

Mechanics of mouse blastocyst hatching revealed by a hydrogel-based microdeformation assay

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Mammalian embryos are surrounded by an acellular shell, the zona pellucida. Hatching out of the zona is crucial for implantation and continued development of the embryo. Clinically, problems in hatching can contribute to failure in assisted reproductive intervention. Although hatching is fundamentally a mechanical process, due to limitations in methodology most studies focus on its biochemical properties. To understand the role of mechanical forces in hatching, we developed a hydrogel deformation-based method and analytical approach for measuring pressure in cyst-like tissues. Using this approach, we found that, in cultured blastocysts, pressure increased linearly, with intermittent falls. Inhibition of Na/K-ATPase led to a dosage-dependent reduction in blastocyst cavity pressure, consistent with its requirement for cavity formation. Reducing blastocyst pressure reduced the probability of hatching, highlighting the importance of mechanical forces in hatching. These measurements allowed us to infer details of microphysiology such as osmolarity, ion and water transport kinetics across the trophoblast, and zona stiffness, allowing us to model the embryo as a thin-shell pressure vessel. We applied this technique to test whether cryopreservation, a process commonly used in assisted reproductive technology (ART), leads to alteration of the embryo and found that thawed embryos generated significantly lower pressure than fresh embryos, a previously unknown effect of cryopreservation. We show that reduced pressure is linked to delayed hatching. Our approach can be used to optimize in vitro fertilization (IVF) using precise measurement of embryo microphysiology. It is also applicable to other biological systems involving cavity formation, providing an approach for measuring forces in diverse contexts.

mammalian embryo hatching | embryo cryopreservation | pressure measurement | hydrogel deformation | mouse blastocyst

Blastocyst formation underpins the foundation of early mammalian development, occurring before embryo implantation into the uterus. During this process, the embryo creates a pressurized cavity within it and eventually breaks out of the protective acellular outer layer, the zona pellucida. Without hatching from the zona pellucida, embryos are unable to implant into the uterus, which therefore represents a stumbling block for in vitro fertilization (IVF) technologies (1). In addition to driving molecular changes to the trophoblast cells and the uterus (2), the timing of embryo hatching is also important, with early hatching resulting in ectopic pregnancies (3) and late hatching causing the window of receptivity to be missed (4). To date, the process has been difficult to study from the mechanobiology perspective due to the lack of suitable tools for imposing and characterizing deformations in 3D tissues. Moreover, embryo assessment is a crucial step during IVF procedures, particularly for evaluating the quality of cryopreserved embryos that have been thawed (5). However, existing methods rely on qualitative scoring, and a well-characterized quantitative methodology could provide more reliable predictive accuracy.

Previous studies have calculated tissue forces by directly observing the deformation of structures of known stiffness, for example by incorporating deformable oil droplets into developing tissues to

measure the anisotropic (shear) stress (6), or by culturing a layer of cells atop a 2D array of bendable hydrogel pillars (7) to measure traction. Atomic force microscopy has also been used to measure local mechanical properties of individual blastocyst cells (8) and the hydrogel shell (9–12). However, integrating the information into a systematic model has proven difficult due to a lack of embryonic force measurements. It is understood how embryonic tissue would respond to forces, but it is unknown what the forces are.

Mammalian preimplantation embryos have been intensely studied from the molecular biology perspective. These studies revealed much detail about the genetic regulation of cell fate decisions (13, 14) and the molecular mechanism of blastocyst cavity development. It has been shown that cavity expansion is driven by active ion transport (15), mediated by Na/K-ATPases in the outer epithelial cell layer sealed by tight junctions (16, 17). Increasing internal osmolarity then results in water transport along the osmotic gradient, which is mediated by aquaporin water channels (18, 19). These components act together to create the system responsible for internal pressurization of the cavity and eventual embryo hatching from its outer shell. The zona pellucida is composed of several glycoproteins, which form an elastic hydrogel layer around the embryo. The width of this layer decreases during embryo expansion as a precursor to hatching, partly in mechanical response to the tangential stretching imposed by the embryo's increasing volume,

Significance

Blastocyst hatching is crucial for implantation of mammalian embryos and a common failure point during in vitro fertilization (IVF). We have little knowledge of the mechanical basis whereby an embryo hatches out of the zona pellucida. We have developed a technique to measure blastocyst pressure, allowing us to quantify physiological parameters and providing additional measures of efficiency in IVF optimization. We find that mechanical stretching of the zona by the blastocyst is essential for efficient hatching. Cryopreservation and thawing of embryos is common during IVF. Our technique reveals significant differences in microphysiology between fresh and thawed embryos. Our experimental and associated mathematical techniques are also applicable to other biological systems involving cavity formation, providing an approach for measuring forces in diverse contexts.

