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ORIGINAL PAPER

Effect of Short-Term Hypergravity Treatment on Mouse 2-Cell Embryo Development

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Abstract Though there are numerous biological experiments, which have been performed in a space environment, to study the physiological effect of space travel on living organisms, while the potential effect of weightlessness or short-term hypergravity on the reproductive system in most species, particularly in mammalian is still controversial and unclear. In our previous study, we investigated the effect of space microgravity on the development of mouse 4-cell embryos by using Chinese SJ-8. Unexpectedly, we did not get any developed embryo during the space-flight. Considering that the process of space experiment is quite different from most experiments done on earth in several aspects such as, the vibration and short-term hypergravity during the rock launching and landing. Thus we want to know whether the short-term hypergravity produced by the launch process affect the early embryo development in mice, and howthe early embryos respond to the hypergravity. In present study, we are mimicking the short-term hypergravity during launch by using a centrifuge to investigate its influence on the development of early embryo (2-cell) in mice. We also examined the actin filament distribution in 2-cell embryos by immunostaining to test their potential capacity of development under short-term hypergravity exposure. Our results showed that most 2-cell embryos in the hypergravity exposure groups developed into blastocysts with normal morphology after 72 h cultured *in vitro*, and there is no obvious difference in the development rate of blastocyst formation compared to the control. Moreover, there were no statistically significant differences in birth rates after oviduct transfer of 2-cell mouse embryos exposed on short-term hypergravity compared with 1 g condition. In addition, the well-organized actin distribution appeared in 2-cell embryos after exposed on hypergravity and also in the subsequent developmental blastocysts. Taken together, our data shows that short-term exposure in hypergravity conditions does not affect the normal development and actin filament structures of mouse embryos.

Keywords 2-cell embryos · Short-term hypergravity · Development · Cytoskeleton

Introduction

Human long duration space flight on the international space station (ISS), colonization of the Moon, exploration of Mars and other new space frontiers will ultimately depend on ability of plants, animals and humans to function and reproduction in space environment. In 1995 Ijiri et al. placed the *Medaka* fish in space flight, and the fish completed mating, fertilization and hatching, which gave the first evidence of the whole reproduction process in space (Ijiri 1995). In mammals, despite numerous biologic experiments, such as effect of space travel on the physiology have been investigated in a microgravity environment, the potential effect of weightlessness on the reproductive system in mammalian is still limited and controversial. There were no mammalian (or even vertebrate) species that have yet been

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raised to sexual maturity from conception in the absence of gravity (Miquel and Souza 1991; Moody and Golden 2000; Wassersug 2001). So far, the experiments of human or animal reproduction, such as fertilization and early embryo development have not been studied clearly in space environment.

Actually, in 2006 in SJ-8 Satellite of China our research group utilized the mouse 4-cell embryos to study the effect of microgravity on the development of preimplantation embryos. Unfortunately, we did not find any developed embryos during space flight (Ma et al. 2008). However, other studies showed that the mouse early embryos can develop well in simulated microgravity (Kojima et al. 2000). So we cannot confirm that microgravity in space was the critical role in the stop of the embryo development. Maybe there were some other possible reasons to this result. As we know, at the beginning of launch process the satellite will be raised at very high speed, which can produce an excess gravity. The rocket carrying the satellite will generate 80–100 g of acceleration gravity for about 10 minutes in rising stage before to be into the orbit. In 2007 Feritas et al exposed the epiphyseal cartilages of 17-days-old mouse fetuses to centrifugation at 3 g for 16 h mimicking hypergravity environment, and found that the extracellular fibrils, cell shape and cell membranes were disrupted in the centrifuge samples, which indicated that hypergravity disturbs cell-to-matrix interaction in the cartilage model (Freitas et al. 2007). In 2003, Sasagawa et al. examined the embryonic development of the nematode *Caenorhabditis elegans* under hypergravity (200 g), the results showed that the embryos after fertilization developed normally whereas the number of eggs laid from an adult hermaphrodite decreased and their hatching rate was reduced under the hypergravity condition (Sasagawa et al. 2003). In 2000 Lwigale et al. reported that simulated microgravity and hypergravity can attenuate heart tissue development in precardiac explants from chick embryos (Lwigale et al. 2000). Taken together, several studies indicated that altered gravity including hypergravity bring some influence to development of cell or organ level.

