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ORIGINAL ARTICLE

Real-Time Micrography of Mouse Preimplantation Embryos in an Orbit Module on SJ-8 Satellite

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Abstract The developmental capacity of mouse embryos in the Chinese SJ-8 Satellite was observed by real time micrography and telecontrol image transmission. Frozen/thawed 4-cell embryos and blastocysts injected with mouse epidemical stem cells were placed in a specially sealed embryonic incubator, and then the incubator was loaded in a space embryonic culture box devised for space-flight. After the satellite launched and arrived at the anticipated orbit, the real time micrography device was opened based on the telecontrol operational technology. Real time micrographs of the mouse embryos were obtained and stored every 3 hours, then the data of images were transmitted at the suitable time. The experiment persisted for 72 hours. The results showed that during space-flight, most mouse embryos cultured in the sealed culture unit kept integrity and natural structure, their location had minor change, but the embryos did not develop. However, the experiment performed on the ground in the same device showed that 4-cell mouse embryos could develop to blastocysts and hatched blastocysts. It may be concluded that the space environment, especially the change of gravity was likely to harm development of the mouse embryo.

Keywords Space-flight · Microgravity · Satellite · Real-time micrography · Image transmission · Embryonic development · Mouse

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Introduction

During space-flight, the functions of many organs or systems of life may undergo changes. Some studies indicated a marked bone loss and disrupted calcium balance of the immune systems of astronauts during space-flight (Rambaut and Johnston 1979, Andrea et al. 1992). Reproduction and development are basic physiological functions of life; such functions can change in the special environment of space. However, knowledge about the influence of the spatial environment on reproduction and development is rather limited, and most of the studies are concentrated on ground-based models to simulate weightlessness. For example, in the tail-suspension model of rats, studies indicated that the weight of testis in male rats decreased significantly, the convoluted tubules atrophied, and the number of spermatocyte decreased markedly as well, after 21 days of tail-suspension (Zheng et al. 2003). Using a horizontal Clinostat device, the effects of microgravity on fertilization and development of preimplantation embryos were studied in vitro. The results suggested that fertilization in vitro was not sensitive to the gravitational vector. However, the possibility still exists that the frequency of early embryonic lethality is increased by microgravity (Kojma et al. 2000). We also studied simulated microgravity on preimplantation embryos' development using a rotating wall vessel bioreactor (RWVB); our study indicat-

ed that embryonic development was significantly retarded in RWVB. At the same time the nitric oxide syntheses activity was significantly elevated compared with those in normal culture, and cell apoptosis was shown in the developmentally retarded embryos in RWVB. So we presume that higher concentration of the nitric oxide in the embryos under microgravity caused cytotoxic consequence (Cao et al. 2007). Due to the rare opportunity of space-flight experiments, there has been only limited data collected correlated with animal reproduction and development. For example, fertilized eggs incubated for 0, 7 and 10 days on earth were subjected to microgravity for 7 days achieved by space-flight. After a 7 day space-flight, only one of the 0-day-old embryos survived, but all ten of the 7-day-old and nine of the 10-day-old embryos survived. This result indicates that microgravity has a lethal effect on early chick embryos, though has little effect on middle and late chick embryos (Kohtaro et al. 1995). There are few research studies about mammal reproduction and development in space-flight. Beginning at the 13th day of pregnancy the rats were under conditions of weightlessness of space-flight for 6 days. After landing, the state of the 18-day-old fetuses' brain development was investigated compared to that of control rats, which were on Earth. Certain deviations in vascularization were observed, the amount of vessels was greater and their volumes were thinner, and such changes were maintained for life (Olenev et al. 1989).

As mankind moves closer to the reality of space habitation and space colonization, the emphasis of space biological research has begun to shift from investigations of acute responses to studies of the chronic effects of altered gravity (Moody and Golden 2000). A more comprehensive understanding of the effects of altered gravity on mammalian reproduction is needed. Based on this purpose, we placed mouse embryos in an orbit module on the Chinese Breeding Satellite, so as to investigate the developmental capacity of mouse embryos in space. Given that the satellite's orbit module would not return to Earth, we used real-time micrography and image transmission utilizing telecontrol operational technology to obtain the images of the mouse preimplantation embryos in space-flight, and do a comparative analysis.

Materials and Methods

Collection and Frozen Preservation of Mouse Four-Cell Stage Embryos and Blastocysts Injected with Mouse Epidermal Stem Cells

Kunming white strain female mice (Experimental Animal Center, the Genetic Institute of Chinese Academy of Sciences) were treated for superovulation with 7.5 IU of pregnant mare serum gonadotrophin (PMSG) and 48 hours later the mice were treated with 7.5 IU of human chorionic gonadotrophin (hCG). Mouse 4-cell stage embryos were flushed from the oviducts with culture medium 31–33 hours after hCG was injected. Mouse 4-cell stage embryos were frozen by programmable freezing equipment (FREEZE CONTROL CL-8000, Cryologic. Pty. Ltd, Australia), then preserved in liquid nitrogen. The embryos were transported to the Satellite Launch Center 10 days before launching.

