—Review—

Effects of Centrifugal Gravity on the Fertilization and Early Development of Mammals

Masao Ito and Sayaka Kita*

Faculty of Bioindustry, Tokyo University of Agriculture, Abashiri-shi, Hokkaido 099-2493, Japan

Studies on amphibians have revealed that the ontogenesis of animals is controlled by a gravitational force. Fertilized amphibian eggs show signs of gravity-dependent polarity, in which the vegetal pole, with a higher density and more highly distributed vitellus assumes a lower position [1]. Such polarity affects the cleavage of the egg and leads to the formation of a fast-dividing micromere area (animal pole) and a slowly-dividing macromere area (vegetal pole), through unequal cleavage after the third segmentation. Back *et al.* [2] reported that tilting the fertilized frog eggs 90 degrees reversed the blastopore invagination site and often caused abnormal development. They also reported that rotating the fertilized frog eggs 180 degrees inhibited the formation of blastopore, and produced embryos similar to the permanent blastura [3]. In addition, another study has reported that applying centrifugal gravity to the eggs immediately after fertilization resulted in the frequent occurrence of double-headed tadpoles (Black *et al.* [4]).

All these studies indicate that the determination of the body axis during amphibian development is dependent on gravity, but it is not known whether the effect of the gravitational force is the same in the embryogenesis of all organisms, as the extent of the effect exerted by the force of gravity (G force) on the embryo differs according to the size, mass and cytoplasmic structure of the embryo. The mammalian egg is much smaller than the amphibian egg and contains virtually no vitellus. Whether or not the mammalian embryo is influenced by G force is a subject of great interest. Centrifugal treatment is often applied in the developmental engineering research of mammals. Because this is an extremely short process; however, it is thought to exert no effect on the developmental potency of the egg [5, 6], but the effect of centrifugal gravity on the embryo when applied during fertilization and embryogenesis is not clear. In this study, we report the effects of centrifugal gravity on the fertilization and early development of mouse eggs to which G force was applied during *in vitro* fertilization and embryonic cultivation by using equipment which can apply a centrifugal G force while incubating fertilized eggs.

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*To whom correspondence should be addressed.

Influence of G force on the Fertilization and Early Development of Mouse Eggs

When the application of G force begins immediately after insemination, *in vitro* fertilization performed under forces of +2G and +3G showed a significantly lower rate of pronuclear formation than that in static culture (control culture method). The spermy rate, including that for fertilized eggs without the pronuclear formation, was extremely high, with a very high polyspermy frequency (30%) (Fig. 1). In previous studies, the fertilization rate (2-cell cleavage rate) for *in vitro* fertilization under +1.4G and +1.6G exceeded that of the control, and suggested that the G force encouraged the entry of the sperm into the egg cytoplasm [7]. When these fertilized eggs are cultured under G force for 72 hours, the rate of embryos developing to the morula stage or higher is significantly lower than in the control, with the rate of those developing to the blastocyst stage leveling off at 40% or less (Fig. 2). We have also reported [7] that more than 80% of the fertilized eggs (2-cell) developed to the blastocyst stage in a culture under a force of +2G. These results show that G force applied after fertilization have a limited impact on the early development of the embryo. When G force are applied from fertilization and throughout the early development stages, the rate of the fertilized eggs developing to the blastocyst stage is lower, indicating that fertilization disorders caused by the application of G force during fertilization affects the subsequent embryogenesis.

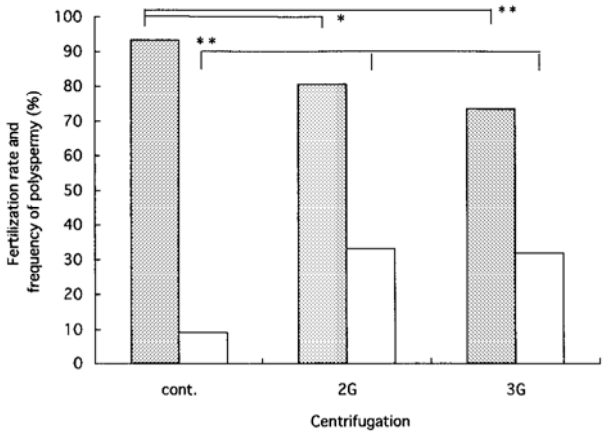

Fig. 1. Rates of fertilization and polyspermy in an environment of various G force. ▨: Fertilization, □: Polyspermy. *: 0.05>p>0.01, **: 0.01>p>0.001.

