Effect of Gravity Load on Cortical Granule Distribution in the Mouse Oocyte

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Abstract: *In this study we investigated the effects of gravity over 1 G on the distribution of cortical granules (CGs) in mouse oocytes. We had found eggs incubated in a centrifuge culture medium showing a high rate of polyspermy, but there had been no difference between 2 G and 3 G loads in the incidence of polyspermy. We focused on cortical granules (CGs) which are known to act as a protection against polyspermy. The CG-free area of mouse oocytes subjected to increased G, when compared to that of control oocytes, was found to be significantly larger. Therefore, it can be assumed that the increased G caused an abnormal CG distribution which resulted in a high incidence of polyspermy in oocytes subjected to increased G. Further, as the centrifugation force was increased, the CGs free area of oocytes did not tend to expand. Observation was facilitated by using FITC-LCA fluorescent dye and TEM.*

Key words: *Gravity load, Cortical granule, Polyspermy, Mouse oocyte, FITC-LCA.*

All living creatures breed and grow in a 1 G environment. But is gravity of absolute necessity for the reproduction of living creatures? It has not yet been proven that mammalian reproduction is dependent on gravity.

We investigated the effects of a gravitational force greater than 1 G on fertilization or development of mouse oocytes and embryos [1].

In previous research it was found that centrifugation at 2 G or less had no effect on the *in vitro* development of 2-cell embryos into blastocysts [1], but there were effects due to centrifugation during *in vitro* fertilization or development of mouse oocytes and embryos [2]. Further, to investigate the effect of centrifugation on the oocyte itself, we compared oocytes that were subjected to a preload of G with those centrifuged during fertilization. A preload of G on the oocyte significantly decreased the fertilization rate and the rate of development to the morula stage, and increased the incidence of polyspermy [2].

We confirmed in previous experiments that centrifugation obstructs fertilization and is related to a high incidence of polyspermy, but the cause remains unknown. It is known that when polyspermy occurs, a large number of sperm penetrate the plasma causing malformation or death during the early development of the embryos [3], but many organisms, including sea urchins and many mammals, are equipped with a mechanism protecting against polyspermy [4]. Cortical granules (CGs) play an important role in preventing polyspermy. There is a CG domain and a CG-free domain in the mouse oocyte cortex at the time of fertilization. Sperm attach to the CG domain and induce a reaction which is facilitated by enzymes present at the time of union with the oolemma. This activation of the oocyte releases Ca^{2+} into the cytoplasm, causing the CGs to unite with the oolemma, which induces CG exocytosis. The zona reaction that then occurs depends on CGs, which then act to block polyspermy.

To investigate the effect of the G load on CG distribution in the oocyte, we examined oocyte subjected to higher than normal G. Observation was facilitated by using FITC-LCA fluorescent dye and electron microscopy [5].

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Materials and Methods

The G-loading incubation (an incubator specially constructed for G loading)

We found that eggs inseminated in a centrifuge culture medium had a high rate of polyspermy at 2 G and more [1, 2]. We verified that this phenomenon is related to the change in CG distribution when subjected

to loads of 3 G and 6 G. Inside the incubator, two 96-hole incubator microplates (Corning, New York, USA) were mounted at such an angle that a combination of gravitational and centrifugal force could act on the microplate incubational tubes directionally at +Gz. The tubes were attached to the top of a 15 cm radius turntable. The speed of rotation of the turntable was monitored with a light sensor, so that the centrifugal force could be kept constant throughout the incubation period. In this study, samples were processed at 3 and 6 G loads for 2 hours.

Oocyte collection and culture

BDF_1 mice (6–12 weeks old) were superovulated with pregnant mare serum gonadotropin (PMSG) (Isei, Yamagata, Japan) and human chorionic gonadotropin (hCG) (Isei), and unfertilized tubal oocytes were recovered 14 hrs after the injection of hCG. The oocytes then were loaded into a centrifuge at 37°C in a humidified atmosphere of 5% CO_2. M16 medium was used for the 3 G and 6 G groups. Dulbecco's phosphate-buffered saline (DPBS) was used for the 10,000 G group.

