Estimating Young's modulus of zona pellucida by micropipette aspiration in combination with theoretical models of ovum

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The zona pellucida (ZP) is the spherical layer that surrounds the mammalian oocyte. The physical hardness of this layer plays a crucial role in fertilization and is largely unknown because of the lack of appropriate measuring and modelling methods. The aim of this study is to measure the biomechanical properties of the ZP of human/mouse ovum and to test the hypothesis that Young's modulus of the ZP varies with fertilization. Young's moduli of ZP are determined before and after fertilization by using the micropipette aspiration technique, coupled with theoretical models of the oocyte as an elastic incompressible half-space (half-space model), an elastic compressible bilayer (layered model) or an elastic compressible shell (shell model). Comparison of the models shows that incorporation of the layered geometry of the ovum and the compressibility of the ZP in the layered and shell models may provide a means of more accurately characterizing ZP elasticity. Evaluation of results shows that although the results of the models are different, all confirm that the hardening of ZP will increase following fertilization. As can be seen, different choices of models and experimental parameters can affect the interpretation of experimental data and lead to differing mechanical properties.

Keywords: ovum; zona pellucida; micropipette aspiration; Young's modulus

1. INTRODUCTION

The mechanical properties of living cells play an important role in helping to understand cell physiology and pathology. As such, a quantitative study in single-cell mechanics needs to be conducted. In this context some mechanical models have been developed to quantitatively evaluate the mechanical properties and responses of living cells when subjected to stimulation and/or perturbation (Lim *et al.* 2006). Recently, considerable biomedical attention has concentrated on determination of the mechanical properties of oocytes as a single cell. Numerous studies concerning the mechanical properties of oocytes have been reported. These studies show that the variation of the oocyte stiffness is strongly influenced by mechanical characteristics of the biomembranes that isolate these structures from their immediate

surroundings (Mitchison & Swann 1955; Hiramoto 1967, 1976; Yoneda & Dan 1972; Nakamura & Hiramoto 1978; Ohtsubo & Hiramoto 1985; Yoshida *et al.* 2000). According to previous reports on various animal species, fertilization of an oocyte leads to a change in the mechanical behaviour of the cell's extracellular biomembrane (Sun *et al.* 2003; Murayama *et al.* 2004, 2006, 2008). Zona pellucida (ZP) is the extracellular layer of glycoprotein enveloping an oocyte. This layer is modified after fertilization in a process described as zona reaction (Braden *et al.* 1954; Wassarman *et al.* 2001; Zhao & Dean 2002). Immediately after a sperm triggers an oocyte, a cascade of calcium (Ca^{2+}) floods the cell and the contents of cortical granules (CGs), a kind of special organelle in the egg, are released into the perivitelline space (PVS; cortical reaction), causing the ZP to become refractory to sperm binding and penetration (zona reaction; Okada *et al.* 1993; Tahara *et al.* 1996). The CG exudates cause zona sperm receptor

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modification and zona hardening thus blocking polyspermic penetration (Ducibella *et al.* 1993; Abbott & Ducibella 2001; Sun 2003). In zona reaction, increased resistance to dissolution by various biochemical agents has been evaluated in many species (Hatanaka *et al.* 1992; Iwamoto *et al.* 1999). This phenomenon has been termed *zona hardening* (Schmell *et al.* 1983). However, although the term zona hardening implies a mechanical change, to date the physical hardness of ZP has been examined with few techniques and is largely unknown.

In general, to measure the mechanical properties of a living cell, if the cell can be deformed in some way with a known force then its deformation can be measured. Until now several techniques have been developed to directly quantify the mechanical properties of single cells (Van Vliet *et al.* 2003; Lim *et al.* 2006). Besides various measurement systems, we have used the micropipette aspiration technique to quantify the ovum's mechanical properties. The micropipette aspiration technique in particular provides a versatile method for determining cellular properties such as Young's modulus (hardness) and viscosity (Evans & Yeung 1973, 1989; Hochmuth 2000), but has not previously been applied to the study of egg cells. With this technique, real-time measurements of pressure and deformation can be made, and in conjunction with theoretical models, can be used to determine the intrinsic mechanical and volumetric properties of a single cell. Here we have applied the micropipette aspiration technique to quantify the elasticity of the ZP layer.

