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Simulated Microgravity Influences Bovine Oocyte *in vitro* Fertilization and Preimplantation Embryo Development

[1]Song-yi Jung, [1]Susan D. Bowers and [1,2]Scott T. Willarda
[1]Department of Animal and Dairy Science,
[2]Department of Biochemistry and Molecular Biology, Mississippi State University,
P.O. Box 9650, Mississippi State, MS 39762, USA

Abstract: The aim of this study was to investigate, whether *in vitro* fertilization and preimplantation embryos exposed to a simulated microgravity environment *in vitro* would improve, or be deleterious to their fertilization and embryonic development. A Rotating Cell Culture System™ (RCCS) bioreactor with a High Aspect Ratio Vessel (HARV) was used to simulate a microgravity environment. *In vitro* Fertilization (IVF) and Culture (IVC) were conducted in standard microdrop culture method conditions (Control) and simulated microgravity conditions; HARV rotated at 34 rpm (high speed) and at 3.7 rpm (Low speed) on a horizontal axis. Embryonic development rates were determined during IVF (experiment 1), during IVC at presumptive zygote stage (experiment 2) and IVC at 2-8 cell stages of embryo development (experiment 3). For IVF studies (experiment 1), 77.3% of bovine oocytes were fertilized in the Control group; however, bovine oocytes and sperm fertilization did not occur in high and low speed groups. Moreover, none of the presumptive zygotes (experiment 2) and 2-8 cell stage embryos (experiment 3) cultured in high and low speed groups were able to develop to the further stages. These results indicate that simulated microgravity environments have a negative impact on bovine *In vitro* fertilization and preimplantation embryo development.

Key words: Bovine embryo, *in vitro* fertilization, *in vitro* culture, simulated microgravity

INTRODUCTION

For several decades, successful space missions have led to growing interests in human space exploration (White and Averner, 2001) and an increasing number of studies have investigated the possibilities of normal animal fertilization and embryo development under microgravity conditions. A variety of animal models have been used, such as medaka fish (Ijiri, 1998), zebra fish (Gillette-Ferguson *et al*., 2003; Shimada and Moorman, 2006), frog (Souza *et al*., 1995), salamander (Gualandris-Parisot *et al*., 2002), mouse (Kojima *et al*., 2000; Schenker and Forkheim, 1998) and rat (Serova and Denisova, 1982) and studies have been conducted during space flights and in simulated microgravity conditions on the Earth. All of the fertilization and embryo development studies in fish and amphibians showed a similar pattern, that their fertilization and development could occur in space and in simulated microgravity conditions even though there were some degrees of cellular and molecular alterations.

In mammals, only mouse and rat studies have been conducted under microgravity conditions and results have not been consistent. Kojima *et al*. (2000) reported that simulated microgravity did not affect mouse oocytes and sperm fertilization *in vitro*. However, they also found that preimplantation embryo development in simulated microgravity conditions was significantly decreased compared to a static culture; although a small portion of 2-cell stage embryos were able to develop to the blastocyst cell stage. In another study, 2-cell mouse embryos were cultured *in vitro* during a flight of the space shuttle Columbia (STS-80). In this experiment, none of the embryos developed to further stages (Schenker and Forkheim, 1998). Moreover in an *in vivo* study, rats were mated in a Cosmos 1129 biosatellite experiment and although 2 out of 5 females experienced fertilization and early embryo development, the females failed to keep the pregnancies (Serova and Denisova, 1982). These researchers speculated that due to the maternal stresses caused by the space flight, the fertilization and embryonic development might be compromised. In their study, one

Corresponding Author: Scott T. Willarda, Department of Animal and Dairy Science, Mississippi State University, P.O. Box 9650, Mississippi State, MS 39762, USA

explanation for this embryonic death could be the disruption of embryonic development when the embryo is exposed to microgravity. Thus, mammalian fertilization and early embryo development under microgravity conditions remains unclear requiring more studies to be completed in various mammalian species to further examine the influence of microgravity on developmental processes. In particular, *in vitro* studies will allow us to determine direct effects of microgravity on the embryo itself without effects associated with an altered maternal environment.

The objective of present study was to determine whether simulated microgravity conditions created by the Rotating Cell Culture System™ (RCCS) bioreactor with High Aspect Ratio Vessel (HARV) (Synthecon, Houston, Texas) affect *in vitro* fertilization and preimplantation embryo development using a bovine model system.