We used the Collagen IV attached method, described as follows. The epidermis is believed to contain two types of proliferating cells: stem cells and cells with a lower capacity for self-renewal and higher probability of undergoing terminal differentiation (transit amplifying cells). Some reports indicated that keratinocytes with characteristics of stem cells can be isolated from cultured epidermis on the basis of high surface expression of beta 1 integrins and rapid adhesion to extracellular matrix (ECM) proteins. Among keratinocytes there was a log linear relationship between the relative level of beta 1 integrins on the cell surface and proliferative capacity; furthermore, the cells with the highest colony-forming efficiency adhered most rapidly to type IV collagen. Proliferating keratinocytes that adhered more slowly had characteristics of transit amplifying cells. Since stem cells can be isolated to greater than 90% purity on the basis of their adhesive properties, this process was then used to purify epidermal stem cells. We obtained mouse epidermal stem cells by collecting the cell which had adhered on murine Collagen IV for 30 minutes (Jones and Watt 1993). We separated and purified murine epidermal stem cells which were obtained from new-born transgenic mice of green fluorescence protein (GFP). Epidermal stem cells were obtained from one new born (born first day) GFP mouse skin, purified by the Collagen IV attached method as described above. Such epidermal stem cells were then injected into blastocysts. About 10–15 epidermal stem cells from mice were injected into every single blastocyst using micromanipulator (Nikon, Japan). Then, the blastocysts were cultured at 37°C, 5% CO_2 in a humidified incubator (Thermo Forma, Model 371, U.S.A) for 2–4 hours. The injected mouse epidermal stem cells would then chimeric with the inner cell mass of blastocyst (Luchuan and Jackie 2000).

Mouse blastocysts were flushed from the uterus with culture medium 64–66 hours after hCG was injected. About 10–15 epidermal stem cells were injected into every blastocyst using a micromanipulator (Nikon, Japan). Then, the blastocysts were cultured at 37°C, 5% CO_2 in a humidified incubator (Thermo Forma, Model 371, U.S.A) for 2–4 hours. Injected mouse epidermal stem cells would then conglomerate with the inner cell mass of the blastocysts. Injected blastocysts were frozen and preserved in liquid nitrogen as for those of 4-cell mouse embryos above.

The Briefness Description of the Space Embryonic Culture Box for Space-Flight

We designed a special space embryonic culture box which is suitable for placing on a satellite during launch and space-flight (Fig. 1 is the sketch map of the space embryonic culture box). The device consists of a sealed embryonic incubator, the telecontrol or programmable control (which dictates the receiving and transmitting system), the real time digital micrography and image data transmitting system, the temperature control and temperature parameter transmitting system.

The sealed embryonic incubator includes four independent culture units. Each culture unit has a porous, minisize embryonic culture tube that can exchange culture medium with the culture unit and embryos can develop under sealed culture conditions. The telecontrol or programmable control can receive the telecontrol operate dictates from the ground via satellite. It can also send telecontrol operate dictates and programmable control dictates to the real time digital micrography and image data transmitting system, and the temperature control and temperature parameter transmitting system. The real time digital micrography and image data transmitting system can receive the dictates above, and can modulate focus and micrography automatically for obtaining clear images of experimental samples, then transmit the image data to the data record system of the satellite. The temperature control and temperature parameter transmitting system can receive the programmable temperature control dictate, and according to the dictate to modulate temperature automatically and real time record the temperature parameters, then transmit the parameters to the data recording system of the satellite. The image data and the temperature parameters are teletransmitted to the receiving station on the ground when the satellite is circulating through Chinese airspace.

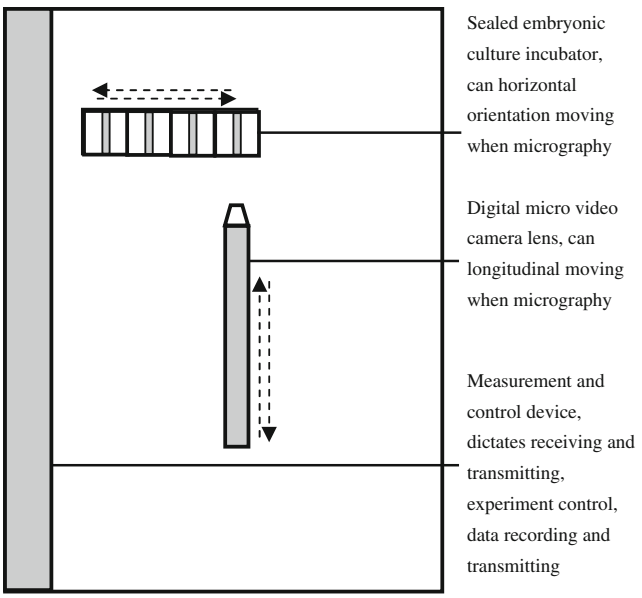

Fig. 1 The sketch map of the space embryonic culture box for space-fight

Experiment Done on the Ground

At the life science laboratory of the Chinese Jiuquan Satellite Launch Center, we have carried out a ground experiment on sealed embryo culture in the space embryonic culture box before satellite launching. The 4-cell stage mouse embryos that were preserved in liquid nitrogen were thawed (Leibo 1977), normal morphological embryos were chosen and every 90 embryos, together with culture medium, were placed into the minisize culture tube as No. 1, No. 2, No. 3 and No. 4 culture units of the embryonic incubator, respectively. The embryonic incubator was sealed and checked-up, confirming that there were no bubbles in every minisize culture tube, and then the embryonic incubator was placed in the space embryonic culture box and sealed. The temperature of the embryonic incubator was kept at 37±0.5°C by the temperature control system. After 2 hours of sealed culture (7 hours of thawing) the first real time embryonic micrography was done under control by the ground controller of the space embryonic culture box, the image data were recorded by computer. Then the real time embryonic micrography was done after 32, 56 and 80 hours of sealed culture. The sealed culture experiment on mouse preimplantation embryos had persisted for 80 hours on the ground.