In recent years, human *in vitro* fertilization (IVF) has become an important issue and better understanding of human oocyte behaviour in different physico-chemical environments is of major interest. As the penetration of the ZP shell by spermatozoa plays a crucial role in mammalian fertilization, hence any inability of spermatozoa to penetrate the ZP inevitably leads to infertility. IVF is the assisted therapeutic technique offered to patients suffering from reproduction difficulties. Therefore, it is inferred that to achieve improved IVF success rates, correctly estimating ZP elasticity may play a key role in optimizing embryo selection with the highest quality embryos as a selection criterion.

The objective of this paper is to study the mechanical properties of animal (mouse) and human ZP during fertilization. To determine Young's modulus of the ZP before and after fertilization, data from testing the ovum in a micropipette aspiration experiment have been evaluated with three different analytical elastic models of the ovum: half-space model, layered model and shell model. More specifically the study focuses on the mechanical change on the hardening of the ZP and mechanical properties of the ZP have been compared using the three models.

2. MATERIAL AND METHODS

2.1. Preparation of human oocytes/embryos

The embryos were donated by infertile couples who did not wish to cryopreserve surplus embryos for future replacement. Female partners were first superovulated following downregulation, as described elsewhere (Porter *et al.* 1984). In brief, suppression of pituitary gonadotrophin secretion with the gonadotrophin-releasing hormone agonist (GnRHa) buserelin acetate (Superfact, Hoechst AG, Germany) at a dose of $500\,\mu g\,d^{-1}$ by nasal spray was commenced in mid-luteal phase of the preceding ovarian cycle (day 21). Once ovarian suppression was confirmed (serum oestradiol $\leq 50\,pg\,ml^{-1}$, $LH \leq 5\,IU\,l^{-1}$), ovarian stimulation was initiated using a subcutaneous injection of $150\,IU\,d^{-1}$ purified human menopausal gonadotrophin (HMG; Pergonal 500, Serono, Italy).

The dose was increased in tandem with ovarian follicular development as monitored by serial vaginal ultrasonography. When at least three follicles reached 18 mm in diameter, GnRHa and HMG were discontinued, and 10 000 IU of human chorionic gonadotrophin (HCG; Organon, The Netherlands) was administered. Oocyte retrieval was performed by ultrasound-guided follicle aspiration, 36–38 h after HCG administration. To remove cumuluse mass around the oocyte, the obtained cumulus–oocyte complexes were first denuded using Synvitro Cumulase (MediCult, Denmark) and gentle pipetting. Synvitro Cumulase is recombinant human hyaluronidase ($80\,U\,ml^{-1}$) in an animal produce-free mammalian expression system and allowed purification of the human glycoprotein to homogeneity with a purity 100-fold greater than the commonly used preparations. Synvitro Cumulase is therefore the safest and has no side effects on the oocyte with its ZP (MediCult, Product Catalogue 2008). The denuded oocytes underwent intracytoplasmic sperm injection (ICSI) and were cultured in G-1 v.3 (Vitrolife, Kungsbacka, Sweden) supplemented with 10 per cent recombinant human serum albumin (rHA; Vitrolife) for 24 h.

