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Softening of the Mouse Zona Pellucida during Oocyte Maturation

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***Abstract*—A change in the elasticity and the resistance to dissolution of the mouse zona pellucida (ZP) was quantitatively evaluated at immature germinal vesicle (GV), mature metaphase II (MII) and fertilized pronuclear (PN) stages. Young's modulus of the ZP was measured using a micro tactile sensor (MTS), a highly sensitive resonator-based sensor for a micro scale elasticity measurement. 0.25% α-chymotrypsin was used for the ZP dissolution assay. The results of measuring the ZP elasticity and the dissolution time clearly showed that the ZP softened during oocyte maturation and the ZP hardened after fertilization. The results indicate that the amount of the zona softening can be a criterion to evaluate oocyte quality for the selection of top quality mature oocyte before *in vitro* fertilization (IVF) treatment.**

I. Introduction

Recent advances in biomedical engineering have made it possible to manipulate cells or tissues in vitro for therapy, and have therefore provided great benefits in human assisted reproductive technology (human-ART), such as in vitro fertilization (IVF). The goal of IVF treatment in humans is to achieve a viable singleton pregnancy followed by vaginal delivery of a healthy child. However, it is still difficult to maintain the natural quality of the embryo in vitro and so the IVF success rate is still quite low. There is also general agreement that twin pregnancy is the most severe complication of IVF resulting in considerable medical risks for both mother and infants, as well as increased obstetric and neonatal costs. Thus, the importance and effectiveness of elective single-embryo transfer (eSET) with top-quality embryos are widely accepted means to reduce the number of multiple births for patients undergoing ART. However, decreasing the number of transferred embryos can reduce the chance of achieving pregnancy. Therefore, it is necessary to develop an appropriate culture device, culture medium, smart cell manipulation system, and a method to evaluate the quality of embryos to select one for eSET to improve the maintenance of embryo quality in vitro and thus achieve higher IVF success rate. To do this, it is of paramount importance to develop a combination of improved selection criteria and improved culture conditions to optimize selection of the embryo with the highest implantation potential. Several studies suggested different types of criteria for the selection of embryos with the highest implantation potential [1, 2]. However, these criteria should be determined morphologically and may differ among physicians and embryologists. Thus, it is necessary to develop a novel sensing technology to estimate embryo quality with quantitative criteria ensuring that the method would be strictly non-invasive and result in no damage to embryos.

The zona pellucida (ZP), the extracellular glycoprotein coat of mammalian oocytes/embryos, which is approximately 15μm in thickness, has been reported to be altered following fertilization in a process described as zona reaction [3]. In a zona reaction, increased resistance to dissolution by lysing biochemical agents, such as α-chymotrypsin, has been recorded in many species [4-6]. This phenomenon has been termed ZP hardening. In the recent past, several techniques have developed to measure the elasticity of the ZP [7-10] and demonstrated that the ZP hardening is also accompanied by an increase in mechanical stiffness, in mouse, pig, bovine and human [11-14].

Those studies are of paramount importance since the embryo quality can be evaluated by measuring the amount of mechanical alteration of the ZP, without any damages, after IVF treatment in human-ART. The authors developed a micro tactile sensor (MTS) [15, 16] that is a highly sensitive resonator-based sensor for a micro scale regional elasticity measurement and measured mechanical ZP hardening in mouse, bovine, pig and human[11, 12]. It was concluded in those studies that the quality of embryos could be evaluated from elasticity parameters [12]. On the other hand, there is also general agreement that the quality evaluation of oocyte before fertilization, *i.e.* IVF treatment, is needed. However, only a few studies investigated the mechanical alteration before fertilization [11, 14] and, to our best knowledge, no study was found investigating dissolution resistance during oocyte maturation before fertilization. In the present study, we measured the ZP elasticity and the ZP dissolution time at immature germinal vesicle (GV), mature metaphase II (MII)

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and fertilized pronuclear (PN) stages and ZP softening during oocyte maturation was discussed. In addition, the relation between the change in the ZP elasticity and the ZP dissolution time on ZP hardening and softening was discussed.