In China, SJ-10 recoverable satellite, which will be launched in the end of 2015 or a bit later, provide a mission of space microgravity experiments including both fields of microgravity science and space life science. We will utilize this precious opportunity to investigate the developmental status of mouse early embryos in space. We will culture the 2-cell embryos in specialized instrument for 96 h, a part of the samples will be captured by microscope to obtain the morphologies of various developmental stages (4-cell, 8-cell, early morula, compacted morula, blastocyst and hatched blastocyst) of early embryos in space, and the others will be returned after chemical fixation to study the mechanism of space environment affecting mouse early embryo development. Thus this investigation will be critical in understanding the beginning of mammalian life, as well as the first step in understanding the entire process of reproduction in space (Hu et al. 2014).

Before real space experiment begins, we need to detect whether the embryo samples which will be used in SJ-10 can tolerate the short-term hypergravity produced by the launch process. 2-cell embryo is the most sensitive stage for its easy blockage, which will induce a various adverse impact during the development of preimplantation embryo in mice (Whittingham 1971). Thus, if 2-cell embryos can survive after exposed in hypergravity condition, we could infer that the embryos in other stages can overcome the effect of excess gravity. So far, there are no reports on the effects of short-term hypergravity exposure on early embryonic development in mice.

In this study, we collected 2-cell mouse embryos and used a centrifuge for mimicking the short-term hypergravity produced during launch to investigate effect of increased gravity on early embryo development in mice. In our study, some 2-cell embryos exposed in hypergravity were collected to test their potential for development *in vitro* and *in vivo*, and other embryos were examined for actin filament distribution by immunostaining. Our results showed that embryo development is unaffected by hypergravity conditions, and short-term exposure on hypergravity does not affect actin filament structure.

Materials and Methods

Animals and Hormonal Stimulation

Adult CD-1mice, aged 6–8 weeks and weighing 20–25 g, were purchased from VITAL RIVER Company (Beijing, China). All experiments involving animals were fed laboratory chow and water under a constant photoperiod (12:12-h light–dark cycle) at a monitored ambient temperature of 22 °C. Female mice were treated with 5 IU pregnant mare's serum gonadotrophin (PMSG, Prospec, Israel), followed 48 h later by 5 IU human chorionic gonadotropin (hCG, Prospec, Israel) injection. Females were paired with same stain males overnight. The presence of a copulation plug was considered as an indication that mating had occurred.

Collection of 2-Cell Embryos of Mice and Hypergravity Treatment

Mated mice were sacrificed 20 h after hCG administration and ampullae were immediately transferred into modified Dulbecco's PBS (mDPBS). The 2-cell embryos were flashed out from the oviduct, and then they were pooled and washed for 3 times with mDPBS. Then centrifuge (Beckman 22R, USA) was used to simulate the short-term

hypergravity during launch, the rotation speed is 1000 rpm, 3500 rpm and 10500 rpm corresponding to 100 g, 1000 g and 10 Kg (approximately) respectively. The 2-cell embryos placed in 1.5 ml tube in CZB solution were subjected to centrifuge at the three level of hypergravity as mentioned above for 20 min. The control group's 2-cell embryo samples were also placed in 1.5 ml tube in CZB for 20 min under normal gravity (1 g). After 20 min short-term exposure to 1 g and hypergravity, those embryos in experiment groups and control group were cultured in incubator *in vitro* or transfered into recipient females for development *in vivo*.

2-Cell Embryo Development *in vitro*

For embryo development *in vitro*, the normal gravity or hypergravity treated embryos were placed into 50 μl CZB medium containing 20-22 embryos, then to be cultured under mineral oil in an incubator at 37 °C, 5 % CO_2 and 95 % humidity for 72h (ThermoForma3110, USA) until developing to blastocysts. The proportion of the blastocyst formation was calculated.