2.2. Retrieval of mouse oocytes/embryos

Six-to-eight-week-old female Naval Medical Research Institute (NMRI) mice were provided from Pasture Institute (Tehran, Iran). The mice were housed in an environmentally controlled room. Female mice were induced to superovulate by intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotropin (PMSG; Intervet, The Netherlands) followed by 7.5 IU HCG (Intervet, The Netherlands) 48 h later. Oocytes were collected from oviducts approximately 14–16 h after HCG injection (48 h after the PMSG). The oocytes were treated with Synvitro Cumulase (MediCult) until the cumulus masses dissociated from the oocytes by gentle pipetting. They were rinsed and incubated at 37°C in T6 medium under 5 per cent CO_2 in air before analysis. Some superovulated female mice were mated with males from the same strain and inspected for the presence of a vaginal plug the following day. Those with vaginal plugs were considered pregnant and were sacrificed 44–48 h post-HCG by cervical dislocation. Embryos were flushed from the oviduct with T6 medium supplemented with $4\,mg\,ml^{-1}$ bovine serum albumin (BSA: Fraction V, Sigma). Morphologically normal embryos were washed and pooled in fresh G-1 v.3 (Vitrolife) with 10 per cent rHA (Vitrolife).
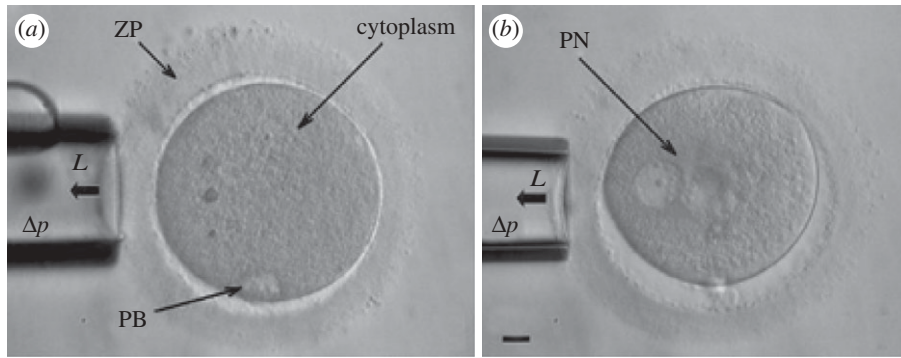
Figure 1. Micropipette aspiration of (*a*) human oocyte at MII stage and (*b*) human embryo at pronuclear (PN) stage. ZP, zona pellucida; PB, polar body; PN, pronuclei; *L*, aspiration length; Δp, applied negative pressure. Scale bar: 10 μm.

2.3. Instruments

The experimental system consisted of an inverted microscope (TE300, Nikon, Tokyo, Japan) equipped with Hoffman modulation optics (Nikon), a Micromanipulator 5173 (Eppendorf AG, Germany) for positioning the micropipette, a manual stage for positioning samples, a Transjector 5246 (Eppendorf AG) for generating well-controlled negative pressures, and a host computer for cell visual tracking and pressure data acquisition. The measurement resolution of the pressure system was 10 Pa. Sterilized glass micropipettes (borosilicate, B 100-75-10; Sutter Instrument Co., Novato, CA, USA) were pulled with heating (setting: ramp value, 500; heat = 750, pull = 55, vel. = 55, time = 200) using a micropipette puller (model P-97, Sutter Instrument Co.). The micropipette tip was cut on a microforge to obtain sharp edges. The system setup was mounted on a vibration isolation table for minimizing vibration.

2.4. Method of micropipette aspiration

The ovum was brought into focus. The movement of the pipette was controlled using a micromanipulator, which allowed positioning of the pipette tip in the appropriate microscopic field. The ovum was held using a holding capillary and then was turned with the aid of a transfer capillary until the polar body (PB) came to rest either above (12.00) or below (06.00). Then the capillary and ovum were sharply focused. When the suction pressure in the pipette was generated, the surface of the ZP was aspirated into a glass pipette by using a controlled suction pressure and the resulting deformation was measured using video microscopy (figure 1). The test was carried out by applying a series of step increases in pressure (200 Pa) up to 2 kPa to the ZP and the ovum. A rest time of 30 s was considered in each step to allow for time-dependent recovery and cell equilibration. The length of the ZP projection inside the micropipette (*L*) was recorded at the end of each pressure interval (Δp).

