Effect of Prefertilization Gravity Load to Mouse Secondary Oocytes on In Vitro Fertilization

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Abstract: *In our previous study, we observed decreases in the rate of fertilization and increases in the rate of polyspermy when mouse ova were exposed to hypergravity during in vitro fertilization. Moreover, the structures in mouse ova were changed by hypergravity. However, it was not determined whether the change in the rate of fertilization was caused by gravitational force acting directly on a process during fertilization or by structural changes in the ovum caused by hypergravity. We investigated the effect of prefertilization hypergravity, which excluded the direct acts on a process during fertilization, on in vitro fertilization. The rates of monospermy, polyspermy, and non-fertilization were investigated under various gravity loading conditions: Pre-G group, 3G-load (centrifugal force) for 2 h before insemination; During-G group, 3G-load for 6 h during fertilization; Pre&During-G group, 3G-load for 2 h before insemination and 6 h during fertilization; Non-G group, 3G-load was not performed. The rates of polyspermy in all G-load groups were significantly higher than in the Non-G group. Furthermore, the rate of polyspermy in the Pre-G group was the highest. Two-hour exposure to hypergravity before insemination increased the rate of polyspermy, and this may be due to structural changes in the cytoskeleton of the secondary oocyte.*

Key words: *Hypergravity, Mouse ova, Reproduction, Polyspermy*

Introduction

Gravitational force has been reported to influence the determination of the dorsal-ventral axis in amphibian embryos at an early developmental stage [1, 2]. This suggests that reproductive mechanisms in the early embryo may be partly influenced by terrestrial gravity. A simulated microgravity study in mice using a clinostat showed that a gravity force below 1G has no influence on fertilization and suggested that mammalian fertilization is not influenced by terrestrial gravity [3]. Unlike amphibians, the gravitational force may not influence mammalian ova because they are very small and include much less yolk.

In contrast to these studies, we observed decreases in the rate of fertilization and increases in the rate of polyspermy when mouse ova were exposed to 2G during *in vitro* fertilization [4]. One possible explanation for these effects is that gravitational force acts directly on a process during fertilization. A second possibility is that critical structures in the ovum are changed by gravitational force. In fact, we observed that the distribution of cortical granules (CGs) which protect the ovum from polyspermy were changed by a 2-h exposure of secondary oocytes to a 3G load [5]. Based on these results, we speculated that a primary mechanical effect of hypergravity might be changes in the formation and distribution of the cytoplasmic organelle. Therefore, we hypothesized that increases in the rate of polyspermy would also be observed after exposure to hypergravity prior to fertilization. To test this hypothesis, we examined the effects of a 3G load for 2 h prior to fertilization on the rate of normal fertilization and polyspermy.

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Materials and Methods

Animals

Female B6D2F1 (C57BL/6xDBA/2) and male ICR mice (Japan SLC Inc., Shizuoka, Japan) were housed and bred under controlled conditions (12 h light 12 h dark cycle, 22 ± 2° C) according to the guidelines of Tokyo University of Agriculture. Food and water were provided *ad libitum*. This animal experiment was approved by the Committee on Animal Experimentation of Tokyo University of Agriculture.

The centrifugal G-load incubator

An incubator with two 96-well microplates (Corning, New York, USA) on a 15 cm radius turntable was specially constructed for generating an artificial gravity load. Two 96-well microplates were mounted so that the resulting vector of terrestrial gravity and centrifugal force would be the Z axis of the microplate wells. The speed of rotation of the turntable was monitored with a light sensor to maintain the centrifugal force. The inside was maintained at 37° C with a humidified atmosphere of 5% CO_2 in air.

Experimental groups

The rates of monospermy, polyspermy, and non-fertilization were investigated under the following four hypergravity conditions: Pre-G group, 3G load (centrifugal force) for 2 h before insemination; During-G group, 3G load for 6 h during fertilization; Pre&During-G group, 3G load for 2 h before insemination and 6 h during fertilization; Non-G group, 3G load was not performed.

Oocyte collection and culture

B6D2F1 mice (6- to 12-week old) were superovulated with 7.5 IU of pregnant mare serum gonadotropin (Isei, Yamagata, Japan) and 7.5 IU of human chorionic gonadotropin (Isei, Yamagata, Japan). The oviducts were excised from euthanized females and were immediately placed in TYH medium (119.37 mM NaCl, 4.78 mM KCl, 12.6 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 1.0 mM sodium-pyruvate, 5.56 mM glucose, 4 g crystalline bovine serum albumin/ml and 50 μg/ml streptomycin sulphate) that was covered with embryo-tested grade mineral oil (Sigma). Freshly ovulated secondary oocytes were released into the TYH medium by puncturing the ampulla of each oviduct. The Pre-G and the Pre&During-G groups were cultivated for 2 h under 3G in the centrifugal G load incubator. The During-G and the Non-G groups were cultivated for 2 h under stationary conditions on a tray outside the turntable within the centrifugal G load incubator.