Immunofluorescence and Laser Scanning Confocal Microscopy

The 2-cell embryos after short-term exposure to hypergravity and 1 *g* were washed with DPBS containing $CaCl_2$, $MgCl_2$ (1 mM each) and 0.01 % BSA and fixed in 4 % paraformaldehyde at room temperature for 40 min. Embryos were permeabilized with 0.5 % Triton X-100 in DPBS for 15 min, incubated for 1.0 h in DPBS containing 1 % BSA at room temperature, incubated with phalloidine (mouse

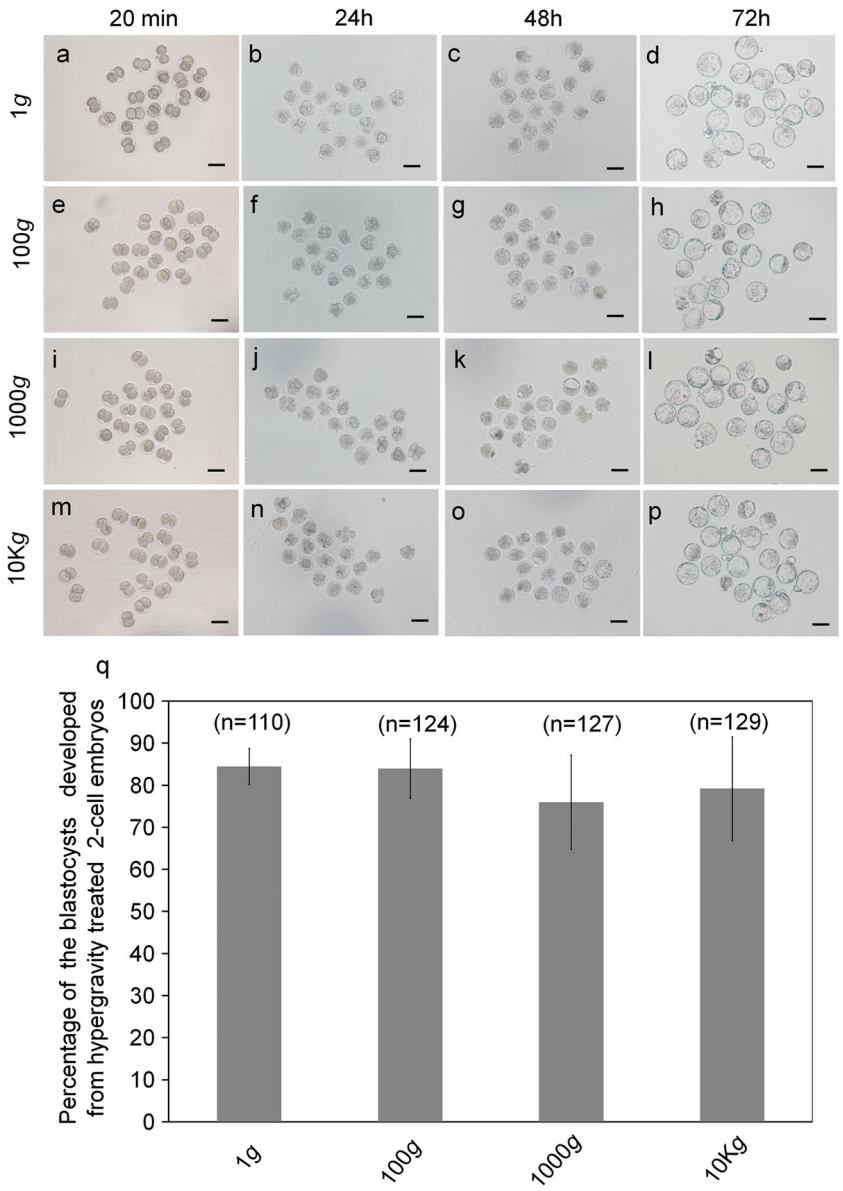

Fig. 1 *In vitro* development of 2-cell embryos after short-term exposed on different gravity conditions (100 g, 1000 g and 10 Kg). a-d Morphology of embryos which were exposed to 1 g for 20 min (**a**) and cultured for 24 h (**b**), 48 h (**c**), 72 h (**d**). e-h represent the morphology of embryos that were exposed to 100 g, i-L for 1000 g centrifuge and m-p for 10 Kg centrifuge. (Bar=100 μm). q Percentage of development rate of hypergravity treated 2-cell embryos to blastocyst after 72 h culture. Note: *P* values for all comparisons were not statistically significant. n = Number of cultured 2-cell embryos

antibody of actin, 1:100) at 4 °C overnight. Nuclei were stained with Hoechst 33342 (1:2000) for 10 min. Finally, the embryos were rinsed in DPBS to remove excess fluorescence reagent, and the images were observed under laser scanning confocal microscope (Zeiss 780, USA). The blastocysts staining which developed from 2-cell embryos after 72 h culture *in vitro* were performed with the same method.