2.5. Equilibrium models

The micropipette aspiration (MA) technique can be used to quantify the elastic modulus of cells by pulling zona with a micropipette: when a pressure difference is applied, the membrane is put under tension and sucked inside the pipette (Evans & Yeung 1973, 1989; Hochmuth 2000). Here we applied the MA technique to quantify the elasticity of the ZP layer surrounding mammalian egg cells. Two different MA continuum models were used for characterizing the mechanical properties of solid-like (e.g. chondrocytes and endothelial cells) and liquid-like (e.g. neutrophils) cells (Needham & Hochmuth 1990; Ting-Beall *et al.* 1993; Discher *et al.* 1998; Jones *et al.* 1999). Nevertheless, the use of previous theories for determination of the ovum's mechanical properties suffers from significant limitations, especially regarding the treatment of sample layered condition which may bias the estimation of the sample stiffness in MA experiments.

To determine Young's modulus of the ZP, experimental length–pressure data were analysed using three different theoretical models of the MA technique: a half-space model (Theret *et al.* 1988; Sato *et al.* 1990; Aoki *et al.* 1997; Haider & Guilak 2002), a layered half-space model and a shell model (Alexopoulos *et al.* 2003, 2005; figure 2). The half-space model, referred to as the punch model derived from Theret *et al.* (1988), assumes that the sample is an isotropic, incompressible, elastic half-space medium. With this model, Young's modulus of the ZP is given by

$$E_{ZP} = \varphi(\eta)\frac{3r_i}{2\pi}\left(\frac{\Delta p}{L}\right) \quad \text{and} \quad \eta = \frac{(r_o - r_i)}{r_i}, \quad (2.1)$$

where $\varphi(\eta)$ is defined as the wall function and is set equal to $\varphi = 2.1$; r_i is the inner micropipette radius and $(\Delta p/L)$ can be determined from the slope of the applied pressure versus the equilibrium length of ZP projection (figure 3).

To determine the influence of the layered structure of the ovum, it is represented by a compressible, homogeneous, elastic layer with a finite thickness *h* (representing the ZP thickness) overlaying an isotropic homogeneous elastic half-space which represents the cytoplasm. The primary advantage of this layered model is that it accounts for the thickness of the ZP and different mechanical properties in two distinct regions of the ovum. Young's modulus is again determined from the slope of the applied negative pressure (Δp) versus the normalized equilibrium length (L/r_i) of ZP projection (figure 3), using the following
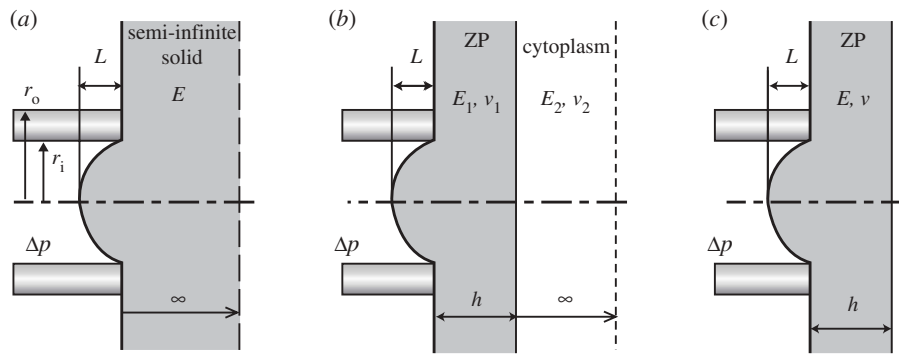
Figure 2. Mathematical models used for the quantification of mechanical properties of the ZP. (*a*) The half-space model assumes that the sample is an isotropic, incompressible, elastic (*E*) half-space medium. (*b*) The layered model represents the sample as a compressible (ν_1), homogeneous, elastic layer (E_1) with a finite thickness *h* (representing the ZP thickness) overlaying an isotropic homogeneous elastic half-space representing the cytoplasm (E_2 and ν_2). (*c*) In the final case, the ovum is modelled as a compressible (ν), layered half-space but under the assumption of zero stiffness for the cytoplasm (i.e. when $E_{ZP}/E_{core} \to \infty$), resulting in a model of the ovum as an elastic (*E*) shell of thickness *h*. *L*, aspiration length; Δp, applied negative pressure; *h*, thickness of the ZP; r_i, inner radius of micropipette; r_o, outer radius of micropipette.
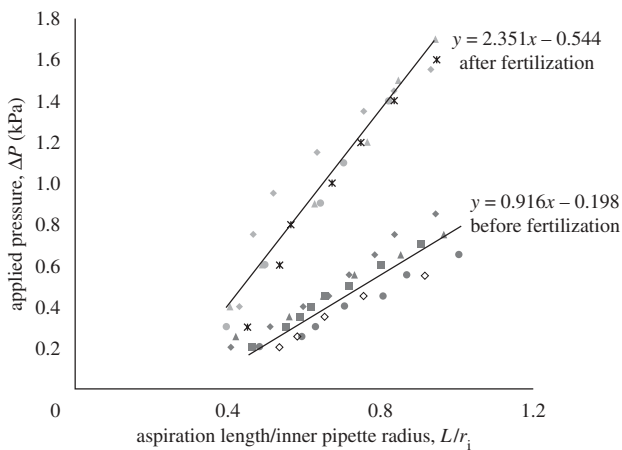