MATERIALS AND METHODS

Oocyte preparation: All the chemicals utilized in the present investigation were purchased from Sigma Chemicals (St. Louis, MO). Bovine Cumulus-Oocyte Complexes (COCs) were purchased from a commercial supplier (BOMED, INC., Madison, WI). They were shipped in 2 mL of commercial maturation medium in tightly closed tubes and were over-night shipped in a 39°C portable incubator.

Rotating Cell Culture System™ (RCCS) bioreactor: Simulated microgravity culture conditions were obtained using the Rotating Cell Culture System™ (RCCS) bioreactor with High Aspect Ratio Vessel (HARV) (Synthecon, Houston, Texas), which was originally developed by NASA. It has become a useful tool to determine the effects of microgravity on a variety of mammalian *in vitro* cell cultures on the ground. Changes observed in cell morphology and functions in the RCCS bioreactor experiments have proven to be very similar to those observed after space flights (Goodwin *et al*., 1993; Ingram *et al*., 1997; Schwarz *et al*., 1992; Unsworth and Lelkes, 1998). One major feature associated with the microgravity environment of space is the lack of sedimentation. On the ground, this situation can be created by rotating samples about the horizontal axis in suspension (Hemmersbach *et al*., 2006; Klaus *et al*., 1998). The HARV, which is completely filled with medium, is rotated about the horizontal axis at a constant velocity. The combined actions of the centrifugal force, gravity and the viscosity of the medium create the condition of maintaining cells or small samples in suspension (Hammond and Hammond, 2001; Klaus, 2001). Gas exchange is achieved by diffusion through a semi-permeable membrane at the back of the HARV. Additionally, it has been shown that the RCCS bioreactor with HARV generates a low shear simulated microgravity of about 10^{-2} g (Unsworth and Lelkes, 1998). In the present study, the HARV-10 mL was rotated at 34 rpm on a horizontal axis for the High Speed group and at 3.7 rpm on a horizontal axis for the Low Speed group.

***In vitro* fertilization and examination of oocytes:** Bovine matured COCs were washed three times in HEPES-TALP (Tyrode's Albumin Lactate Pyruvate; Bioniche, Pullman, WA) wash medium and once in IVF-TALP (Millipore, Billerica, MA) fertilization medium supplemented with pyruvate (0.2 mM), Fatty-Acid-Free Bovine Serum Albumin (BSA-FAF, 6 mg mL^{-1}), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), heparin (10 μg mL^{-1}), hypotaurine (0.5 μg mL^{-1}) and epinephrine (0.5 μg mL^{-1}) (Miller *et al*., 1994). The matured COCs were placed in groups of n = 70 in 100 μL of IVF-TALP fertilization medium drops under mineral oil in 60×15 mm sterile polystyrene disposable petri dishes (Fisher Scientific, Pittsburgh, PA). For fertilization in simulated microgravity conditions, matured COCs were transferred in microdrops of 100 μL of IVF-TALP covered with mineral oil in 10 mL High Aspect Ratio Vessels (HARV) of the Rotary Cell Culture System™ (Synthecon; Houston, TX). The culture medium and mineral oil were equilibrated at 39°C and with 5% CO_2 in air overnight. All the air bubbles and head space were removed by adding mineral oil in the HARV. All the matured COCs in fertilization medium were placed in a 5% CO_2 incubator at 39°C, until spermatozoa were added. The sperm preparation for IVF used the BoviPure™ gradient (Nidacon International AB, Gothenburg, Sweden) and was performed according to the protocol provided by the manufacturer. Briefly, in a 15 mL conical centrifuge tube, 2 mL of BoviPure™ Bottom Layer was placed and then carefully layered with 2 mL of BoviPure™ Top Layer on the top of the BoviPure™ Bottom Layer to make a gradient. Two 0.5 mL straws of frozen semen from a single Angus bull were thawed in a 37°C water bath for 120 sec. The thawed semen were gently mixed with BoviPure™ Extender and brought to a 2 mL volume. The 2 mL of the prepared semen was gently layered on top of the BoviPure™ gradient and centrifuged for 20 min at 500× g. After the centrifugation, the supernatant was carefully removed and the sperm pellet was resuspended with 5 mL of BoviPure™ Wash and centrifuged for 5 min at 300× g. Then, the pellet was suspended in the IVF-TALP fertilization media. The spermatozoa concentration and motility were then assessed. The spermatozoa were added to the matured

oocytes at a final concentration of approximately 2×10^6 spermatozoa per 1 mL. Spermatozoa and COCs were co-incubated at 39°C and with 5% CO_2 in humidified air for 18 h.