Embryo Transfer

For embryo development *in vivo*, 2-cell embryos after short-term hypergravity and 1 g treatment were incubated in CZB for 2 h, then embryos collected from each group were randomly transferred to the oviduct of surrogate mother. The recipient mice were maintained until term pregnancy and the number of pups sired was documented at term pregnancy.

Statistical Analysis

Experiments were repeated three times under the same conditions. The data were presented as the means ± SD, and differences were evaluated by Student's *t*-test. Values of $P < 0.05$ were accepted as significant.

Result

The Development Potential of 2-Cell Mouse Embryos after Short-Term Exposure to Hypergravity

We previously investigated the development capacity of mouse early embryos in the Chinese SJ-8 Satellite, our results showed that the development of preimplantation embryos stopped during the space flight; however they developed normally in ground experiment (Ma et al. 2008). Thus, we wonder whether increased gravity during launch affects preimplantation embryo development. To address this question, centrifuges were used to obtain the desired hypergravity with a speed range from 100 *g* to 10 K*g*. First, we exposed 2-cell embryos on various hypergravity conditions (1 g, 100 g, 1000 g and 10 Kg) respectively. After 20 min exposure, those embryos were cultured in an incubator at 37 °C with 5 % CO_2 in air. Figure 1 shows the preimplantation development of 2-cell embryos after 20 min exposure under normal gravity (1 *g*) or different hypergravity conditions (100 g, 1000 g and 10 Kg). Most of the embryos in the 1 g group can developed to 4-cell (Fig. 1b), morula (Fig. 1c) and blastocysts (Fig. 1d) with normal morphology after culture of 24 h, 48 h and 72 h. Those hypergravity treated embryos showed similar development capacity to 1 g group, and had comparable morphologies to those in the 1 g group (Fig. 1e-p). The embryos in the hypergravity exposure groups had good development, with most of embryos having developed to blastocyst stage after 72 h cultured (Fig. 1d, h, i, and p). The percentage of blastocysts developed from hypergravity treated 2-cell mouse embryos after 72 h culture as shown in Fig. 1q. The number of embryos reaching the blastocyst stage was not statistically significant after 72 h culture in the normal gravity control group and altered gravity groups. This result indicates that embryo development is unaffected after short-term exposed in hypergravity conditions.

Effect of Short-Term Exposure to Hypergravity on the Actin Cytoskeleton of 2-Cell Embryos and Subsequent Developmental Blastocysts

The actin cytoskeleton is quite sensitive to the change of gravity. To investigate whether short-term hypergravity treatment affects the cytoskeleton distribution, we analyzed the actin cytoskeleton of 2-cell embryos after short-term exposure on various hypergravity. Interestedly, no significant difference of actin filament distribution was observed by comparing the control and various treated groups. Intense actin staining of 2-cell embryos in control group (Fig. 2a)

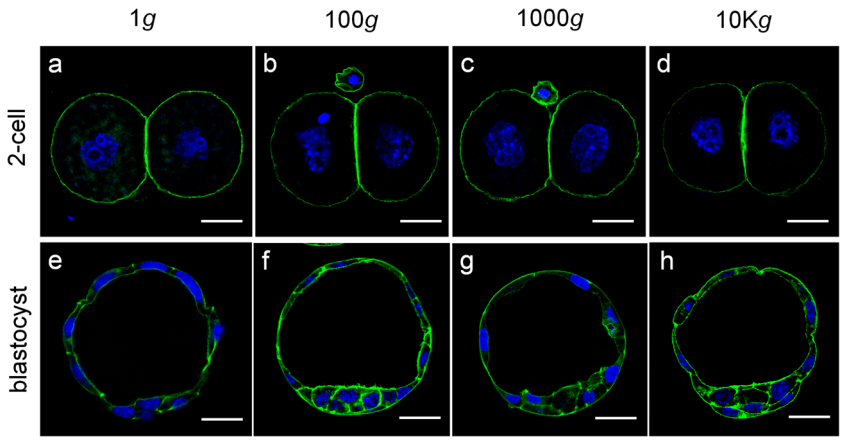

Fig. 2 Photomicrographs to illustrate the actin cytoskeletal distribution of hypergravity treated 2-cells embryos and subsequent developed blastocysts using a confocal microscope. The embryos were stained with Alexa Fluor 488-phalloidin (*green*) to visualize actin filaments and DAPI to stain the cell nuclei (*blue*). Bar= 20μm