Figure 3. Typical equilibrium response of the human ZP subjected to a series of step increases in aspiration pressure (Δp). Young's modulus of the ZP is determined from the slope of the length versus pressure curve, as described in the methods section. Each symbol represents an individual ovum for first donor ($n = 5$ cells). The second embryo was degenerated one day after injection then excluded from the study ($p < 0.05$). First donor, dark filled diamond, oocyte 1; dark filled square, oocyte 2; dark filled triangle, oocyte 3; dark filled circle, oocyte 4; open diamond, oocyte 5; light filled diamond, embryo 1; light filled triangle, embryo 3; light filled circle, embryo 4; asterisk, embryo 5.
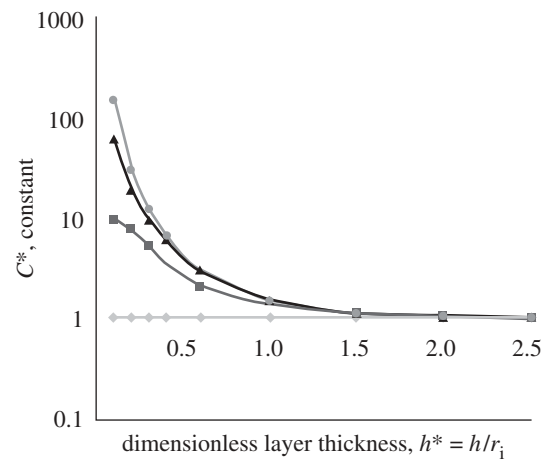

Figure 4. Effect of the layer thickness *h** and Young's moduli ratio E_{ZP}/E_{core} in the calculation of the constant *C** (filled diamond, 1 half space; filled square, 10; filled triangle, 100; filled circle, ∞).

equation:

$$E_{ZP} = 2C(1 + \nu_{ZP})\left(\frac{\Delta p}{L/r_i}\right) \qquad (2.2)$$

or

$$E_{ZP} = C^*\left(\frac{\Delta p}{L/r_i}\right), \qquad (2.3)$$

where $C^* = 2C(1 + \nu_{ZP})$. In figure 4, the effect of the layer thickness *h** and Young's moduli ratio E_{ZP}/E_{core}, in the calculation of the constant *C** for the Young's modulus of the ZP (equation (2.3)) by

assuming incompressibility ($\nu_1 = \nu_2 = 0.5$), are evaluated. If $E_{ZP}/E_{core} = 1$ or $h^* \to \infty$, we obtain the half-space solution ($C^* = 0.98$). The most important parameters for the calculation of the constant *C** are the dimensionless thickness of the layer $h^* = h/r_i$ and Young's moduli ratio E_{ZP}/E_{Cyt}. It is also known that approximately 97 per cent of the ovum is water; therefore, it can be assumed that $E_{ZP}/E_{core} \to \infty$, and thereby the layered model reduces to the aspiration of a shell (described as a shell model).

