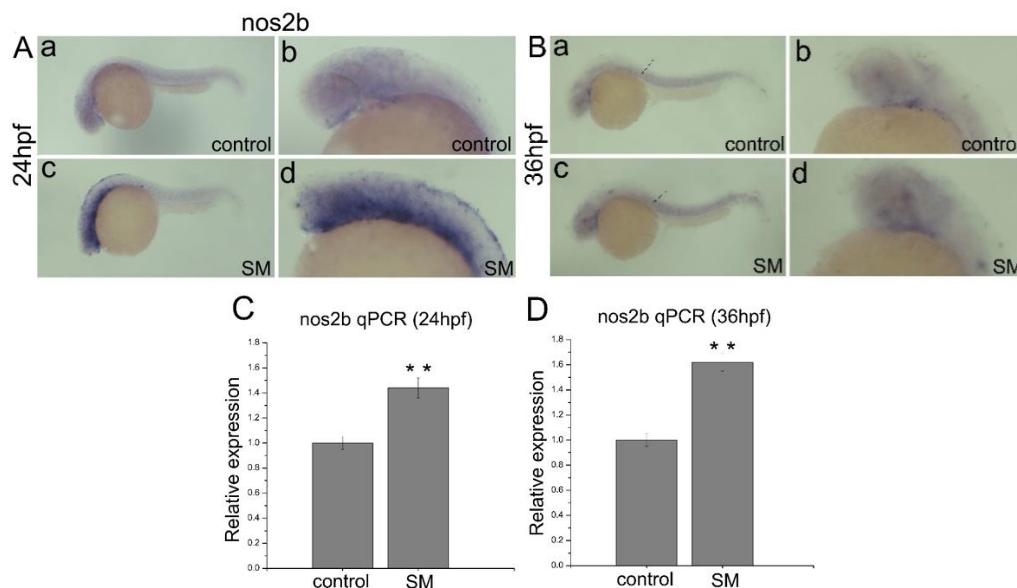

Figure 6. Zebrafish nos2b gene expression was increased significantly under SM conditions, indicating that it may be involved in vascular regulation processes. A) Whole-mount *in situ* hybridization images of nos2b gene. The right picture shows the tail enlarged. The arrows point at the dorsal vessel of zebrafish, the location for nos2b gene expression. (B) qPCR results of nos2b gene relative expression at 24 hpf (no significant difference). C) qPCR results of nos2b gene relative expression at 36 hpf. *t*-test: *indicates significant difference ($p<0.05$), **indicates highly significant difference ($p<0.01$).

4 Discussion

During manned space flights where the human body is exposed to microgravity several cardiovascular dysfunction phenomena have been observed [Antonuttoand Prampero (2003)]. Mesenchymal stem cells (MSCs) flk1 expression was higher at SM conditions [Zhang, Nan, Wang et al. (2013)]. The endothelial differentiation potential of MSCs was improved under SM compared with the normal gravity group. In the present study, we researched the development of the vasculature under SM *in vivo*. The results showed that SM inhibited vein vascular development and promoted arterial vascular angiogenesis in zebrafish. Compared to studies on the vasculature *in vitro*, the vascular development in the present study was closer to the situation of human exposure to microgravity and therefore may provide better support for human research and cardiovascular disease treatment.

In the present study, we found that SM had effect on the angiogenesis but not the process of vasculogenesis. In the vascular development process of zebrafish, the main arterial and venous blood vessels, cardiac primordia and the original embryonic capillary network are formed from 12 hpf to 24 hpf [(Yancopoulos, Davis, Gale et al. (2000)]. Zebrafish heart starts to beat and blood starts circulating in the whole body at 24 hpf. Blood flow plays an important role in vascular development. At the beginning of the establishment the blood circulation, primitive vascular plexus showed a great deal of plasticity during the process of vascular development. Large blood vessels became widen and bold, somesmall blood vessels will disappear directly, while some fragments of the blood vessels began to formed new blood vessels [Buschmann, Pries, Styp-Rekowska et al. (2010), Le, Moyon, Pardanaud et al. (2004)].Different mechanical environment may lead to the development of arteriovenous inconsistent in zebrafish [Stabley, Dominguez, Mora Solis et al. (2012)]. Several studies demonstrated that microgravity affect blood flow caused by the change of the release of blood cells from the bone marrow [Aubert, Beckers and Verheyden (2005), Antonutto and Prampero (2003)]. Blood cells and plasma volume changes could alter blood flow by microgravity, which was possible affect on the vascular development. Therefore, SM may alter the mechanical environment of blood vessels in zebrafish, causing the differences in the tail vascular development in angiogenesis but not the vasculogenesis.

Our *insitu* hybridization results confirmed that SM affected vascular development. The ephrinB2 gene expression was increased by SM and the tail arterial vessel development was more perfect and plumb. On the contrary, the flt4 gene expression was decreased and *in situ* hybridization images indicated that tail venous vessel was obvious deformity and slow development in SM group. In a word, SM had influence on the vascular development *in vivo*, which was different between arterial vessel and venous vessel. In this study, the vascular change became from 24 hpf to 36 hpf, which indicated that SM had a significant effect on angiogenesis, had little effect on vasculogenesis in zebrafish. VEGF-A receptors promoted embryos growth and angiogenesis in zebrafish, which was increased in hypoxia environment [Yang, Xue, Wang et al. (2009)]. Decreased VEGF-A expression induce less ephrinB2 expression and less formation of intersegmental vessels but has little effect on venous development. [Lawson, Vogel and Weinstein (2002)] The vascular development changes were likely linked with the dissolved oxygen in the

container.

SM affected the vascular related gene expression, which suggests that gene regulation may be involved in the development of the vaculature. SM-induced gene changes may be a protection mechanism, research indispensable to disclose the impact of gravity on biological processes and organism in SM. Zebrafish vascular development may be associated with cells gravity sensing factor under SM conditions. Gravity sensing factor nos2b was mainly expressed in the head before 24 hpf, which affect the synthesis of NO in zebrafish. The nos2b gene whole-mount *in situ* hybridization images showed no significant differences in the hypothalamus but increase at the dorsal near the vascular at 36 hpf in SM group. We speculate that nos2b may participate in the angiogenesis process. In future studies, we will try to knockout and inject nos2b mRNA to study whether nos2b take part in vascular development in zebrafish angiogenesis. Vascular smooth muscle cell glycocalyx-hepararan sulfate proteoglycans (HSPG) was altered by gravitational stimulation [Andrew, Giuseppe and Antonio (2012)]. HSPG concentration could significantly impact on the expression of some genes, perhaps affect the nos2b expression in SM. These gravity sensing factor changes may play an important role in the development of blood vessels under SM conditions.

Our research used transgenic zebrafish as an animal model to study the effect of microgravity on zebrafish vascular development. The results showed that SM had an important impact on the angiogenic process. Based on the study of zebrafish vascular response to microgravity, it can provide a theoretical basis for human cardiovascular disease research in weightless conditions of space.

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Conflict of interest: The authors declare that there are no conflicts of interest.

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