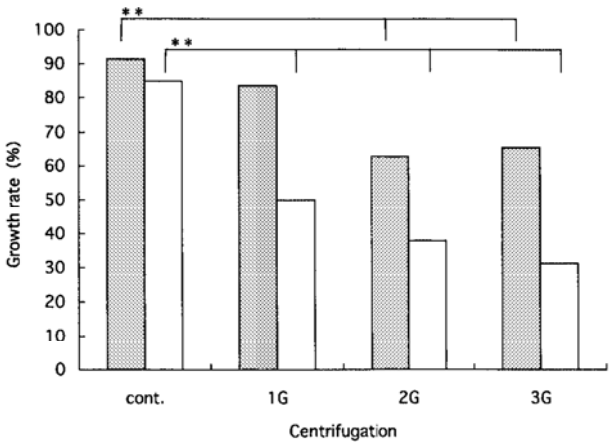

Fig. 2. Embryonic development from fertilization in an environment of various G force. ▨: Morula, □: Blastocyst. **: 0.01>p>0.001.

Influence on Fertilization when G force are Applied Before Insemination

The application of centrifugal gravity during *in vitro* fertilization is thought to push both eggs and sperms to the bottom of the dish, and raises the sperm concentration around the eggs. It is known that an excessively high sperm concentration during *in vitro* fertilization causes a higher occurrence of polyspermy. It is also thought that the frequent occurrence of polyspermy observed with *in vitro* fertilization under G force is associated with changes in the sperm concentration. When, to verify this assumption, normal *in vitro* fertilization was carried out after a force of +3G was applied for 2 hours before insemination, an extremely high fertilization rate was observed with the pronucleus formation in almost 100% of the eggs, although 50% of the eggs were polyspermy. In contrast, fertilization under G force caused a significantly lower fertilization rate, regardless of whether or not G force were applied prior to fertilization (Fig. 3). On one hand, these findings means that the polyspermy resulted from the effect of centrifugal gravity on the morphology or functioning of the oocytes, but not on the sequence of fertilization processes. On the other hand, the rate of fertilization is thought to decrease as a result of damage to fertilization processes when G force are applied during fertilization.

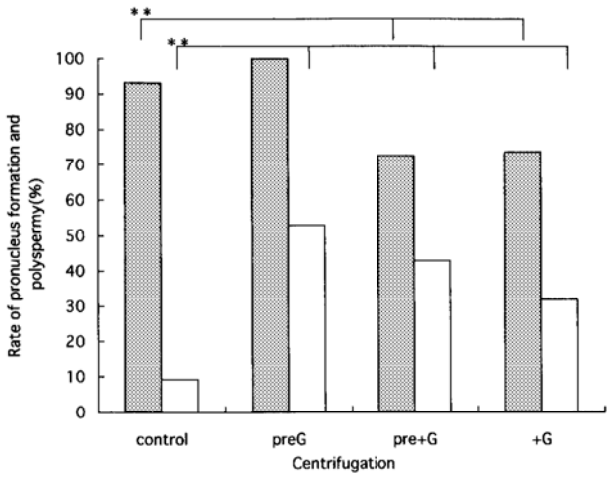

Fig. 3. Effect of centrifugation on pronucleus formation and incidence of polyspermy. ▨: Rate of pronucleus, □: Rate of polyspermy. **: 0.01>p>0.001.

Behavior of Cortical Granules (CGs) in Eggs under G Force

We assumed that the frequent occurrence of polyspermy under the influence of centrifugal gravity during fertilization was attributable to the effect of G force on the morphology and functioning of the eggs. In this way, we examined whether or not centrifugal gravity affects the distribution of CGs. Our study focussed on the cortical granule, an important mechanism in the rejection of polyspermy [8, 9].

Eggs were collected 4 hours after ovulation and cultured together with cumulus cells under a force of +3G and +6G for 2 hours. The oocytes were stained with 0.1% FITC-LCA immediately after the removal of the G force, and distribution of the CGs was examined.

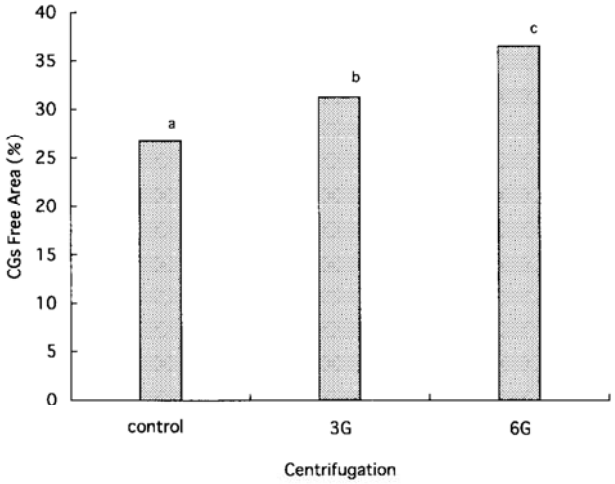

Fig. 4. Effect of centrifugation on CG-free domain. a, b, c: Different superscripts indicate significant, differences at 0.05>p>0.01.