CG and chromosomal staining

After centrifugation, the cumulus cells were removed by treatment with 0.05% hyaluronidase (Sigma Chemical, St. Louis, MO, USA) in saline, and the zonae pellucidae were removed by treatment with HCl (Kanto Chemical Co. Inc., Tokyo, Japan) bringing the pH of the Tyrode solution to 2.5. The naked oocytes were fixed in DPBS containing 4% paraformaldehyde and 0.1% tannic acid (Sigma) for 30 min at room temperature. They were then washed 3 times in a blocking solution composed of 3 mg bovine serum albumin (BSA, Sigma), 7.5 mg glycine (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 1 ml DPBS, immersed in DPBS containing 0.1% Triton X-100 (Nakarai Tesque, Kyoto, Japan) for 5 min at room temperature and replaced in the blocking solution. The oocytes were immersed in a staining solution composed of 10 μg FITC-LCA (EY Laboratories Inc., San Mateo, USA), 3 mg BSA and 1 ml DPBS, for 45 min at room temperature. After being washed 3 times in DPBS containing 0.75% glycin and 0.3% BSA, the oocytes were incubated with 0.27 mg/ml propidium iodide (PI) (Sigma) in DPBS for 15 min at 5°C in order to facilitate fluorescent chromosomal staining. The oocytes thus treated were thoroughly washed in DPBS containing 0.75% glycin and 0.3% BSA and placed on glass slides to be photographed under a reflected-light fluorescence microscope. The CG-free domain was then observed (Figs. 1, 2).

Electron microscopy

Because we do not know CG movement with a G load, we examined CG movement with an extremely high G (10,000 G) load by electron microscopy. The sample was processed in micro tubes for 15 min at 37°C under a load of 10,000 G in a centrifuge (MRX-150, Tomy, Tokyo, Japan). For thin sectioning, the oocytes with cumulus cells were fixed for 1 hr at 22°C with Karnovsky's paraformaldehyde-glutaraldehyde fixative containing 2% tannic acid (Sigma) after the oocytes had been loaded at 10,000 G. After the specimens were washed with 0.1 M cacodylate buffer solution (pH 7.2), they were left overnight at 4°C in the same buffer solution and then postfixed for 1 hr at 4°C with Dalton's chrome-osmium fixative (pH 7.2). Fixed specimens were dehydrated in an ethanol series and embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate.

Results

The percentages of CG-free domain area of the oocyte were 32.2% and 31.0% for the 3 G and 6 G groups, respectively. The percentage of the CG-free domain area of the control oocytes was 22.4% (Fig. 1). The CG-free domain areas of the 3 G and 6 G oocytes were both significantly larger than that of control oocytes ($P<0.001$) (Fig. 3), but there was no significant difference between the percentages of the 3 G and 6 G oocytes (Fig. 3).

Electron microscopical observation confirmed that CGs move to the inner cytoplasm with a G load (Fig. 4).

Discussion

In order to test whether gravity is required for normal animal development, several different species of organisms were subjected to a microgravity environment on board several different orbital spacecraft in a series of experiments. It was found that gravity is not required for embryonic development of brineshrimps and drosophilae [6–8]. It was also found that vertebrates can also develop to a nearly normal free-living stage, despite some abnormalities in early embryonic stages in the three species experimented on: a small killifish (Medaka), frog and Japanese red bellied newt [9–16].

Of particular significance was the success of the first microgravity experiment throughout the entire duration of the reproduction process, from copulation, ovulation, fertilization through to birth, with Medaka as the research specimen.