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and partly due to the action of degrading enzymes (20–22). Although both mechanical and enzymatic factors have been suggested to be important, making a distinction between them has proven difficult due to the lack of experimental techniques.

Here, we therefore extend the idea of measuring mechanical forces using deformable media and develop an approach focused on confining embryos in hydrogels. This allows us to measure not only the pressure but also the dynamics of other important underlying processes, such as ion and water transport, and quantify the mechanical components of the hatching process. We use this approach to characterize the microphysiology of embryos and discover previously unknown blastocyst cavity pressure differences between freshly collected embryos and embryos thawed after cryopreservation.

Results and Discussion

Relating Hydrogel Deformation and Tissue Pressure. Developing E3.5 mouse embryos were embedded in microfabricated cylindrical cavities within hydrogels of known Young's modulus (E). Optical bright-field microscopy (Fig. 1A and Movies S1 and S2)

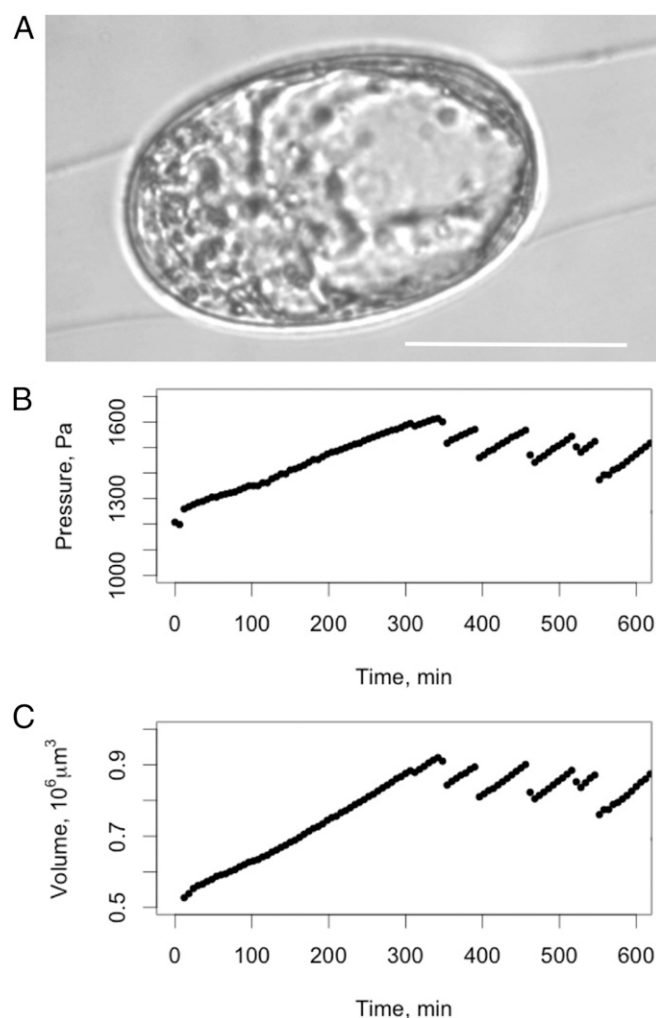


Fig. 1. Summary of embryo development in hydrogel cavities. Pressures in developing embryos were measured by using deformable hydrogels (Movies S1 and S2) (A). The embryos were cultured from early cavity stage ~3.5 d after fertilization in KSOMaa Evolve media of osmolarity of 0.252 Osm. (Scale bar: 50 μm .) The elastic hydrogel made it possible to calculate the pressure (B), which was generated during volumetric expansion (C). The time lapse was taken over a period of 2 h with a resolution of 6 min.

was then used to measure channel dilation by the embryo from a (undeformed) to $a + h$ (maximum dilated radius; SI Appendix, Fig. S14). A simple elastic model of the channel then allowed us to calculate the pressure exerted and hence the pressure P within the embryo as follows (Eq. 1: Calculating pressure exerted upon the hydrogel):

$$P = \frac{E}{6} \left(1 + 2 \ln(\tilde{R}) - \frac{1}{\tilde{R}^2} \right), \tilde{R} = \frac{a + h}{a}. \quad [1]$$

Our elastic model is exact for a channel dilated evenly from a to $a + h$ and remains a good approximation if h varies slowly along the channel. To confirm the validity of Eq. 1 in the experimental regime, we replaced embryos with oil droplets of known surface tension and hence known internal pressure, and confirmed that the model correctly reproduces droplet pressure (SI Appendix, Fig. S1B). Details of the elasticity calculation and droplet verification are in Materials and Methods.