In the final case, the ovum is modelled as described above for a compressible, layered half-space but under the assumption of zero stiffness for the cytoplasm (i.e. when $E_{ZP}/E_{Cyt} \to \infty$), resulting in a model of the ovum as a shell of thickness h_{ZP}. For calculation of the E_{ZP}, it is assumed that the properties of the substrate (cytoplasm) are similar to those of pure water (Saul & Wagner 1989; Smith *et al.* 2005). In addition, the Poisson ratio of the ZP is set equal to that of pericellular matrix (PCM; $\nu_2 = 0.04$; Alexopoulos *et al.* 2003, 2005).

Young's modulus of the ZP (E_{ZP}) is given by the equation

$$E_{ZP} = 2C(h^*_{ZP})(1 - \nu_{ZP})(1 + \nu_{ZP})\frac{\Delta p}{L/r_i}. \qquad (2.4)$$

In this case, the constant C^* is only a function of the dimensionless thickness $h^* = h/r_i$ and can be approximated for $h^* > 0.1$ by the following equation (see Alexopoulos *et al.* 2003, 2005):

$$C(h^*) = \begin{cases} \dfrac{\begin{aligned}&a + c\ln(h^*) + e\ln^2(h^*) \\ &\quad + g\ln^3(h^*) + i\ln^4(h^*)\end{aligned}}{\begin{aligned}&1 + b\ln(h^*) + d\ln^2(h^*) + f\ln^3(h^*) \\ &\quad + h\ln^4(h^*) + j\ln^5(h^*)\end{aligned}}, & 0.1 \le h^* \le 50, \\[4pt] 0.64395655 & h^* \ge 50, \end{cases}$$

$a = 1.070275412$, $b = 0.592405186$, $c = -0.44373783$,

$d = 0.126723221$, $e = 0.721290633$, $f = 0.074985305$,

$g = -0.14390482$, $h = 0.027220129$, $i = 0.040156098$,

$j = 0.00132358$.

$$(2.5)$$

3. RESULTS

3.1. Human case studies

In this study, 15 surplus donated oocytes from three infertile couples were used. Oocytes did not exhibit major differences in shape or size based on the patients. Micropipette aspiration of the cells was carried out at metaphase-II (MII) and one day after insemination at the pronuclear (PN) stages. All ova exhibited linear deformation behaviour in response to a series of step increases in pressure (figure 3). The average value of r_i for all micropipettes employed was $40 \pm 5\,\mu\text{m}$, and the average value of h_{ZP} was $18.5 \pm 4.5\,\mu\text{m}$. Using the layered model, embryos from donors exhibited a higher Young's modulus ($E_{ZP} = 14.19 \pm 1.18\,\text{kPa}$) than the oocyte ($E_{ZP} = 7.47 \pm 1.29\,\text{kPa}$). Both the shell and layered models predicted similar Young's moduli. Using the shell model, ZP of embryos have a mean Young's modulus equal to $E_{ZP} = 13.18 \pm 1.17\,\text{kPa}$, which was again higher than Young's modulus of ZP of the oocytes, $E_{ZP} = 7.34 \pm 1.36\,\text{kPa}$. The half-space model predicted a mean Young's modulus lower than that of the shell and layered models; ZP of embryos exhibited a mean Young's modulus $E_{ZP} = 2.38 \pm 1.24\,\text{kPa}$, which was higher than the Young's modulus of ZP of oocytes, $E_{ZP} = 1.05 \pm 0.51\,\text{kPa}$ (figure 5).