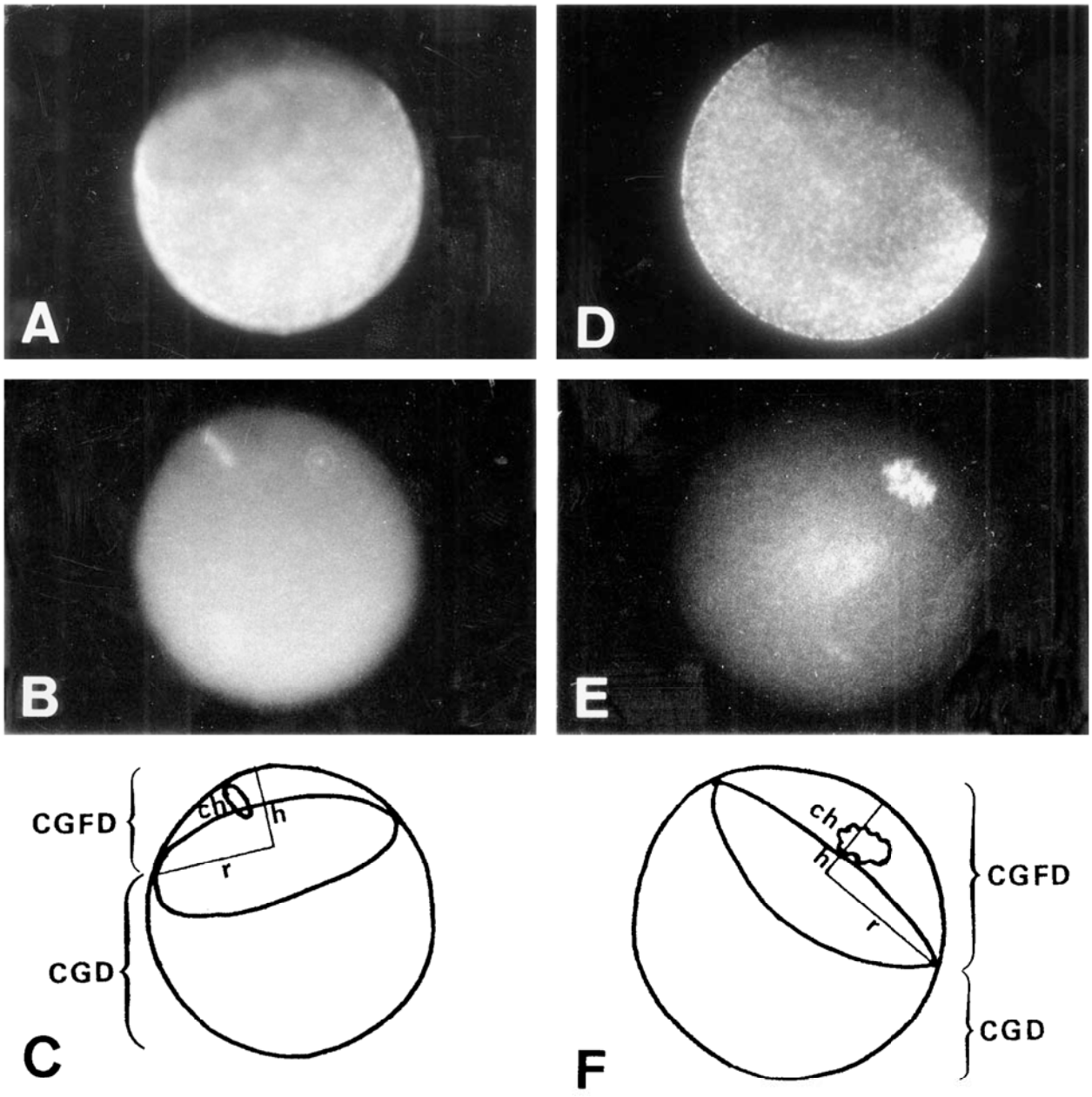

Fig. 1. Cortical areas: Double labeling (FITC-LCA (A, D) and PI (B, E)). (A–C) Control group. (D–F) 6 G-load group. (C, F) These figures were synthesized from tracings of the two above photographs. CGFD: cortical granule-free domain. CGD: cortical granule domain. Ch: chromosome.

From this it was impossible, however, to draw the absolute conclusion that development of vertebrate embryos is not dependent on gravity, as the experiment was only conducted once and the Medaka specimens used were of an advantageous genetic type. Furthermore, it is still not determined what effect a microgravity environment would have on genes that could be manifest in succeeding generation.

In a famous experiment on the effects of centrifugal force on *Xenopus laevis* eggs, there was an abnormally high occurrence of conjoined twins with one body [17, 18]. This was thought to be a clue to the formation of a second blastopore on the opposite side of the axis brought about by centrifugal force applied at an angle of 90°.