In vitro fertilization

For *in vitro* fertilization, sperm were obtained from the cauda epididymides of a mature (8- to 14-week old) ICR male. They were cultivated for 2 h under stationary conditions for capacitation in 400 μl TYH medium. Sperm were suspended in a droplet of the THY medium containing oocytes at (1 to 3) × 10^5 sperm/ml. Immediately after this insemination, the During-G and the Pre&During-G groups were subjected to a 3G load for 6 h, and the Pre-G and the Non-G groups were incubated under stationary conditions for 6 h.

Observation of eggs

After 6 h of fertilization, some of the ova were removed from the incubation medium, fixed in neutralized aldehyde for 16 h in a refrigerator (5 to 10° C), and stained with lacmoid (0.25%). The stained and the other fresh ova were observed using Hoffman type and phase contrast microscopes, respectively. The degree of monospermy, polyspermy, and non-fertilization were assessed by observation of the pronuclei. Normal fertilization (monospermy) was defined as an ovum with a female pronucleus, a male pronucleus, and a second polar body. Polyspermy was defined as an ovum with a female pronucleus, two or more male pronuclei and sperm tails, and a second polar body.

Statistical analysis

The rates of monospermy, polyspermy, and non-fertilization were analyzed by the χ^2-test. A p value < 0.05 was considered statistically significant.

Results

Polyspermy in an ovum 3G loaded for 2 h before insemination is shown in Fig. 1. The rates of monospermy, polyspermy, and non-fertilization following the various treatments are shown in Fig. 2. The rates of normal fertilization (monospermy) were 83.7% in the Non-G group, 66.3% in the Pre-G group, 57.5% in the During-G group, and 54.8% in the Pre&During-G group, and the rates of normal fertilization for all groups receiving a G load were significantly lower than that of the Non-G group. The rates of polyspermy were 6.5% in the Non-G group, 29.2% in the Pre-G group, 21.9% in the During-G group,

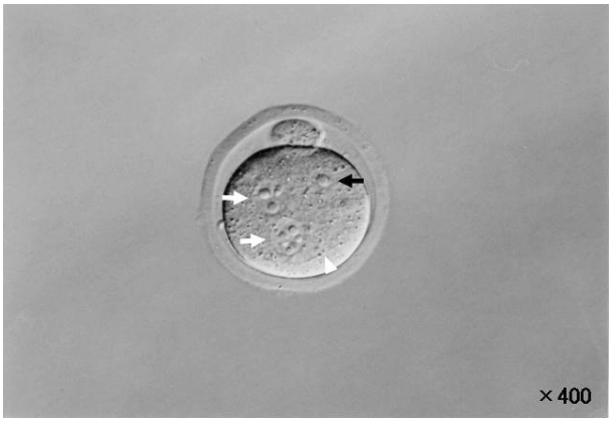

Fig. 1. A mouse ovum that was inseminated after a 3G load for 2 h and formed three pronuclei. A black and two white arrows show a female pronucleus and two male pronuclei, respectively. The arrowhead shows a sperm tail.

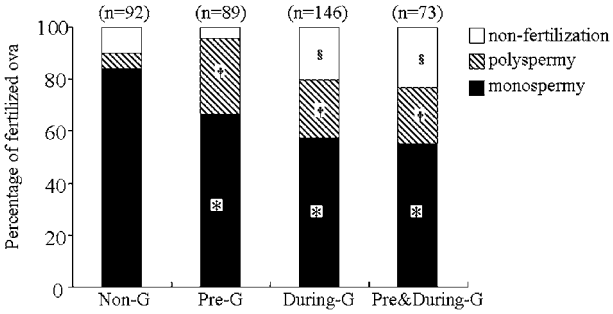

Fig. 2. The percentages of fertilized ova with monospermy and polyspermy under various gravity conditions. Non-G group, 3G load was not performed; Pre-G group, 3G load for 2 h before insemination; During-G group, 3G load for 6 h during fertilization; Pre&During-G group, 3G load for 2 h before insemination and 6 h during fertilization. *, $P<0.05$ *vs.* the Non-G group for monospermy; †, $P<0.05$ *vs.* the Non-G group for polyspermy; §, $P<0.05$ *vs.* the Non-G group and the Pre-G group for non-fertilization.

and 21.9% in the Pre&During-G group, and the rates of polyspermy for all groups receiving a G load were significantly higher than for the Non-G group. Finally, the rates of non-fertilization were 9.8% in the Non-G group, 4.5% in the Pre-G group, 20.5% in the During-G group, and 23.3% in the Pre&During-G group, and the rates of non-fertilization in the During-G and the Pre&During-G groups were significantly higher than those in the Non-G and Pre-G groups.

Discussion

There were two novel findings in the present study. First, exposure to hypergravity at 3G prior to fertilization increased the rate of polyspermy, even without exposure to hypergravity during fertilization. Second, exposure to hypergravity at 3G during fertilization increased the rates of non-fertilization.