3.2. Animal (mouse) case studies

Mouse zona elasticity was measured at the MII ($n = 22$) and PN ($n = 20$) stages. The average value of r_i for all micropipettes employed was $28 \pm 5\,\mu\text{m}$, and the average value of h_{ZP} was $7.5 \pm 1.3\,\mu\text{m}$. Using the shell model, Young's modulus of MII and PN stages was 11.8 ± 1.47 and $36.9 \pm 2.34\,\text{kPa}$, respectively. The ZPs of all eggs showed significant hardening in the PN stage. The calculated hardening rates (3.1-fold) were almost the same as those characterized in previous studies.
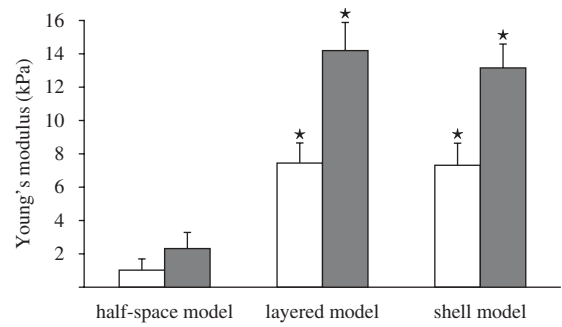

Figure 5. Young's modulus of the human ZP determined using the half-space model, the layered model and the shell model. Significant differences were found between the layered model and the half-space model (*$p < 0.05$ versus the half-space counterpart) as well as between the shell model and the half-space model (*$p < 0.05$ versus the half-space counterpart). Filled bar, embryo; unfilled bar, oocyte.

4. DISCUSSION

The objective of this study is to use an appropriate method to measure the mechanical properties of the ovum's extracellular envelope (ZP) and to determine whether these properties vary with fertilization. In this regard, we have used suitable mathematical models of micropipette aspiration to calculate the elastic properties of the ZP. Our findings support the hypothesis that there is a significant increase in Young's modulus of the ZP of the ovum after fertilization as compared to the non-fertilized ovum.

To calculate the mechanical properties of the ZP, we have used the available theoretical models of micropipette aspiration of an elastic layered structure, a half-space model, a layered half-space model and a shell model (Alexopoulos *et al.* 2003, 2005). The primary advantage of these models over previous models is that they account for different mechanical properties in two distinct regions of the ovum, a peripheral layer (ZP) and a substrate (cytoplasm). Young's modulus of the human embryo ZP at the PN stage (from the layered model $E_{ZP} = 14.19 \pm 1.18\,\text{kPa}$ and from the shell model $E_{ZP} = 13.18 \pm 1.17\,\text{kPa}$) is found to be approximately 1.8-fold higher than that of Young's modulus of the oocyte ZP at MII stage (from the layered model $E_{ZP} = 7.47 \pm 1.29\,\text{kPa}$ and from the shell model $E_{ZP} = 7.34 \pm 1.36\,\text{kPa}$). However, both the layered model and the shell model yield similar results. The important consideration in theoretical modelling of micropipette aspiration of the ovum is the incorporation of the ovum's layered geometry and compressibility of the ZP in the model. Significant differences have been found in the apparent modulus of the human ZP using different models, and the study suggests that neglecting ZP thickness and compressibility in an incompressible, elastic half-space model (PN: $E_{ZP} = 2.38 \pm 1.24\,\text{kPa}$ versus MII: $E_{ZP} = 1.05 \pm 0.51\,\text{kPa}$) may lead to underestimation of the elastic modulus of the ZP by over 50 per cent. However, both the layered model and the shell model yielded close results. This study used human ovum ZP from different couples ($n = 15$ oocytes from the donors) but the ova were similar in terms of shape and size. Although the

Table 1. Comparison of hardening rates of mouse ovum ZP studied by different techniques.

cell types	technique	data	hardening rates	source
mouse oocyte	capillary suction apparatus	$L_{\mathrm{p}}/\Delta p = 41$ (μm kPa^{-1})	1.8	Drobnis *et al.* (1988)
mouse embryo		$L_{\mathrm{p}}/\Delta p = 23$ (μm kPa^{-1})		
mouse oocyte	microinjection	$E = 17.9$ (kPa)	2.4	Sun *et al.* (2003)
mouse embryo		$E = 42.2$ (kPa)		
mouse oocyte	microtactile sensor	$E = 8.26$ (kPa)	2.7	Murayama *et al.* (2006)
mouse embryo		$E = 22.3$ (kPa)		
mouse oocyte	micropipette aspiration	$E = 11.8$ (kPa)	3.1	current study
mouse embryo		$E = 36.9$ (kPa)		

mechanical properties of the ZP might be influenced by genetics, age or other parameters (De Felice & Siracusa 1982; Cohen *et al.* 1990; Schiewe *et al.* 1995; Rankin & Dean 1996; Stanger *et al.* 2001), the study did not consider those parameters and left them for a future study.