II. Materials and Methods

A. Retrieval of mouse oocyte/embryo

Four to six-week-old female F1 hybrid mice (ICR female × BDF1 male) were given an intra-peritoneal injection of 5 IU pregnant mares' serum gonadotrophin (PMSG; Serotropin, Teikokuzouki, Tokyo, Japan) to induce follicular recruitment and to increase the number of oocytes collected. Forty-eight hours after the PMSG injection, the animals were killed by cervical dislocation and the ovaries were dissected and placed in M2 culture medium containing 4 mg/mL human serum albumin (HSA). Immature germinal vesicle (GV) oocytes were released from the follicles using a 16-gauge needle and collected into the M2 medium. Mature MII oocytes (at the metaphase stage of the second meiotic division) were collected from the oviducts 16 h after human chorionic gonadotropin (HCG) injection (48 h after the PMSG), and then cumulus cells were removed by digestion with hyaluronidase (80 units/mL) in M2 medium.

Four to five-week-old female F1 hybrid mice (ICR female × C57BL male) were superovulated with i.p. injections of 5 IU PMSG and 5 IU HCG, mated with BDF1 males and checked for copulation plugs the next morning. Mated females were killed by cervical dislocation and their oviducts flushed with an M2 culture medium. Fertilized pronuclear (PN) embryos were collected at 15 h after HCG. The elasticity and the dissolution time of each oocyte ZP was measured immediately after collection.

B. Elasticity measurement of zona pellucida

The elasticity of ZPs was measured using a micro tactile sensor and an exclusive measurement platform. The MTS is a highly sensitive resonator-based contact impedance meter capable of estimating the elastic modulus of soft tissues in micrometer scale. It consists of a cylindrical piezoelectric transducer (PZT) that generates ultrasound (Fuji Ceramics, Shizuoka, Japan) and a glass needle with a 20 μm tip spherical point which made contact with the ZP surface (Fig.1). As can be seen in Fig. 1, the MTS was made similar to a microinjection needle so that it could be mounted easily on a standard intracytoplasmic sperm injection (ICSI) system with a smart stepping motor (P and M Co., Ltd., Fukushima, Japan) with a resolution of 10 nm movement (Fig.2). The PZT transmits a very weak longitudinal ultrasonic wave (frequency 100-200 kHz) into a glass needle that makes contact with the measurement object. A PLL (phase-lock loop) phase shift circuit is used to drive the MTS to obtain high signal to noise ratio. Details of the composition, the electronics and the detection principle of the MTS are given in the publications [13], [14]. They show that the change in the oscillation frequency Δf (Hz) for a certain indentation or $\Delta f/\delta$ (Hz/μm) correlated highly to E. Also, it was studied both theoretically and experimentally that the MTS test does not cause any damage to embryos [12].

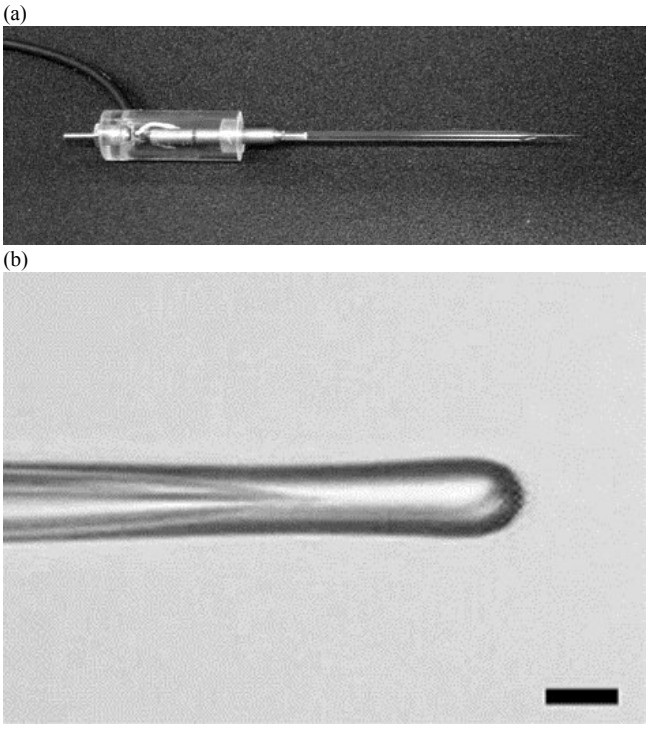

Fig.1. (a) Micro Tactile Sensor and (b) its 20 μm tip point. Scale bar is 20 μm.