In order to examine fertilization rates, the presumptive zygotes were denuded from cumulus cells by vortexing for 6 min and were fixed in acetic acid and ethanol (1:3/v:v) for 24 h at room temperature. The presumptive zygotes were mounted on slides with coverslips and stained with 1% aceto-orcein (w:v) for examination by light microscope at a magnification of ×400. Oocytes containing swollen sperm head or pronuclei were considered as fertilized oocytes. Oocytes that were not matured or were degenerated oocytes were not counted.

***In vitro* culture:** The cumulus cells of the presumptive zygotes were removed by vortexing for 2 min and the denuded presumptive zygotes were washed 3 times with HEPES-TALP wash medium. Synthetic Oviductal Fluid (SOF) (Millipore, Billerica, MA) culture medium supplemented with pyruvate (0.4 mM), 100× MEM (20 μL mL^{-1}), 50× BME (10 μL mL^{-1}), BSA-FAF (8 mg mL^{-1}), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) was used for culture medium (Edwards *et al*., 1997). Groups of n = 70 fertilized oocytes were cultured in 100 μL drops of SOF with mineral oil overlay in 60×15 mm sterile polystyrene disposable petri dishes for the static culture condition or 10 mL HARV of the RCCS for the embryo culture in simulated microgravity conditions. Bovine embryos were incubated at 39°C and with 5% CO_2 in humidified air and blastocyst rates were assessed at 9th day post insemination (pi). The day of insemination was defined as 0 day.

Experimental designs

Experiment 1: microgravity simulation during *in vitro* fertilization: The aim of this experiment was to examine the effect of simulated microgravity on bovine *in vitro* fertilization (0-18 h pi). Bovine COCs and spermatozoa were co-incubated in the following fertilization conditions. (Control) groups of n = 70 COCs in 100 μL microdrops of medium with 10 mL of mineral oil overlay in petri dish; (high speed) groups of n = 70 COCs in 100 μL microdrops of culture medium with mineral oil overlay in 10 mL of High Aspect Ratio Vessel (HARV) rotated at 34 rpm on a horizontal axis; (Low Speed) groups of n = 70 COCs in 100 μL drops of culture medium with mineral oil overlay in HARV rotated at 3.7 rpm on a horizontal axis. This experiment was replicated three times (Table 1 for numbers (n) of oocytes examined) and fertilization rates were assessed at 18 h pi.

Table 1: Effect of simulated microgravity obtained by RCCS rotation with 34 rpm (high speed) and 3.7 rpm (low speed) on bovine oocyte and sperm fertilization

Treatment groups	Replicates	No. oocytes examined	Fertilized oocytes*
Control	3	185	77.3±3.7[a]
High speed	3	176	0.0±0.0[b]
Low speed	3	160	0.0±0.0[b]

[a, b]Values with different superscripts in the same column are significantly different ($p<0.05$). *Oocytes containing swollen sperm head or pronuclei were considered as fertilized oocytes

Table 2: Effect of simulated microgravity obtained by RCCS rotation with 34 rpm (high speed) and 3.7 rpm (low speed) on development of presumptive bovine zygotes

Experimental group	Replicates	No. presumptive zygotes cultured	Embryos developing*	
			Morulae	Blastocyst
Control	3	683	11.0±1.7[a]	3.6±1.3[a]
High speed	3	601	0.0±0.0[b]	0.0±0.0[b]
Low speed	3	598	0.0±0.0[b]	0.0±0.0[b]

[a, b]Values with different superscripts in the same column are significantly different ($p<0.05$). *Number of total presumptive zygotes developing to morulae and blastocyst at 9th day pi (%)

Experiment 2: microgravity simulation at the 1-cell stage: The aim of this experiment was to examine the effect of simulated microgravity on bovine preimplantation embryo development from the presumptive zygotes to the blastocyst stage (from 18 h pi to 9th day 9 pi). After IVF, groups of n = 70 presumptive zygotes were randomly allocated into 100 μL microdrops under mineral oil in a petri dish for the Control group, for the HARV group rotated horizontally at 34 rpm for the high speed group and for the HARV group rotated horizontally at 3.7 rpm for the low speed group. This experiment was replicated three times (Table 2 for numbers (n) of oocytes examined) and the development rates were assessed at 9th Day pi.