Table 1 Result of embryos transferred of 2-cell embryo exposed in short-term hypergravity conditions

Group	No. of transferred embryos (recipients)		No.(%) of newborns from transferred embryos	
1 g	60 (3)	20	9	35 (53)
		20	9	
		20	12	
100 g	60 (3)	20	16	35 (58.3)
		20	7	
		20	12	
1000 g	75 (4)	20	11	42 (56)
		20	9	
		20	12	
		15	10	
10 Kg	55 (3)	20	11	26 (47.2)
		20	9	
		15	6	

Note: There were no statistically significant differences about birth rates in each group for all comparisons (1 g vs 100 g, p =0.765; 1 g vs 1000 g, p =0.742; 1 g vs 10 Kg, p = 0.418).

and different experiment groups (Fig. 2b, c and d) were observed in the cortex, the cell borders and cell-cell contact were clearly staining. This expression pattern was considered as a standard for 'good' actin cytoskeleton morphology (Overstrom et al. 1993). We also investigated the distribution of actin cytoskeleton of blastocytes developed from short-term hypergravity treated 2-cell embryos. As shown in Fig. 2e-f, there is no difference of actin staining in control and experimental group, the cytoskeleton of blastocyst in each group was typified by precise, sharp restriction of actin staining to the cell borders. Collection, this result indicates that short-term exposure to hypergravity does not affect actin filament structures of mouse embryos. The well-organized actin distribution appeared both in 2-cell embryos and subsequent developmental blastocysts.

Development After Oviduct Transfer of 2-Cell Embryos in Short-Term Hypergravity Treatment

We performed additional experiments to investigate whether the 2-cell embryos exposed on a hypergravity condition for short time would implant and develop to term normally in an *in vivo* environment after oviduct transfer. A total of 60 embryos (1 g), 60 embryos (100 g), 75 embryos (1000 g), and 55 embryos (10 Kg) were transferred to 3-4 recipients per conditions (Table 1). The recipients gave birth to a total of 32 pups (1 g), 35 pups (100 g), 42 pups (1000 g) and 26 pups (10 Kg). There were no statistically significant differences in birth rates after oviduct transfer of 2-cell embryos in each group for all comparisons.

The newborns, which were developed from 2-cell embryos in short-term exposure in 1 g condition (Fig. 3a), 100 g condition (Fig. 3b), 1000 g condition (Fig. 3c), and 10 Kg condition (Fig. 3d), with the foster mother, were shown in Fig. 3. There were no statistically significant differences in the average body weight of offsprings among the four conditions (data not shown).

Discussion

In the whole space experiment process, there are several different processes from normal experiment in ground, including vibration on launch of the shuttle, short-term hypergravity on launch and landing, and radiation exposure throughout the flight time line. Although vibration and hypergravity are of duration in the spaceflight protocol, their individual contribution to the flight samples is unknown. In this study, we detected the development of the 2-cell mouse embryos after short-term exposure to 100 g, 1000 g and 10 Kg conditions, respectively. Actually, 100 g for 20 min treatment is used to consist with the hypergravity and duration generated in the real shuttle launch, just a little more severe. Interestingly, our results showed that the 2-cell embryos subjected to this treatment fulfilled the subsequent developmental process normally, thus we infer that the early mammalian embryos can tolerate the hypergravity during launch, and such hypergravity do not produce adverse effect to development of early embryos. However, whether the early embryos bear much heavier acceleration, and what extent acceleration can the embryos tolerate? So we further investigated the early embryo development after exposed in 1000 g and 10 Kg condition for 20 min. Surprisingly, our results indicated that the 2-cell embryos treated in 10 Kg