It is known that dynamic changes in metabolism and structure continue during the 10 to 12 h of fertilization. For example, Ca^{2+} oscillation and a rise of pH occurs, CGs, which prevent polyspermy, are released, and mitotic apparatus, nucleus, and cytoskeletal elements move inside the cytoplasm [6, 7]. Gravity may have some influence on this important early phase of reproduction. Previously, we observed that exposure to hypergravity above 2G increased the rate of polyspermy and decreased normal fertilization when a G load was performed during fertilization [4]. Furthermore, using a LCA (*Lens culinaris* agglutinin) and FITC (fluorescein isothiocyanate)-lectin dyeing technique, we observed that the distribution of CGs in the secondary oocytes was changed by a 2-h exposure to 3G [5].

The secondary oocytes which exist between ovulation and fertilization store most of the factors for early development and fertilization. In particular, in the periphery of the cytoplasm, a mitotic apparatus appears that prepares the oocyte for resumption of the second metaphase, and CGs undergo a substantial change in distribution to prepare for fertilization [8, 9]. The CGs are released to protect against polyspermy during fertilization. Exposure to hypergravity before insemination may induce changes in the structure of the cytoskeleton, such as disruption of the distribution of organelles or CGs and polymerization of actin filaments. Misdistribution of CGs and structural changes in the cytoskeleton in the second oocyte may impair the preparation for fertilization and may increase the rate of polyspermy. In fact, in the present study, exposure to hypergravity before insemination in the Pre-G group increased the rate of polyspermy and decreased the rate of normally fertilized ova. Thus, it appears that the misdistribution of CGs and structural changes in cytoskeletons induced by hypergravity remained until fertilization of the oocytes, thereby causing the high rates of polyspermy.

In the present study, while the range of sperm concentrations did not affect the frequency of the polyspermic rate, we found that 30% of the ova receiving a G load and penetrated by sperm had polyspermy. Although this high incidence of

polyspermy may have been caused by hypergravity, other factors might have been involved, such as excessive sperm concentration [10, 11], temperature [12], and various factors or conditions at fertilization [13]. The absolute value of the rate of polyspermy may differ in a hypergravity study with different changes in these factors. However, in the current study, we precisely controlled the environmental factors. Thus, the tendency of hypergravity to increase the incidence of polyspermy would be expected to occur even if environmental factors were changed.

It is unclear whether a polyspermic embryo induced by hypergravity would be able to undergo normal ontogeny. This is because whether a normal birth can be achieved with a polyploid ovum is unclear. It is generally accepted that chromosomal non-disjunction happens in polyploid ova with polyspermy and that they cannot progress to normal fetuses [14]. However, Yu *et al.* [15] and Yoshizawa [16] have reported that polyploid ova with polyspermy send surplus chromosomes out of the nucleus during early development, and suggested that there is a possibility that polyploid ova with polyspermy might become normal embryos. We have previously cultivated polyploid ova with polyspermy as a result of hypergravity to blastocysts and observed their chromosome composition. We found that 80% of the embryos were polyploid and 20% were mosaic, including polyploid and diploid blastomeres. In addition, there were no embryos that were composed only of a diploid blastomere [17]. Furthermore, when the morulae developed from ova with polyspermy were transplanted into uteri of recipient mice, approximately 15% reached at least implantation [18]. Thus, we cannot rule out the possibility that polyploid and mosaic embryos can develop to offspring. However, there have been no reports of polyploid mammals. Therefore, we speculate that there is a very low possibility that ova with polyspermy as a result of hypergravity can develop to live offspring.

Oocytes are often exposed for 3 to 5 min to hypergravity above 10,000G to improve cytoplasmic visibility prior to micromanipulation. Such hypergravity treatment does not appear to influence fertilization or early development if the duration is short [19, 20]. However, low-level hypergravity of 3G for 2 to 6 h in the present study clearly effected fertilization. Thus, the duration of the exposure to hypergravity may be more important than the gravitational force.

In the present study, the rates of non-fertilization in the During-G and the Pre&During-G groups were significantly higher than those in the Non-G or Pre-G groups. Thus, exposure to hypergravity at 3G decreases the penetration rate of sperm to ova, suggesting that a 3G load during fertilization impairs sperm binding to the zona pellucida or the cytoplasmic membrane, and also that it possibly reduces sperm penetration into the cytoplasm. These current results contrast with our previous study [4, 21], which showed that hypergravity at 1.4 or 1.6G increased rates of fertilization. Further studies will be needed to determine the detailed dose-response relationship between hypergravity and sperm penetration. This could lead to the development of novel approaches for increasing rates of *in vitro* fertilization.

In summary, we showed that a 6-h exposure to hypergravity during fertilization increased rates of non-fertilization and polyspermy and that a 2-h exposure to hypergravity before insemination increased the rate of polyspermy. These effects may be due to induction of structural changes in the cytoskeleton of the secondary oocyte. Induction of polyspermy in ova by hypergravity may prevent the embryo from developing to birth, and hypergravity at this level may increase the incidence of sterility.

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