Experiment 3: microgravity simulation at the 2-, 4- and 8-cell stages (2nd day pi): The aim of this experiment was to examine the effect of simulated microgravity on bovine early embryo development from the 2-cell stages to the blastocyst stage (at 2-9 day pi) of embryos. Following IVF, presumptive zygotes were cultured in 100 μL microdrops of the culture medium covered with mineral oil in a petri dish for 2 days. Cleaved embryos (2-8 cell stage embryos), at 2nd day pi, were equally distributed to control, high speed and low speed groups. All the conditions of these three groups were the same as in experiment 2. All experiments were replicated three times (Table 3 for numbers of oocytes examined) and the development rates were assessed at 9th day pi.

Statistical analysis: All the experiments were repeated three times and data were analyzed using ANOVA, followed by Fisher's Protected Least Significant

Table 3: Effect of the simulated microgravity obtained by RCCS rotation with 34 rpm (high speed) and 3.7 rpm (low speed) on 2-8 cell stages bovine embryos

Treatment groups	No. 2-8 cell embryos cultured	Different stages of embryos at day pi		
		2-8 cell	> 8-cell	Blastocyst
Control	508	67.7±4.1[a]	15.8±3.1[a]	16.5±2.6[a]
High speed	508	100.0±0.0[b]	0.0±0.0[b]	0.0±0.0[b]
Low speed	508	99.6±0.4[b]	0.4±0.4[b]	0.0±0.0[b]

[a, b]Values with different superscripts in the same column are significantly different ($p<0.05$)

Difference (PLSD) test using the StatView program (Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as mean±SEM. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Experiment 1: microgravity simulation during *in vitro* fertilization: A total of n = 521 COCs were fertilized with spermatozoa in control, high speed and low speed groups and examined for evidence of fertilization after 18 h pi. As shown in Table 1, 77.3% of bovine oocytes were fertilized in the Control group. However, none of the bovine oocytes in both the high (0.0%) and low (0.0%) speed groups were fertilized *in vitro* and no sperm penetration was observed in either of the high or low speed groups.

Experiment 2: microgravity simulation at the 1-cell stage: A total of n = 1,882 presumptive zygotes were investigated for their development in simulated microgravity conditions with High (34 rpm) and Low (3.7 rpm) speed rotation. As shown in Table 2, while, the Control group exhibited 11.0 and 3.6% of presumptive zygotes reaching the morulae and blastocyst stages, respectively at 9th day pi, none of the presumptive zygotes that were cultured in the RCCS bioreactor at high (0.0%) and low (0.0%) speed groups developed to any other stages of embryo formation. All the embryos failed to undergo even a single cell division in both the high and low speed groups.

Experiment 3: microgravity simulation at the 2-, 4- and 8-cell stages (day 2 pi): A total of n = 1,524 2-8 cell stages of embryos were cultured to examine the development of cleaved embryos (2-8 cell stages) from 2-9 days pi in simulated microgravity conditions with high (34 rpm) and low (3.7 rpm) speed rotation. As shown in Table 3, the proportions of 2-8 cell stage embryos in the high speed and low speed groups (100.0 and 99.6%, respectively) were significantly higher ($p<0.05$) than 2-8 cell stage embryos in the Control group (67.7%) on 9th day pi; with only 1 embryo reaching the morulae stage for the low speed group. Thus, in the Control group, a greater ($p<0.05$) proportion of morulae and blastocyst stage (15.8 and 16.5%, respectively) embryos were achieved than in the high (0%) and low (0% blastocyst and 0.4% morulae) groups. There were no significant differences ($p>0.05$) in embryo development rates between the high and low speed groups with respect to any of the embryo stages noted on 9 day pi.

DISCUSSION

In the present study, bovine *in vitro* fertilization and preimplantation embryo development have been determined in a simulated microgravity environment. The results have shown that embryonic development was disrupted by the simulated microgravity applied during bovine *in vitro* fertilization and at the presumptive zygotes and 2-8 cell stages of development. It has been reported that *in vitro* fertilization in the mouse normally occurred in simulated microgravity (Kojima *et al*., 2000). However, the results from experiment 1 (Table 1) in this study shows that bovine *in vitro* fertilization did not occur in simulated microgravity conditions in either the high or low speed groups and it appears that none of the bovine spermatozoa were able to penetrate the zona pellucida under these conditions. It is usually likely that sperm fail to penetrate the zona pellucida *in vitro* due to decreased motility (Puglisi *et al*., 2004) or the defective binding of sperm to the zona pellucida (Amann *et al*., 1999; Braundmeier *et al*., 2004). The disruption of sperm penetration under simulated microgravity might be related to these two factors. In support of this, human sperm motility has been examined in microgravity environments using the clinostat and parabolic flight models, in which sperm motility was found to decrease compared with a 1 g environment (Ikeuchi *et al*., 2005). Additionally, Andrews and Winters-Hilt (2004) reported that bovine sperm lost their motility within 3 h under simulated microgravity conditions. Also, they observed abnormal sperm nuclear membranes and mitochondria in sperm under simulated microgravity condition compared to those in a 1 g environment. A review of the study found no study investigating the effect of microgravity on sperm zona pellucida binding in mammals. However, Tash *et al*. (2001) reported that the rates of sea urchin sperm binding to oocytes and fertilization were disrupted in a hypergravity environment. Thus, in the present study, the failure of bovine sperm penetration of the oocytes and fertilization under simulated microgravity conditions might be the result of decreased sperm motility