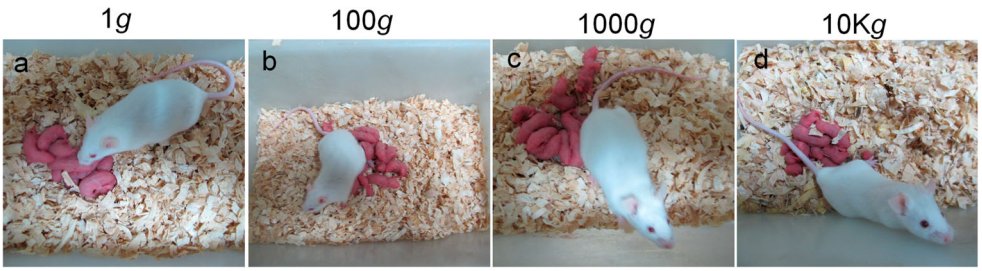

Fig. 3 Offsprings derived from 2-cell embryos in short-term exposure in the different conditions, and with the foster mother. **a**: in 1 g condition; **b**: in 100 g condition; **c**: in 1000 g condition; **d**: in 10 Kg condition

for 20 min, such extreme acceleration, could still accomplished the whole process of preimplantation development from 2-cell to blastocyst. More importantly, the blastocyst formation rate is not significant difference when compared with the 1 g condition. In addition, our result demonstrates that 20 min exposure to 100 g, 1000 g or 10 Kg does not affect actin filament structure.

In 2009, Machado et al. evaluated the effect of the force of centrifugation on sperm quality used in vitro-produced (IVP) bovine embryo. The bovine sperm were yielded in 700 g for 20 min and 5000 g for 5 min, and both treatments did not affect sperm quality, and subsequent embryo development, and sex ratio of in IVP bovine embryo (Machado et al. 2009). Actually we also have examined the effect of 20 min exposure in 3 extent hypergravity (100 g, 1000 g and10 Kg) on the viability of the mouse sperm and mouse embryonic stem cells (ESCs), preliminarily (data not shown). Our data indicated that the mouse sperm were subjected to 10 Kg for 20 min, which due to the abnormal morphology and few sperm could move, while the 100 g and 1000 g treatment had no effect in sperm's viability likely. For mouse ESCs, 100 g, 1000 g and 10 Kg treatment would induce about 0 %, 3 % and 10 % mortality, respectively. Different cell types have different response to the exposure of hypergravity or microgravity. In 2000 Wakayama et al. examined the mouse fertilization and preimplantation embryo development subjected to the microgravity induced by a three-dimensional (3D) clinostat. They found that fertilization can occur normally under simulated microgravity, but preimplantation embryo development was little worse than 1 g control (Wakayama et al. 2009). By the way, three-dimensional (3D) clinostat with large volume of medium could generate shear force, which had negative effect in results they obtained.

Cytoskeleton is the support of cell structure, in case of damage of the cytoskeleton, cells will hard to maintain normal morphology, which lead to subsequent cell death or apoptosis. Cytoskeleton contains three components, microtubules (MT), intermediate filaments (IF) and microfilaments (MF), actin and tubulin are two important proteins for consisting of MT and MF. Also, actin and tubulin have other crucial functions, including the transport of organelles and the process of cell division of cell. As well as action and microtubules are known to be required for correct localization of developmentally important mRNAs and proteins during embryogenesis and oogenesis (Lantz et al. 1999). Some studies showed that microgravity and altered gravity will lead to cytoskeleton changes, and cytoskeleton is quite gravity sensitive (Crawfod-Young 2006). Apoptosis may be affected when cell cytoskeleton is disorganized. Abnormal patterning may either cause premature cell death or the lack of death at inappropriate times (Zahir and Weaver 2004). Searby et al. have detected the influence of increased mechanical loading by hypergravity on microtubule cytoskeleton in primary osteoblasts. They found that a stimulus of 10 g for 3h decreased microtubule network height in immature osteoblasts, but without affecting cell viability, as well as osteoblasts differentiated sensitivity to hypergravity declined (Searby et al. 2005). In present study, we ask whether the cytoskeleton of 2-cell embryos changed after short-term exposed in hypergravity condition, and does it respond to this physical force? So we detected the actin filament expression pattern of the treated 2-cell embryos and their subsequent blastocysts. Our results indicate that there are no differences in the pattern of actin expression in each treated groups.

In generally, our findings indicate that early embryo development is unaffected after short-term exposed on hypergravity conditions, and short-term exposure to hypergravity also does not affect actin filament structures. Subsequently, the development of early mouse embryos in real microgravity in space flight will be further performed, while Chinese SJ-10 satellite will provide us a precious opportunity.

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