The percentage of the CGs-free domain within the total egg surface area was 33.3% under a force of +3G and 37.5% under a force of +2G. These figures were higher by 5.3% and 9.5%, respectively, than those of the control eggs (Fig. 4). This result indicates that movement of the cortical granules is influenced by centrifugal gravity. From this it may be concluded that this movement damages the mechanism for CG release and the zona reaction does not function correctly, thereby inducing polyspermy. Nevertheless, it can also be concluded that, in addition to the effect on the cortical granules, the G force caused uneven distribution of the organelles associated with the cytoskelton, destroyed the cortical network, and obstructed the release of CGs. Okada *et al.* [10] used an electron microscope to examine the micro-structure of mouse oocytes under G force, and found that the number of CGs just under the membrane was reduced, and that the ratio of these CGs to the CGs located at a distance from the membrane was 1/3 that of the control. Frequently observed collapses or deformation of the membrane and large vacuoles in the cytoplasm of eggs subjected to a force of 10,000G for 15 minutes suggest that the distribution and behavior of actin fibers in the cytoskelton are disturbed by G force.

Behavior of Actin Fibers in Eggs under G Forces

It is known that the microscopic fertilization of equine and swine eggs with a high lipid content is easier when lipids are pushed aside unilaterally by the application of a force of 12,000 to 15,000G. But the observation of the distribution of altered CGs accompanied by morphological changes immediately after applying a force of 10,000G suggests that the distribution and behavior of actin fibers are disturbed. In order to examine the effects of G force on the polymerization and de-polymerization of actin fibers in the oocyte, we inspected the behavior of oocytes collected 14 and 16 hours after the injection of hCG, cultured under G force for 2 hours, and stained with FITC-phalloidin, a substance which combines specifically with actin.

It is well known that actin forms a contractile ring during cell division [11]. On the cleavage device, actin polymerization was observed in all oocytes 14 hours after the injection of hCG, whereas de-polymerization and re-polymerization were observed after 16 hours and 18 hours, respectively [12]. These results suggest that repeated polymerization and de-polymerization in the polar body formation zone adjust the shape of a polar body and induce the release of most of the actin into the polar body. De-polymerization of actin fibers is suppressed in the *in vitro* culture system, and the application of G force intensifies the suppression (Fig. 5) [13, 14]. This suggests that G force accelerate the polymerization of actin. The application of G force encourages the entry of a sperm into an egg cytoplasm, as described in section (1), and actin fibers are also associated with the phagocytosis of sperms in the oocyte. The actin polymerization thus promoted by G force may increase the rate of spermatozoon entry into an ovum. The actin polymerization promoted by G force was also observed in osteoblasts [15, 16].

Effects of G force-induced Morphological Changes in the Distribution of CGs and the Behavior of Actin

We have reported that G force applied during *in vitro* fertilization led to a higher occurrence of polyspermy and the obstruction of fertilization, and that the distribution of CGs and the behavior of actin associated with fertilization were altered by G force, but, as these phenomena are accompanied by morphological changes in the oocyte, the potential effects of G force pressure on the oocyte cannot be denied. By using a culture solution with a density gradient effected by layering of percoll [17, 18], the distribution of CGs and the behavior of actin were compared with the results obtained by using conventional methods. As mentioned earlier, the CG free domain is enlarged when a G force is applied, but we