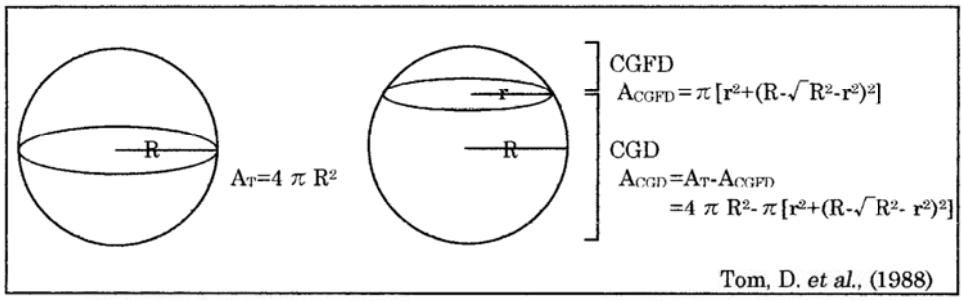

Fig. 2. CG quantification. R: The radius of the oocyte. r: The radius of the cross section of the CG-free area at the line of demarcation. CGFD: CG-free domain. CGD: CG domain.

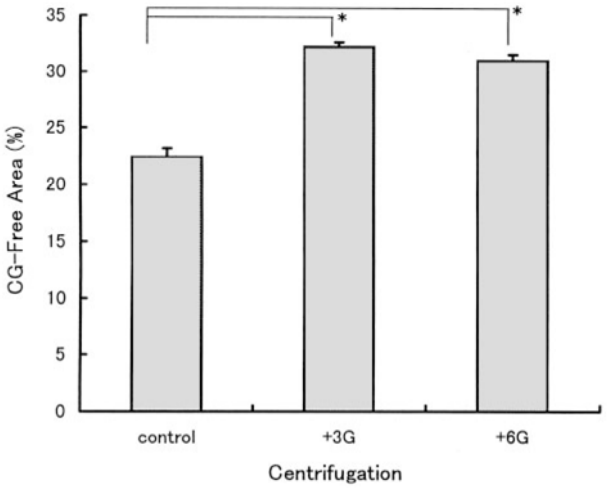

Fig. 3. Effect of centrifugation on CG-free domain area. *There is a significant difference between the control and both centrifugations ($P<0.001$). Data were analyzed by Student's t-test. The numbers of oocytes examined were 89, 72 and 67 for the 3 G, 6 G and control groups, respectively.

There, has however not been any conclusive experiment done on how centrifugal force may influence the formation and distribution of the cytoskeleton such as microtubule and microfilament. Furthermore, there is the question as to what extent inferences on smaller mammalian embryo formation can be drawn from this experiment, as the size of the embryo and embryo formation are different, as well as the types of cleavage in mammals and amphibians and between the respective species. We had proven, however, in previous studies that centrifugal force had an effect on the early development of embryos [1, 2]. In this paper, we investigated the movement of the CG within the oocyte when subjected to centrifugation.

The release of CGs is related to increased Ca^{2+} which is released after the fusion of sperm and oocyte membranes, a process which depends on enzymes such as phospholipase C, which, through mediation of inositol 1,4,5-trisphosphate (IP_3), triggers the release of Ca^{2+} [19, 20]. We reported the no difference between 2 G and 3 G loads in the incidence of polyspermy [2]. In this study, the CG free areas of oocytes were observed under 3 G and 6 G experimental conditions. The CG free areas of oocytes were similar with 3 G and 6 G loads. These results suggest that in *in vitro* fertilization under G loading, the increase in the CG free area parallels the incidence of polyspermy. The increase in polyspermy which occurs with enlargement of the CG-free domain might be caused by an incomplete release of CGs at fertilization, but there are no reports about the effect of the G load on CG moves, and it was difficult to forecast. Therefore, we carried out the tentative experiment about CG movement with 10,000 G loading by electron microscopy. Though under extreme load conditions, this conclusion was further supported by the movement of the CG from the cortical cytoplasm to the inner cytoplasm with a 10,000 G load as seen in the electron microphotograph (Fig. 4). Therefore, it can be assumed that increased G interferes with the process of fertilization. We need to do the experiment on CG movement under G load conditions, but the CG-free domain does not tend to enlarge proportionately with the G load. Further, the G loading changes the CG-free domain, which could also affect the distribution of other cell organelles. The release of Ca^{2+} begins from the endoplasmic reticulum (ER) and its cell cortical network [19, 20]. Therefore, we believe that there is a maldistribution of cell organelles in the cytoplasm, due to the effect of increased G, which presumably affects the distribution of ER, and, in turn, CG release.