Sample Loaded, Checked-Up and Located on the Satellite

Thirty-eight hours before the satellite was launched, 4-cell stage mouse embryos and blastocysts which were injected with GFP epidermal stem cells that were preserved in liquid nitrogen were thawed. Normal morphological embryos were chosen for experimentation. Ninety 4-cell mouse embryos, together with culture medium, were placed into minisize culture tubes as No. 2, No. 3 and No. 4 culture units of the embryonic incubator, respectively. Similarly, the blastocysts, which were injected with mouse epidermal stem cells marked by GFP, were placed into a minisize culture tube as No. 1 culture unit. The embryonic incubator was sealed and checked-up, confirming that there were no bubbles in any of the microsize culture tubes, then the embryonic incubator was placed in the space embryonic culture box. Embryo sample images were obtained using the real time micrography system, just as for the ground experiment. The space embryonic culture box was sealed and batteries were used to maintain the temperature. It was then sent to the inspection center for an electrical associated examination. Embryo sample images were obtained by real time micrography system again after the electrical associ-

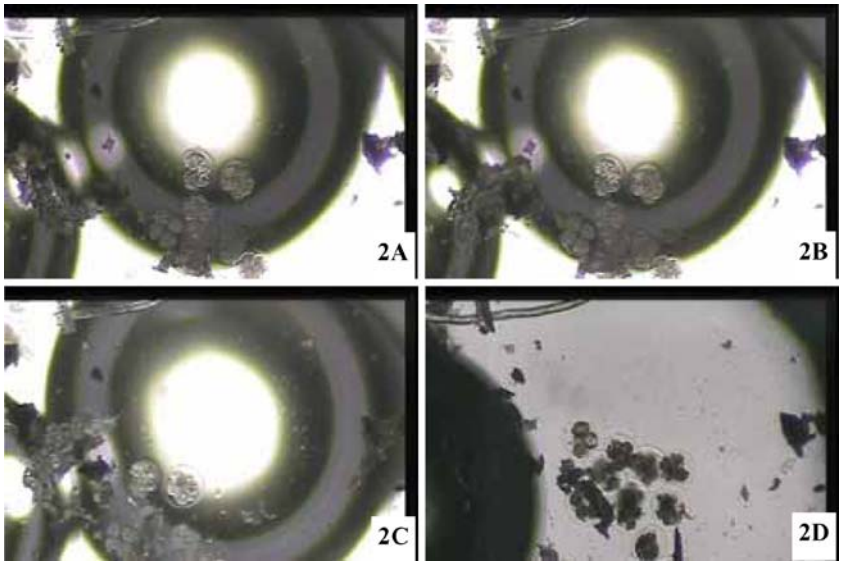

Fig. 2 The embryonic sample images of the No. 1 culture unit in ground Experiment. **A**: culture for 2 h (2 h, 120 min, 7200 s); **B**: culture for 32 h (32 h, 1920 min, 115,200 s); **C**: culture for 56 h (56 h, 3,360 min, 201,600 s); 2**D**: culture for 80 h (80 h, 4,800 min, 288,000 s)

ated examination. The space embryonic culture box was sent to the satellitic launch tower and located in the satellite's orbit module at 30 hours before launching. After the embryonic culture box was placed on the satellite, temperature was maintained by power from the satellite.

Satellite Launched, Real Time Micrography Image Transmitted in Space-Flight

At 3:00 P.M., 9 September 2006, Chinese SJ-8 Satellite (The 8th Practical Satellite) was launched from the Jiuquan Satellite Launch Center of China. We used telecontrol operational technology to open the real time micrography device every 3 hours, and recorded the images of mouse preimplantation embryo samples after the satellite arrived at the anticipated orbit. The images of the samples and the temperature data were transmitted back to the ground when the satellite was circulating through Chinese airspace. The experiment lasted for 72 hours after the satellite was launched.

Results

Success Culturing the Mouse Preimplantation Embryos on the Ground under the Same Condition as in Space-Flight

Initially, we performed the '4-cell mouse embryos in sealed culture' experiment on the ground at the Satellite Launch Center. The process of the experiment has been described above. The space embryonic culture box and the embryonic

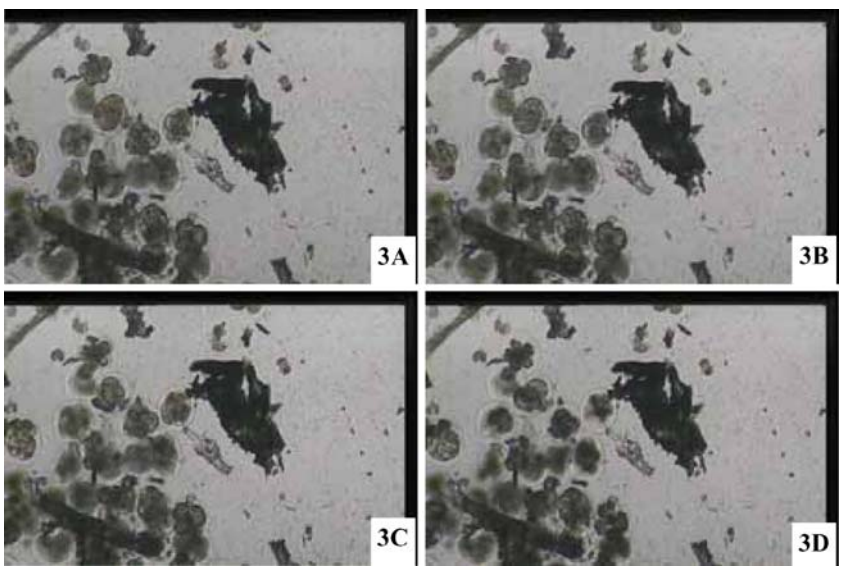

Fig. 3 The embryonic sample images of the No. 2 culture unit in ground Experiment **A**: culture for 2 h (2 h, 120 min, 7200 s); **B**: culture for 32 h (32 h, 1920 min, 115,200 s); **C**: culture for 56 h (56 h, 3360 min, 201,600 s); **D**: culture for 80 h (80 h, 4,800 min, 288,000 s)