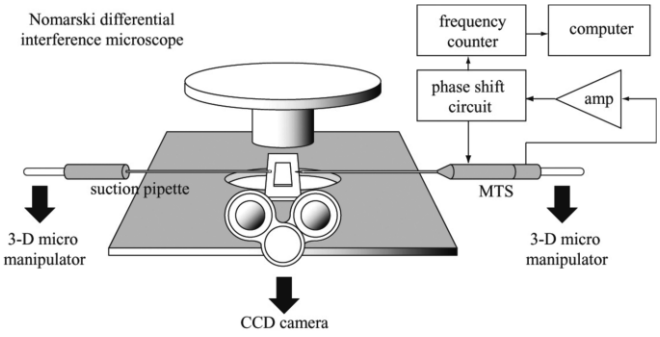

Fig. 2. Schematic diagram of the micro-mechanical sensing platform. Contact of the MTS with the embryo was observed on a monitor and recorded on video via CCD camera.

The schematic diagram of the MTS setup used to measure Young's modulus of ZP is shown in Fig. 3. The oocyte/embryo was transferred to a drop of Dulbecco's phosphate- buffered saline (DPBS) solution on a slide under a glass cover. The space between the slide and the glass cover was approximately 4 mm. Either end of the glass cover was supported by PDMS silicone blocks. The suction pipette was introduced through one end of the chamber, and the oocyte/embryo was held with the pipette using gentle suction. The MTS was introduced through the other end of the preparation uniaxial to the suction pipette. The oocyte/embryo was gradually pressed against the MTS up to an approximately 10 μm indentation at a constant velocity (10μm/s) under computer control (Fig. 4). The change in

resonance frequency from 0 to 5 μm tip displacement was measured and the slope values (Hz/μm) were then calculated to obtain a single value (Fig. 5). Before each measurement, the MTS was calibrated using bovine gelatin (G-9382, Sigma, St Louis, MO, USA) as a known elasticity standard. The elasticity of the gelatin gel was modified by varying the water content to 4, 6 and 8%. Resonance frequency changed as the gelatin was pressed against MTS and was plotted vs. tip displacement. As described above, these slope values (Hz/μm) were then plotted against Young's moduli of the gelatin samples, which were 21.8, 42.3, and 63.8 kPa for the 4, 6, and 8% samples, respectively. These Young's modulus values of the gelatin samples were determined by the force-deformation method using a metal rod 1 mm in diameter, which was pressed against the gelatin blocks, each 5 cm thick.

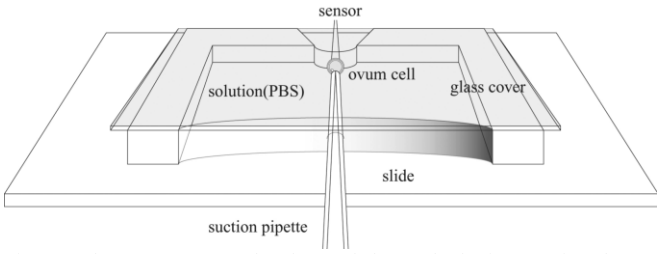

Fig. 3. The measurement chamber and the method of measuring the ZP elasticity of oocytes/embryos. Under microscopic control, a suction pipette supported the oocyte/embryo in the same horizontal axis as the MTS.

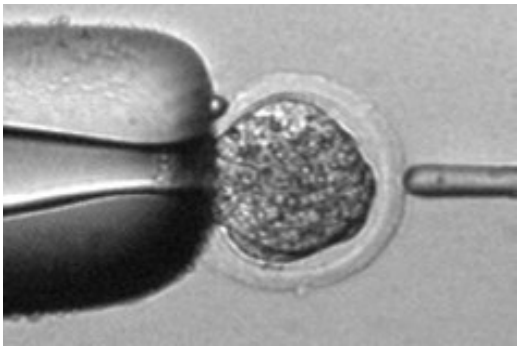

Fig. 4. Mouse morulae was pressed against the MTS with 5 μm indentation.

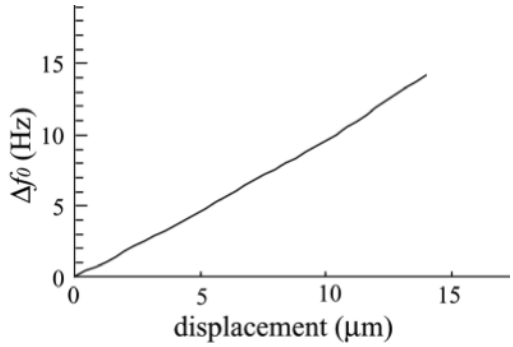

Fig. 5. Δf_0 response of the MTS when applied to the mouse ovum.