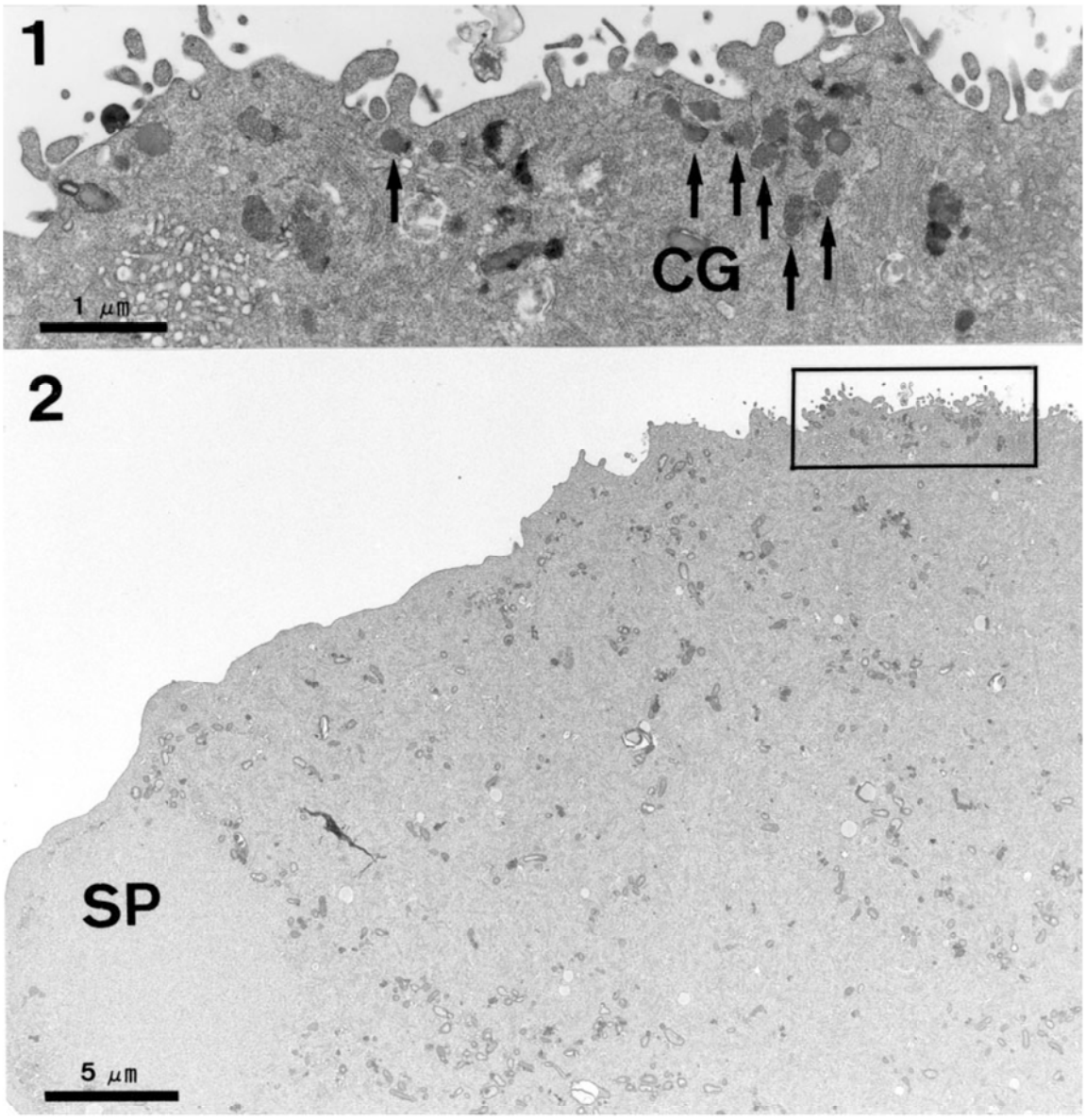

Fig. 4. Electron microphotographs of the CG-free domain area and CG domain area in the oocyte. A magnification of the portion outlined by the square in B. The arrows show the movement of the CG within the inner cytoplasm with a 10,000 G load. SP: spindle. CG: cortical granule.

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