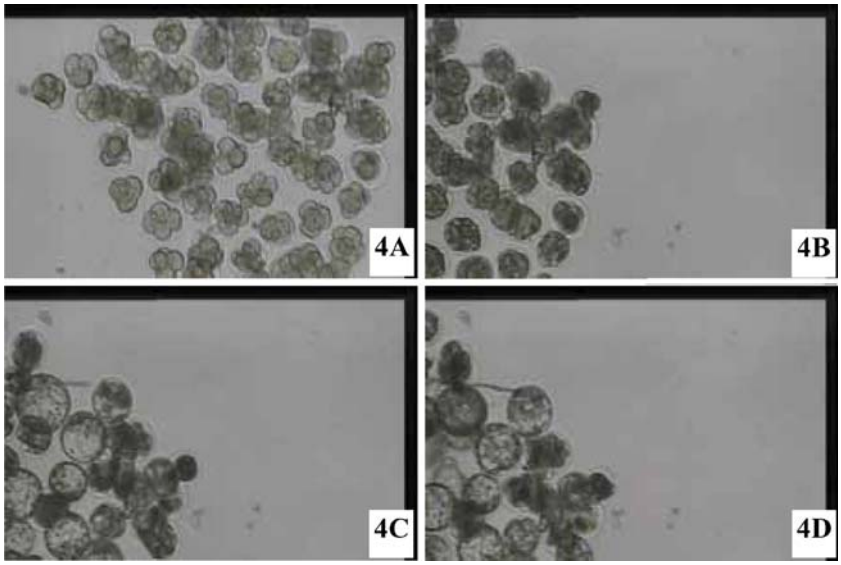

Fig. 4 The embryonic sample images of the No. 3 culture unit in ground Experiment **A**: culture for 2 h (2 h, 120 min, 7200 s); **B**: culture for 32 h (32 h, 1920 min, 115,200 s); **C**: culture for 56 h (56 h, 3360 min, 201,600 s); **D**: culture for 80 h (80 h, 4,800 min, 288,000 s)

incubator were the same as those located on the satellite in the following space-flight. The real time micrography results from different mouse developmental stages during the experiment were shown as greyscale pictures. The embryo sample of No. 1 culture unit is indicated in pictures 2A, 2B, 2C and 2D (Fig. 2); The sample of No. 2 culture unit is exhibited in pictures 3A, 3B, 3C and 3D (Fig. 3); The sample of No. 3 culture unit is exhibited in pictures 4A, 4B, 4C and 4D (Fig. 4); The sample of No. 4 culture unit is exhibited in pictures 5A, 5B, 5C and 5D (Fig. 5). The samples in No. 1 and No. 2 culture units did not fully develop, as many embryos stopped in 4-cell stage, some embryos developed to 8-cell and morula, but most embryos had degenerated when cultured 80 hours (Figs. 2 and 3). The samples in No. 3 and No. 4 culture units had good development, with many embryos having developed to 8-cell, morula, blastocyst and hatched blastocyst stage during culturing (Figs. 4 and 5).

Mouse preimplantation embryos cultured in the space environment have the special requirement that the embryos can develop under sealed culture conditions without gas exchange. We examined the current medium for mouse preimplantation embryo culture, and made an improvement to a component of it; successfully satisfying the requirements of nutrition and gas in sealed culture condition. The experiment on the ground indicated that some bubbles appeared in No. 1 culture unit; mouse 4-cell embryos which appeared in the visual field did not develop; Mouse 4-cell embryos in No. 2, No. 3 and No. 4 culture units had a persistent development that accompanied with the time of culture, a part of mouse 4-cell embryos had been developing to the blastocyst and hatched blastocyst stage.

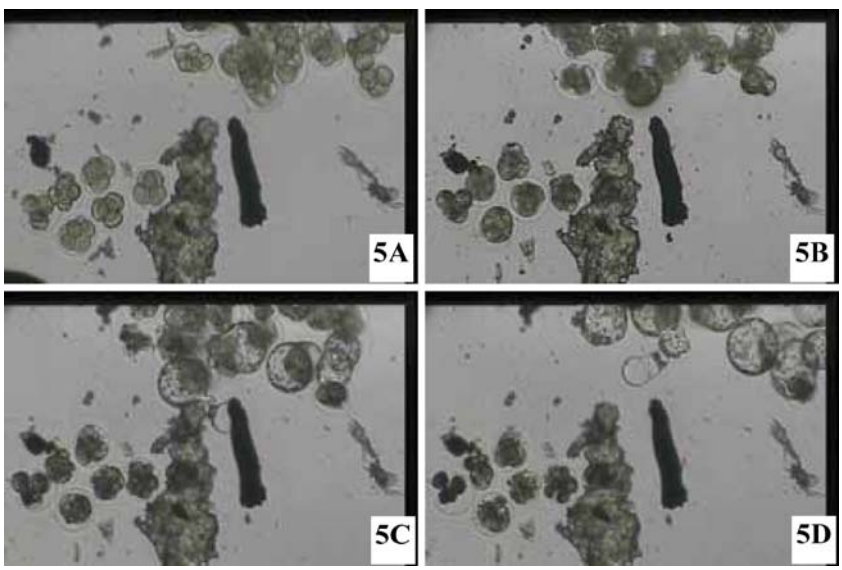

Fig. 5 The embryonic sample images of the No. 4 culture unit in ground Experiment **A**: culture for 2 h (2 h, 120 min, 7200 s); **B**: culture for 32 h (32 h, 1920 min, 115,200 s); **C**: culture for 56 h (56 h, 3360 min, 201,600 s); **D**: culture for 80 h (80 h, 4,800 min, 288,000 s)