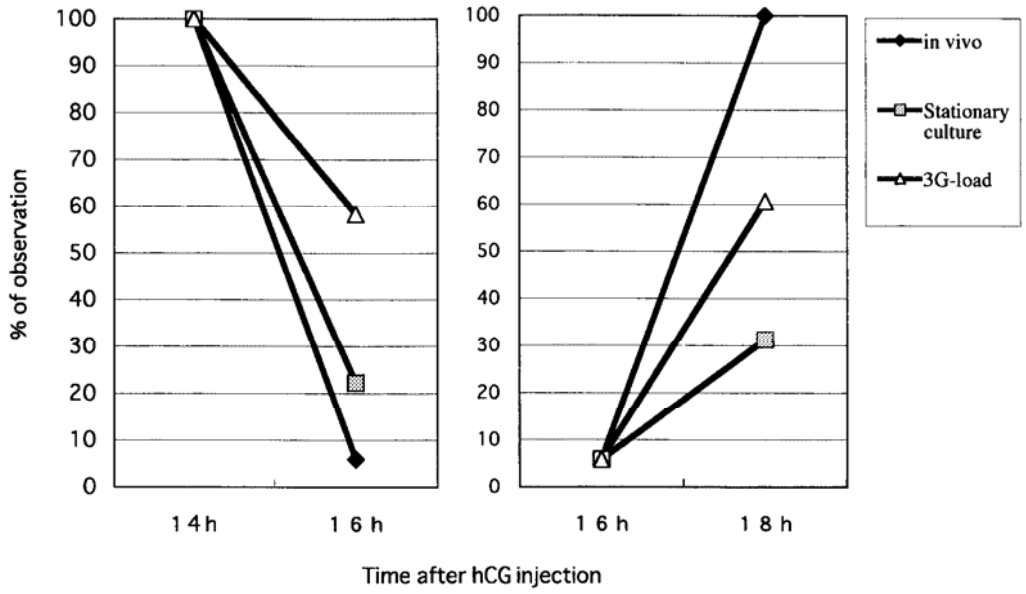

Fig. 5. Actin polymerization on mitotic apparatus.

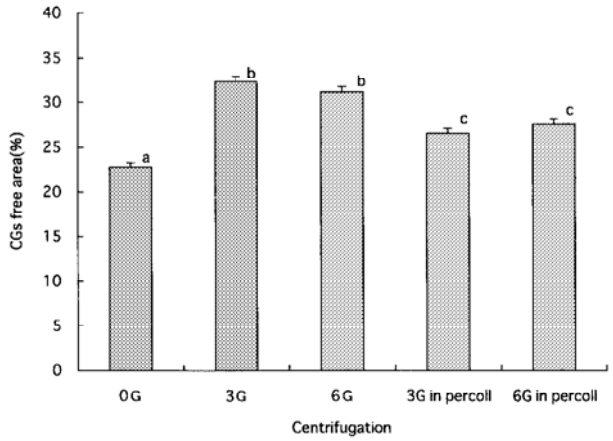

Fig. 6. Effects of centrifugation on CG-free domain. a:b, c: Significant differences at $p<0.01$, b:c: Significant differences at $p\leq0.01$.

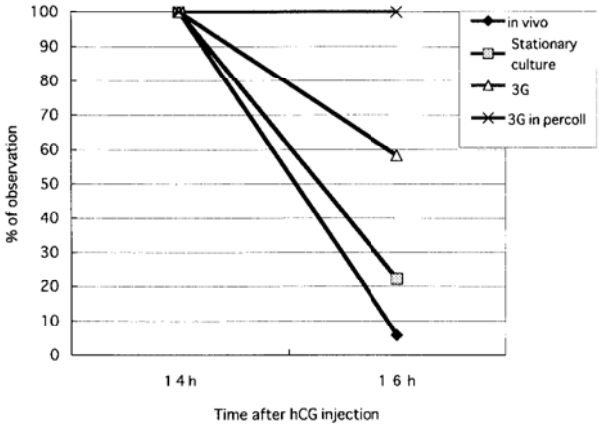

Fig. 7. Actin polymerization on mitotic apparatus.

observed that the extent of enlargement caused by G force was smaller when a density-gradient culture solution was used. This shows that the effect of G force on floating oocytes is weaker than that when a conventional method is used (Fig. 6). The de-polymerization of actin on the cleavage device 14 to 16 hours after the injection of hCG is prevented by G force. This prevention is particularly strong when the G force is applied to floating oocytes (Fig. 7). In view of the observation that the effect of a G force on the floating oocytes is different from that on the compressed oocytes, the effect of the compression attributable to G force must be taken into account when examining the effects of G force.

Effects of G force on the Development of Fertilized Eggs

Ito *et al.* (1993) [7] reported that a force of 2G or less had virtually no effect on the *in vitro* development of 2-cell eggs to the blastocyst stage, but the number of blastocyst cells decreased when a force of 1.2G or more was applied, indicating that a relatively low G force retards embryo development. It has been reported that the cell cycle of *in vitro* cultured embryos became slower

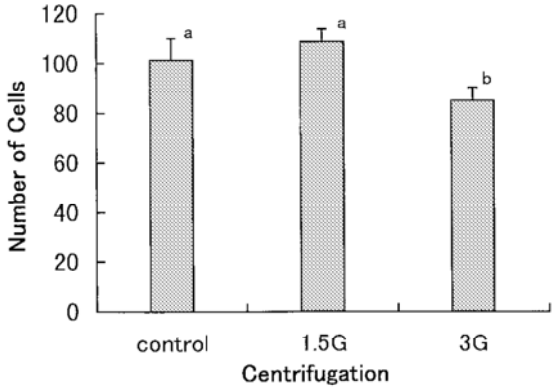

Fig. 8. Number of cells in blastocysts. a, b: Different superscripts indicate significant differences at 0.05>P>0.01.