C. Zona dissolution assay

Oocytes/embryos were transferred to a drop of medium of 0.25% α-chymotrypsin (C-7762, Sigma, St Louis, MO, USA) in PBS under oil at room temperature. Each group of oocytes/embryos were continuously observed until an end point when the ZP was considered lysed as it completely disappeared.

III. Results

A. Elasticity measurements of zona pellucida

The elasticity of the ZP was measured in 13 immature GV oocytes, 15 matured MII oocytes and 4 fertilized PN embryos in vitro. The average values of Young's modulus at each stage are reported in Table.1. Young's modulus of the ZP of matured MII oocyte was significantly lower than that of the immature GV oocyte ($P < 0.01$). On the other hand, Young's modulus of the ZP of fertilized PN oocyte was significantly higher than that of the matured MII oocyte ($P < 0.01$).

TABLE 1. Young's modulus of ZP at immature GV, mature MII and fertilized PN stages.

Stage	Number	Young's modulus (kPa) (mean±S.D.)
Immature GV	13	25.42 ± 8.68
Mature MII	15	8.39 ± 3.81
Fertilized PN	4	17.51 ± 3.15

B. Dissolution assay of zona pellucida

The dissolution time was measured in 6 GV oocytes, 5 MII oocytes and 6 fertilized PN embryos. The average values of dissolution time at each stage are reported in Table.2. Dissolution time of matured MII oocyte was significantly lower than that of the immature GV oocyte ($P < 0.01$). On the other hand, dissolution time of fertilized PN oocyte was significantly higher than that of the matured MII oocyte ($P < 0.01$).

TABLE 2. Dissolution time of oocyte/embryo at immature GV, mature MII and fertilized PN stages.

Stage	Number	Dissolution time (min.)
Immature GV	6	326 ± 24
Mature MII	5	4.2 ± 1.7
Fertilized PN	6	447 ± 31

IV. Discussions

The experimental results clearly demonstrated that the mouse ZP mechanically and biochemically softened following oocyte maturation, in the same manner with the term ZP hardening means both mechanical hardening and increase resistance to dissolution. Furthermore, it was also shown that the mouse ZP hardened following fertilization as

several studies have already demonstrated. It has been considered that a release of cortical granules (cortical reaction) induces zona reaction followed by structure alteration resistance to dissolution by various chemical agents and the ZP mechanical hardening. However, on the other hand, mechanism of ZP softening is totally unknown. Our results showed that the ZP softened as it matured by approximately 3 times and it hardened following fertilization by approximately 2 times, whereas the dissolution time decreased by 78 times and increased by 106 times, respectively. Put simply, larger elasticity change was shown following maturation whereas larger dissolution time changed was shown following fertilization. This result may indicate different mechanisms for ZP hardening and softening.

Recent advances in biomedical engineering have made it possible to manipulate cells or tissues *in vitro* for therapy, and have therefore provided great benefits in human assisted reproductive technology (humanART), such as *in vitro* fertilization (IVF). The goal of IVF treatment in humans is to achieve a viable singleton pregnancy followed by vaginal delivery of a healthy child. However, it is still difficult to maintain the natural quality of the oocyte/embryo *in vitro* and so the IVF success rate is still quite low. There is also general agreement that twin pregnancy is the most severe complication of IVF resulting in considerable medical risks for both mother and infants, as well as increased obstetric and neonatal costs. Thus, the importance and effectiveness of elective single-embryo transfer (eSET) with top-quality embryos are widely accepted means to reduce the number of multiple births for patients undergoing humanART. However, decreasing the number of transferred embryos can reduce the chance of achieving pregnancy. Therefore, it is necessary to develop an appropriate culture device, culture medium, smart cell manipulation system, and a method to evaluate the quality of embryos to select one for eSET to improve the maintenance of cell quality *in vitro* and thus achieve higher IVF success rate. To do this, it is of paramount importance to develop a combination of improved selection criteria and improved culture conditions to optimize selection of the embryo with the highest implantation potential. Several studies suggested different types of criteria for the selection of embryos with the highest implantation potential. Several studies have shown that the amount of the change in the elasticity of ZP following fertilization can be one of the indicators to assess the quality of embryos. In the same way, our preliminary results may indicate that, before fertilization, ZP softening test can be another criterion to investigate oocyte quality for the selection of top quality mature oocyte for IVF treatment.

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