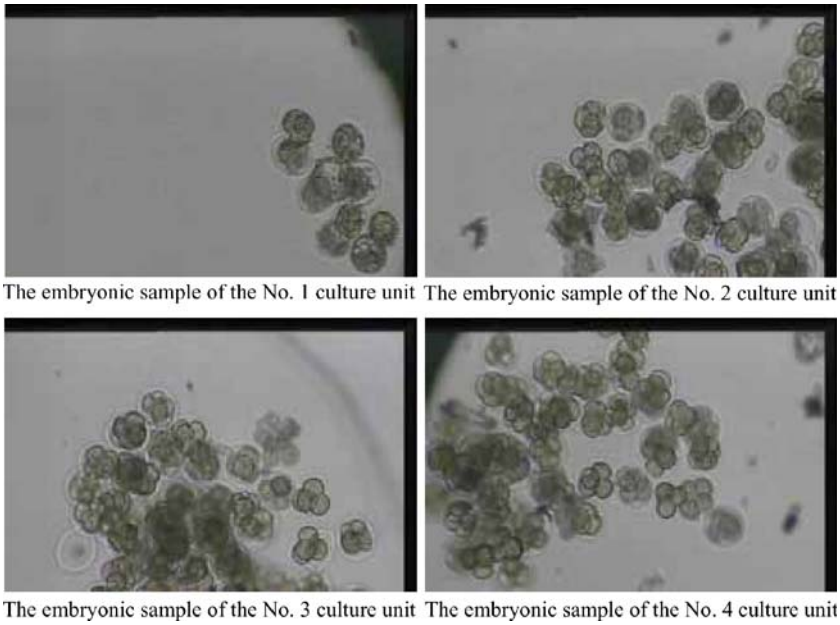

Fig. 6 The embryonic sample images before the space embryonic culture box was located on the satellite

The above results show that we have successfully cultured mouse preimplantation embryos under the sealed culture conditions required by space-flight. Not all embryos in every culture unit were recorded by the real time micrography system, because the visual field of the system could not cover the entire minisize culture tube in which the embryos were placed. This disadvantage impeded analysis of the results of the experiment on count lever.

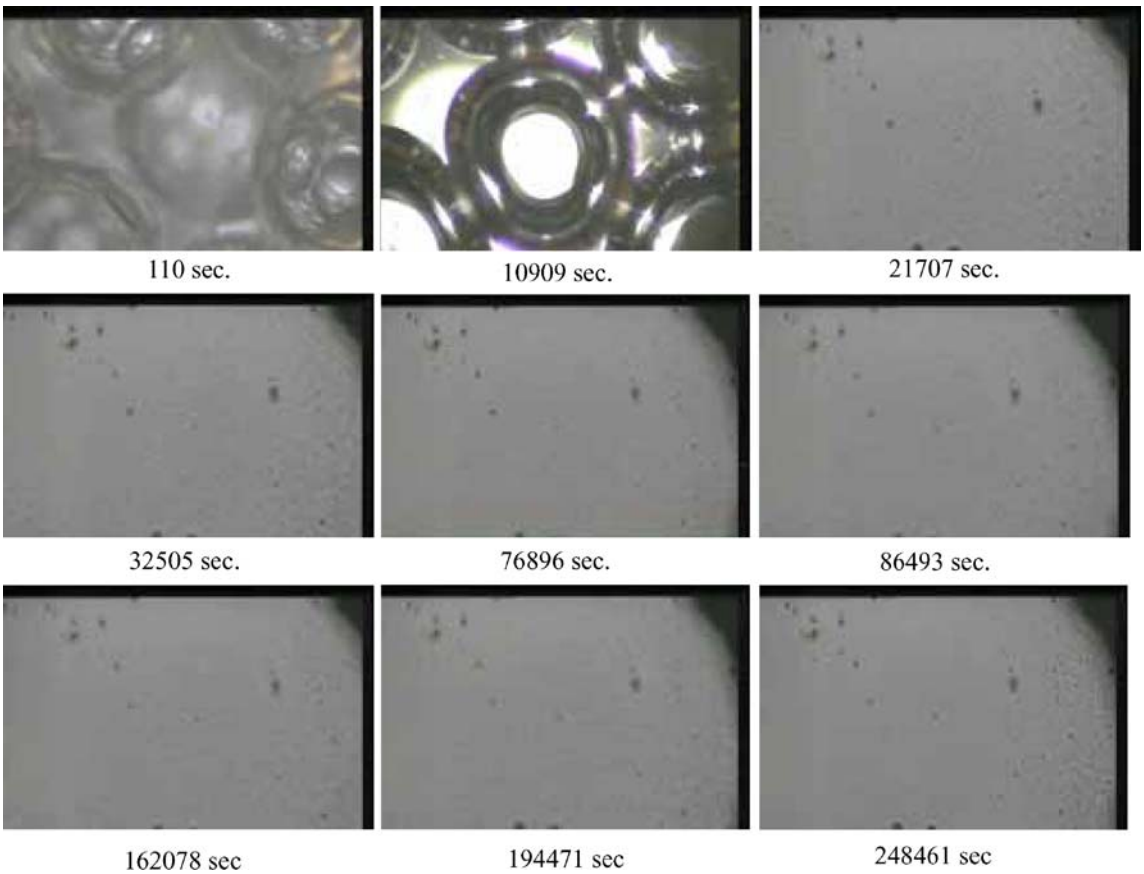

Fig. 7 The sample images of the No. 1 culture unit during the satellite circulating in orbit. The time at the bottom of each picture is the representation of continuous working time during the data recording in the satellite