and sperm and zona pellucida binding ability. Otherwise, it is possible that exposure to simulated microgravity for 18 h during *in vitro* fertilization may lead to bovine sperm and oocyte death since none of the bovine oocytes in the high and low speed groups at 18 h pi showed any sign of fertilization. Additionally, the results of experiment 2 (Table 2) also showed that none of the presumptive zygotes cultured in the RCCS bioreactor for high and low speed groups reached any other stages of embryo development. In the development of 2-8 cell stage embryos (Table 3), only one embryo reached the morulae stage for the low speed group. However, we were unable to exclude the possibility that cell divisions had taken place prior to transfer of embryos from the static culture to the simulated microgravity culture condition. Present results indicated that the simulated microgravity culture conditions were lethal to bovine preimplantation embryos. However, it has been reported that although, simulated microgravity can have a negative effect on preimplantation embryo development in mice, 2-cell stage mouse embryos were able to reach blastocyst stages in simulated microgravity conditions obtained by 100 rpm clinostat rotation on a horizontal axis (Kojima *et al*., 2000). These different results may be explained by either variation of sensitivity towards the microgravity between mouse and bovine species or differing technical aspects of the simulated microgravity. Only one live embryo experiment has been conducted under microgravity conditions of space. *In vitro* culture of 2-cell stage mouse embryos were performed on board the space shuttle Columbia (STS-80) resulting in death of all embryos (Schenker and Forkheim, 1998). The researchers concluded that microgravity, as well as possibly other space environment factors, may have caused embryonic death in these mouse embryos.

Because little information is available regarding the effects of microgravity on cellular and functional alterations of spermatozoa, oocytes and early embryos following exposure to microgravity, it is difficult to explain causes of failed fertilization, development and apparent mortality in the present experiment. However, many *in vitro* culture experiments of various mammalian cells during space flight and under simulated microgravity conditions have shown that microgravity conditions caused decreased cell proliferation, loss of mitochondrial activity and induction of apoptosis (Bucaro *et al*., 2004; Cogoli *et al*., 1984; Lewis *et al*., 1998; Yang *et al*., 2002). It has been suggested that disruption of the cytoskeleton structure is one of the major causes of cellular changes in microgravity (Lewis, 2004) and is likely to compromise mammalian embryonic development in microgravity as well (Crawford-Young, 2006). Several studies have reported alterations of cytoskeleton structures in response to microgravity in mammalian cell culture *in vitro*. The cytoskeletal structures of papillary thyroid carcinoma cells (Infanger *et al*., 2006) and glial cells (Uva *et al*., 2002) in culture were damaged <30 min after simulated microgravity. In addition, Lewis *et al*. (1998) reported that space flown Human T lymphoblastoid cells exhibited diffuse, shorter microtubules extending from poorly defined microtubule-organizing centers that led to cell death. Thus, the somatic cell death caused by the disruption of cytoskeleton structures described here are likely to occur in sperm, oocytes and embryos exposed to simulated microgravity *in vitro*; although, this was not confirmed as a component of the present investigation. Furthermore, Tabony *et al*. (2007) showed that self-organized microtubule structures lost ability to transport organelles and chromosomes under brief periods of simulated microgravity and also proved that the round shapes of cells like embryos were more sensitive to simulated microgravity environments than square shaped cells. The relationship between embryonic development and functions of cytoskeleton under microgravity conditions have not been investigated; however, these somatic cell culture studies under microgravity conditions clearly indicate that disruption of cytoskeleton is probably a major cause of the inhibition of bovine fertilization and embryonic development in the present experiments; although, this requires further study to confirm.

CONCLUSION

The present study showed that *in vitro* fertilization and preimplantation embryo development in the bovine was inhibited by simulated microgravity conditions. Further studies are needed to determine the mechanisms, through which short term (acute) microgravity conditions can affect *in vitro* fertilization and early embryo development.

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