To validate the animal (mouse) results, we compared our findings with previous attempts and observed a close agreement, as shown in table 1. In the first attempt, a quartz fibre method was used to measure the zona hardness of hamster ova by Green (1987, 1997), which concluded that sperm cannot penetrate the ZP only by force. The second attempt to measure zona hardness was made by Drobnis *et al.* (1988), who developed a ZP capillary suction apparatus. Hamster and mouse oocyte/embryos were deformed by decreasing pressure in a suction pipette and zona hardness was estimated from the zona deformation. The authors concluded that the ZP of mouse embryos was 1.8-fold harder than that of oocytes (Drobnis *et al.* 1988). The next attempt to measure zona hardness was made by Sun *et al.* (2003) with the benefit of recent developments in microelectromechanical system (MEMS) technology. Sun *et al.* (2003) developed a MEMS-based force sensor to measure forces applied to cells and concluded that mouse embryos were 2.4-fold harder than the oocytes. Although the previous two studies brought great advances in understanding zona hardening, the theories used improper models for the oocytes/embryos (the bilayer membrane was considered to be coupled with the ZP). Thus, in such a case, it was not theoretically suitable to calculate the elastic modulus of the ZP because the results should also relate to, for example: aspiration pressure, zona thickness and the entire cell size. More recently, Murayama *et al.* (2004, 2006, 2008) developed a noticeably less invasive tip, a microtactile sensor, to measure mouse zona hardness and concluded that the mouse embryos were 2.7-fold harder than oocytes.

In this study, in order to determine the equilibrium elastic modulus of the mouse ZP, we used a theoretical model, the shell model. Young's modulus of the mouse embryo ZP ($E_{\mathrm{ZP}} = 36.9 \pm 2.34$ kPa) was found to be approximately 3.1-fold higher than that of the oocyte ZP ($E_{\mathrm{ZP}} = 11.8 \pm 1.47$ kPa). The observed discrepancy between results of different studies could be attributed to the differences in test techniques, the type and extent of deformation, the time that the deformation was applied and respective theoretical models.

After fertilization of mouse, hamster and human oocytes, CG exudates accumulate in the PVS and form a new coat, termed the CG envelope, around the fertilized eggs (Okada *et al.* 1993; Tahara *et al.* 1996). This envelope is possibly related to blocking polyspermic penetration at the level of the PVS or the oolemma by hindering gamete interaction or modifying an incoming sperm (Dandekar *et al.* 1995). CG release is dependent on Ca^{2+} rise and calcium-dependent proteins. A relatively low Ca^{2+} rise is sufficient to induce a partial cortical reaction, while a higher level of Ca^{2+} is required to complete the cortical reaction (Raz *et al.* 1998). Murayama *et al.* (2008) have examined the correlation between mechanical ZP hardening and the amount of CG release in mouse eggs. The results indicate that the ZP in the PN stage significantly hardened following natural fertilization as was shown in our results, and the partial CG release after partially activating (partial activation) slightly hardened the ZP. However, mechanical ZP hardening has been shown to be dependent on the amount of CG released into the PVS and oocyte activation (maturation, fertilization) may be evaluated by changes in ZP elasticity. Consequently, these findings may suggest that Young's modulus of the ZP can be applied in human assisted reproductive technology (ART) to embryo selection with the highest hatching and implantation potential as a selection criterion.

5. CONCLUSION

The outcome of this study may be used for better understanding of the biomechanical role of the ZP in the ovum. The study provides a method to examine mechanical changes on the ovum ZP during fertilization. Also the above approach demonstrates a step towards a more accurate estimation of the layered sample stiffness by using appropriate models. It is hoped that obtaining more realistic models will provide a good starting point for working towards a better understanding of ovum mechanics.

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