Success in Obtaining Real Time Micrography Images of the Mouse Preimplantation Embryos in Space-Flight

At 3:00 P.M., 9 September 2006, Chinese SJ-8 (The 8th Practical Satellite) was launched from the Jiuquan Satellite Launch Center of China. The real time micrography device, which was loaded in the space embryonic culture box, was opened after the satellite arrived at the anticipated orbit, and we made a real time micrography operation on each of the four culture units every 3 hours for the following 72 hours, recording images and the temperature of the embryonic samples. The data was teletransmitted to the receiving station on Earth when the satellite was circulating through Chinese airspace. Real time micrography images of the embryonic samples in the space embryonic culture box before placement in the satellite are shown in Fig. 6. Embryonic examples from No. 1 culture unit are shown in Fig. 7; embryonic examples from No. 2 culture unit are shown in Fig. 8; embryonic examples from No. 3 culture unit are shown in Fig. 9; embryonic examples in No. 4 culture unit are shown in Fig. 10.

During the 72 hour space-flight of the satellite, we successfully obtained a series of real time micrography images of mouse preimplantation embryos for the first time in the world. The images showed that there were no images of embryos collected in No. 1 culture unit, a few bubbles were observed in visual field at the beginning of the space-flight, but the bubbles vanished as the space-flight prolonged; images of embryos were successfully collected in No. 2 and No. 3 culture units, distribution of the embryos were congregative, only a limited change in the distribution of embryos had been observed during the space-flight, most of the embryos maintained integrity and natural conformation. In contrast, the ground experiment, focused on different layers of embryos. Utilizing real time micrography we got more than one clear focus of each embryo sample. A varying number of bubbles of different sizes appeared in No. 4 culture unit during most of the space-flight, the number and the shape of bubbles were continuously changing. A few of the embryos were observed accompanying bubbles. No development of mouse embryos in No. 2, No. 3 and No. 4 culture units was observed during the 72 hours of space-flight. We could not analyze the change in embryo distribution and bubble appearance because images could not be obtained between the time that samples were placed in the satellite to the satellite arriving orbit.

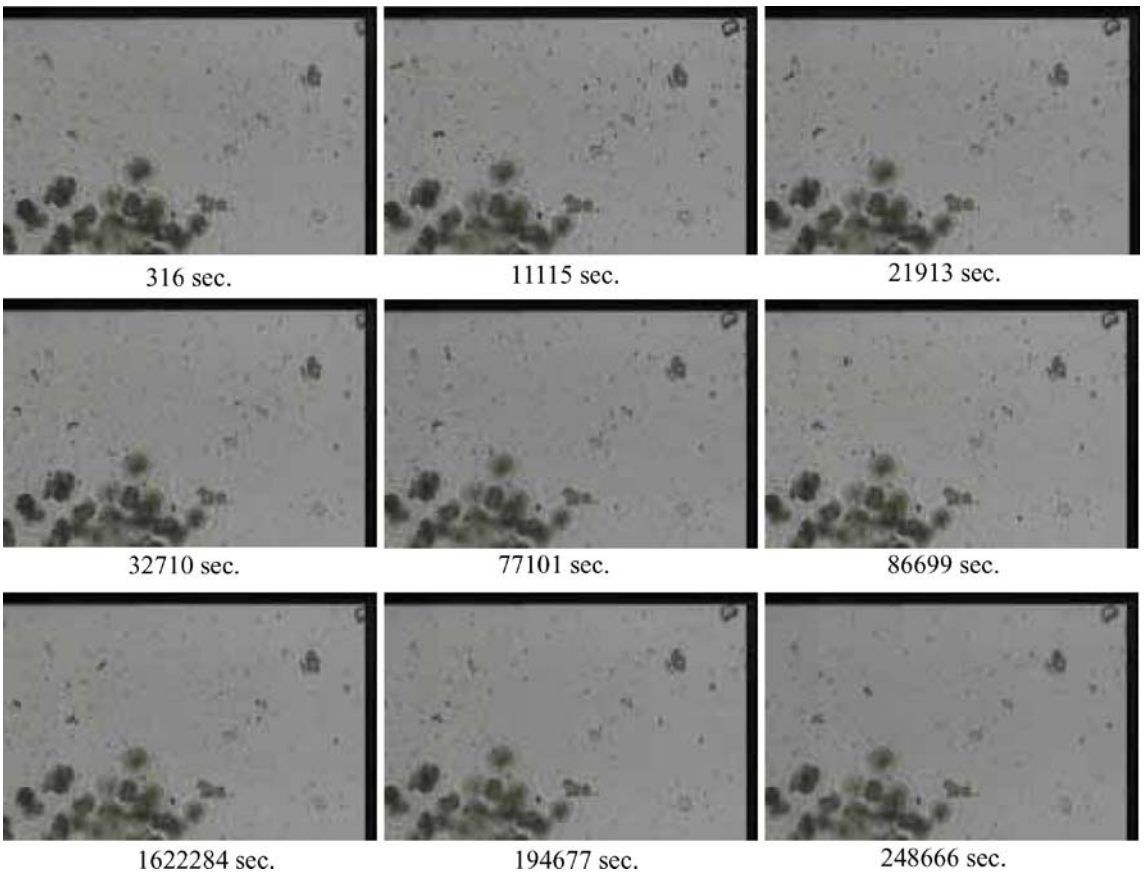

Fig. 8 The sample images of the No. 2 culture unit during the satellite circulating on the orbit. The time underside each picture is the representation of continuous working time during the data recording in satellite