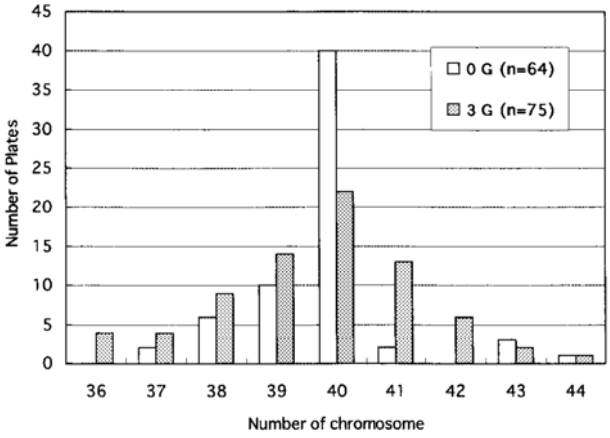

Fig. 9. Number of chromosomes in loaded mouse oocytes.

than that of *in vivo* development because of the involvement of various factors [19]. In view of this, we concluded that the cell cycle was retarded by centrifugal G force, but there is another possibility: that centrifugal G force damage parts of the blastomeres and then form blastocysts consisting of fewer cells. To verify this possibility, 2-cell eggs were cultured under a force of 3G for 72 hours and the number of cells and dead cells in the blastocysts so produced were counted with the Hoechst and trypan blue staining methods.

The result was that the number of cells cultured under a force of 3G was significantly lower than the number cultured without a G force. This clearly indicates that G force retarded embryo development (Fig. 8). In contrast, the average number of dead cells in the blastocysts was 0.4 under a force of 3G and 0.5 in the control, with no significant difference between the two. This indicates that a reduction in the number of cells constituting the blastocyst due to the application of centrifugal G force in the early development process can be explained by the delayed cell cycle. It is not yet known what process in the cell cycle is affected by centrifugal G force. Nevertheless, if G force affect not only the behavior of the CGs and actin, but also organelles such as endoplasmic reticulum and microtubules, it can be inferred that the behavior of cellular calcium ions and spindle formations, both of which are associated with control of the cell cycle, is disturbed by G force, resulting in delay of the cell cycle.

Effects of G force on Chromosomes

When a force of 2–3 G is applied from the time of fertilization through the early development stages, only 40% of eggs develop into blastocysts despite approximately 80% of the eggs being fertilized. Moreover, we observed that the cell cycle of the fertilized egg was delayed under G force, suggesting that G force can affect cell division. The effect of G force on the occurrence of chromosomal aberration was investigated through the chromosomal analysis of blastocysts obtained by culturing under a force of 3G.

Fig. 9 shows the frequency distribution of the number of chromosomes in blastocysts under a force of +3G and in the control. We observed a high occurrence (70.7%) of aneuploidic nuclear plate in the blastocysts under G force. This suggests that the application of G force led to the frequent occurrence of quantitative chromosome aberration. We previously reported that a force of 4G or higher seriously affected embryogenesis and significantly reduced the rate of embryo development to the blastocyst stage, and that the number of cells in these blastocysts was extremely small [7]. Although it is not clear whether or not the interruption of embryogenesis by G force is associated with chromosome aberration, it is clear that G force has a serious effect on embryogenesis.

Conclusion

Centrifugal treatment commonly used in developmental engineering studies of mammals is a very short process, and is thought to have no effect on the developmental potency of the ova, but G force have an unexpectedly profound effect on the reproduction phenomena such as fertilization and embryogenesis. The effects of temporary G force on the distribution of CGs, the behavior of actin, or on the cytoskelton may be reversed when the G force is removed, but the latent

effect may remain. Recent advancements in space development projects have made long-term and research in microgravity environments possible. Studies of animal reproduction in the space environment are done with insects [20], fish [21], amphibian [22] and birds [23]. These results suggest that animal reproduction in space is possible, but systematic experiments in space on mammalian reproduction are not found [24, 25].

We therefore anticipate that experiments on earth in environments under hyper gravity or simulated microgravity [26, 27] and experiments in space under microgravity will clarify how animal reproduction is associated with gravitational forces.

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