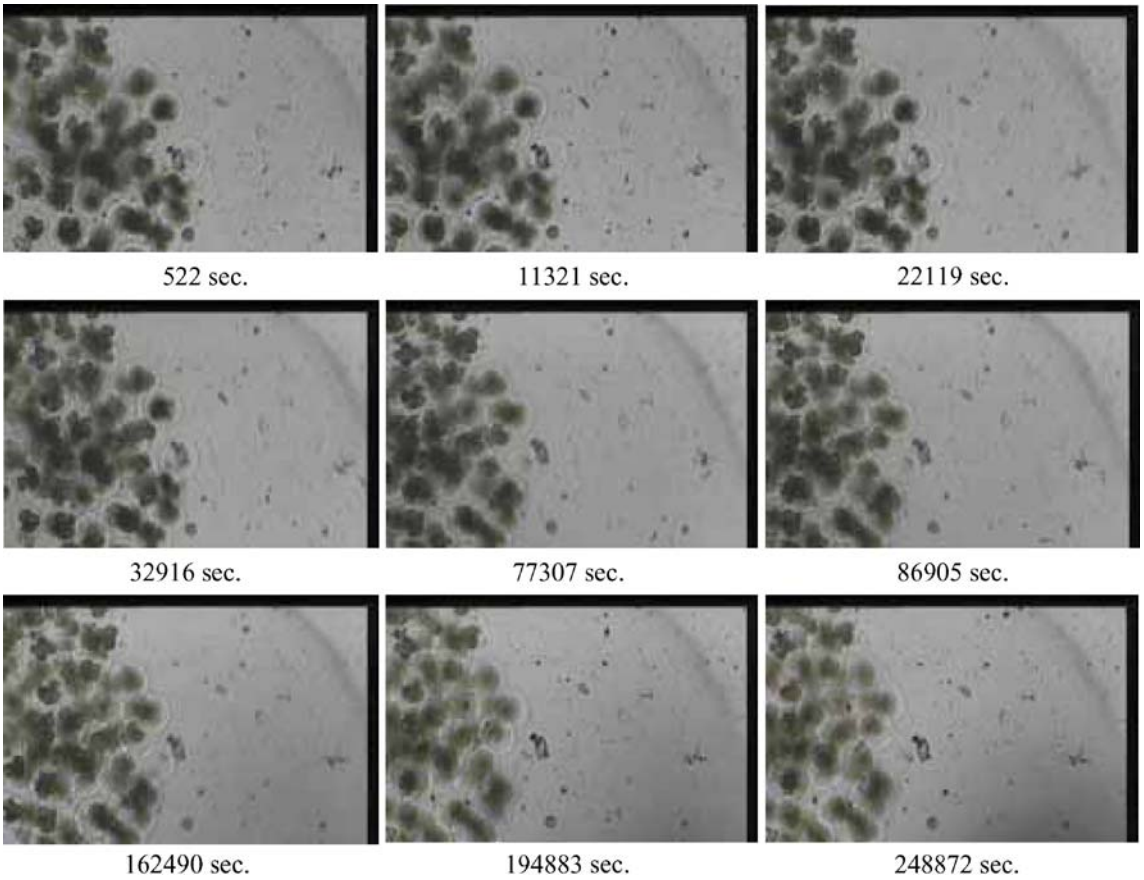

Fig. 9 The sample images of the No. 3 culture unit during the satellite circulating on the orbit. The time underside each picture is the representation of continuous working time during the data recording system in satellite

Discussion

Is gravity required for normal embryonic development? Many researchers used the clawed toad Xenopus laevis to study the role of gravity on the early development of animals. In 1994 Souza tested whether gravity is required for normal amphibian development. Xenopus laevis females were induced to ovulate aboard the orbiting Space Shuttle. Eggs were fertilized in vitro, and although early embryonic stages showed some abnormalities, the embryos were able to regulate and produce nearly normal larvae. These results demonstrate that a vertebrate can ovulate in the virtual absence of gravity and that the eggs can develop to a free-living stage. Their study showed that gravity is not necessary for early amphibian embryogenesis (Souza et al. 1995).

There is little study of the development of mammal embryos in vitro during space-flight. Dr. Jerusalem studied the early development of mice embryos in microgravity in 1996 (http://www.iami.org.il/projectsEng.htm), the experiment was done on space shuttle Columbia (STS-80) -IAMI, 2-cell and 8-cell mice embryos continued to grow in culture media throughout the space-flight. After Columbia landed, the experimental embryos were compared with similar ones grown at the same time on the Earth. They found embryos that were exposed to microgravity stopped growing and did not reach the stage at which they would have implanted in the uterus in normal development. In contrast, the Earth-based control embryos had grown normally. However, this result has not been published.

On account of the fact that satellite's orbit module did not come back to the Earth, we could not obtain the samples when the experiment was ended, except the series of real time micrography images. The development of mouse preimplantation embryos had stopped in the space-flight experiment, but they had developed normally in the ground experiment. This result is similar to the experimental results on Columbia.

The stop in development of the embryos during the space-flight experiment was perhaps due to several reasons. The satellite was not launched until the embryo samples had been put into the sealed embryonic incubator for 34 hours. It was too long for the development of embryos on the ground, but not in space-flight; Classical studies showed that mouse embryo develop to 4-cell by ~56 hr

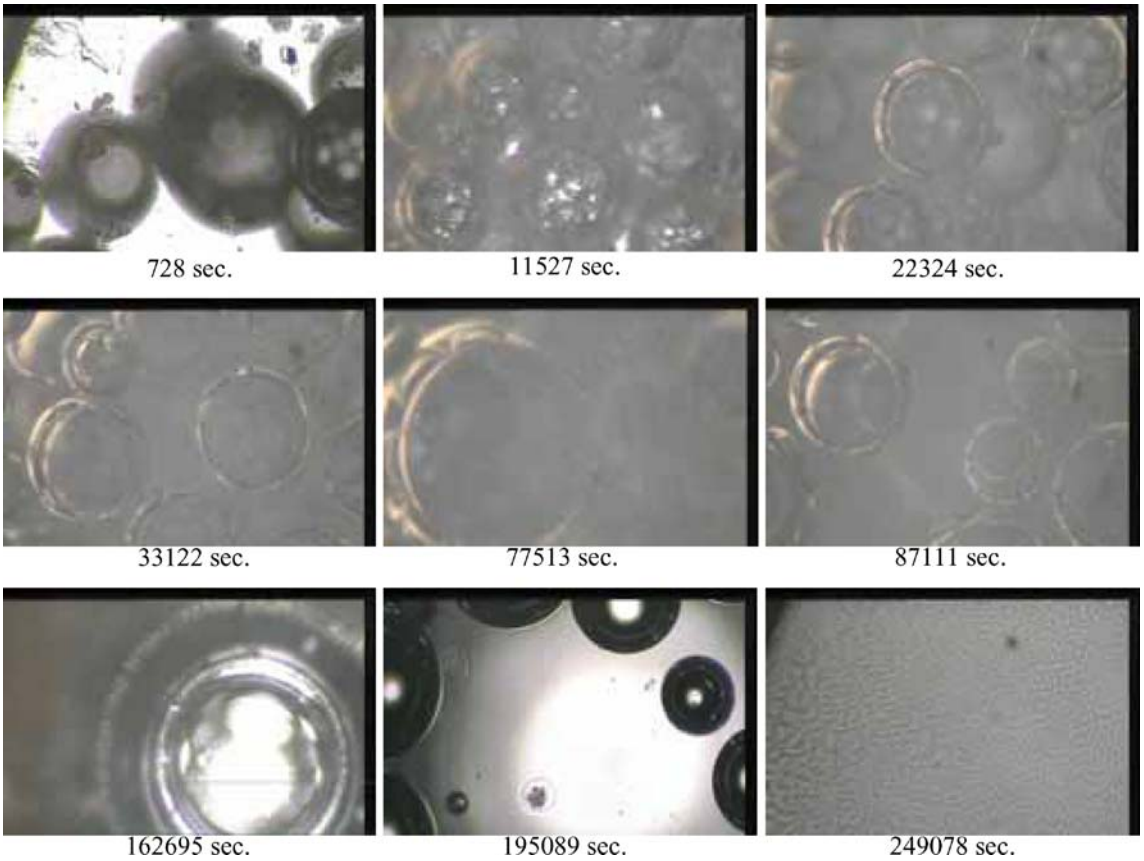

Fig. 10 The sample images of the No. 4 culture unit during the satellite circu1lating on the orbit. The time underside each picture is representation of the continuous working time during the data recording system in satellite

post-hCG, then develop from 4-cell to hatched blastocyst during ~72 hr. This process included 5–8 cell stage, morula, blastocyst and hatched blastocyst (Andras et al. 2003). On the other hand, because the visual field of the real time micrography system could not cover the entire minisize culture tube in which the embryos were placed, the space embryo culture box was upside down many times in order to ensure that as many embryos as possible were captured in the visual field. Electrical associated examination on the ground was required before the space embryo culture box was placed on the satellite. Both the inversion of the space embryo culture box and the electrical associated examination on the ground required that the power be turned off to the space embryo culture box. These operations could have led to a rise and fall in the temperature of the cultural environment and, produced harmful affects on the development of the embryos. Many researches were concerned with the affects of temperature on mammal embryos. When eight-cell embryos at day 5 after insemination were exposed to heat shock of 41°C for 6 hours, the result was decreased development to the blastocyst stage and the number of cells per embryo (Paula-Lopes et al. 2003). There are not any studies about the effects on the development of mouse embryos caused by the temperature of the cultural environment rising and falling, so we can only do some hypothesis on it. Relative studies will be done on the ground soon. Some research studies have investigated the effects of increased gravity and simulated microgravity on the development of animals. For example, one studied how increased gravity affects amphibian development. Uncleaved Xenopus laevis eggs were raised at 2 g and 5 g, while controls were raised in normal gravity. These findings suggested that high gravity suppressed certain gene functions and induced abnormal apoptosis in the brain and eyes, resulting in developmental retardation and various morphological abnormalities (Kawakami et al. 2006). Another study on sea urchin embryos indicated that spicule formation of sea urchin embryos was inhibited to 68% in an experiment under simulated microgravity as compared with the 1g control. The author thought that the microgravity had some negative effects on skeletogenesis, and this was the possible inhibitor mechanism on spicule formation (Izumi-Kurotani et al. 2006). Increased gravity produced during rocket launch, abruptly changed from increased gravity to weightlessness when the satellite arrived at the anticipated orbit, and the environment of space was

perhaps harmful to the development of mouse preimplantation embryos. What is the true reason will need more research in the future.

Conclusion

From this experiment we may draw some conclusions as follows: (1) The real time micrography images of mouse preimplantation embryos are obtained successfully utilizing telecontrol operation technology and teletransmittal technology for the first time in the world, this enables people to observe mouse embryos in space-flight and has a diameter of about 60–80 microns directly on the ground. It indicates the potentiality for the improvement of the device for space biological research not only for the reliability but also for the precision. (2) Mouse preimplantation embryos can develop normally in a sealed embryonic incubator devised for space-flight by ourselves and has provided a good base for researching reproduction and development in space-flight in the future. (3) The development of mouse preimplantation embryos stopped during the space-flight experiment, but they had developed normally during the ground experiment, more research will be done deeply to find the true reason for this result.

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