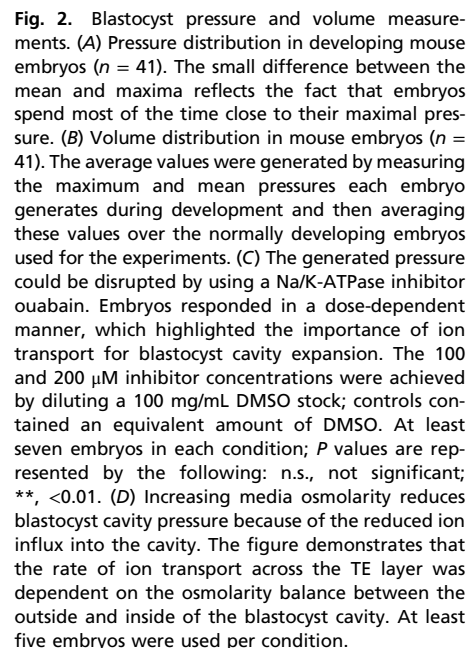
To test whether culturing embryos within the hydrogel impairs viability, we analyzed the number of inner cell mass and trophoblast (TE) cells, as well as the number of fragmented nuclei. Across various sets of experimental conditions, we found no significant differences between embryos cultured within hydrogels and control unconfined embryos, suggesting that our method was not harmful (SI Appendix, Fig. S1 C–E).

The Effects of Embryo Compression. Embryo compression itself is likely to generate some additional internal pressure based on elongating an embryo from the spherical shape. This requires the zona pellucida to stretch, which is an additional source of pressure. Therefore, internal embryo pressure is the sum of measured pressure acting on the hydrogel and the added pressure due to deformation from spherical to elongated shape. To estimate the pressure originating solely from the deformation, we consider that, on average, embryos increase pressure with increasing surface area at the rate of 0.03 Pa/ μm^2 . This can be estimated by tracking embryo area and pressure increases with time. It can also be estimated that compression results in surface area increase of less than 3,000 μm^2 , which indicates that internal pressure may be higher than measured by as much as 90 Pa. Since this is below the measurement error of 150 Pa, we can assume that pressure exerted on the hydrogel represents the internal embryo pressure. Also, the measurement error of 150 Pa made it impossible to measure zona pellucida free and hatched embryos, which could no longer significantly deform the hydrogel (SI Appendix, Fig. S2). Intact outer shell was required for generating a significant, measurable pressure.

Microphysiology of Blastocyst Cavity Development. To build a mathematical model for embryo hatching, it is crucial to look at the details of how all of the different components of the system create a pressurized cavity. By measuring blastocyst pressure and volume dynamics (Fig. 1 B and C), we could see that pressure and volume rises were linear over the course of development, but interrupted by sudden drops, which likely corresponded to TE cell divisions or epithelial cell junction cycling (SI Appendix, Fig. S3). Overall, developing mouse blastocyst-stage embryos generated pressures of around 1.4 kPa and had an average volume of $0.66 \times 10^6 \mu\text{m}^3$ (Fig. 2 A and B). These pressure and volume measurements allowed us to derive other important physiological parameters, such as blastocyst osmolarity using the van't Hoff (ideal gas) law (Eq. 2), where R is the ideal gas constant, V is cavity volume, and c_{media} is 0.252 Osm:

$$P = \Delta c RT = (c_{\text{cavity}} - c_{\text{media}}) RT. \quad [2]$$

This leads to the conclusion that typical pressurized embryo cavities have $\Delta c \sim 0.8$ mOsm of additional electrolytes. Furthermore, by



the osmolyte flux, or $3.3 \times 10^8/\text{min}$. Using confocal imaging, we have estimated experimental embryos to contain 60 cells on average, 42 of which belong to the TE layer (*SI Appendix, Fig. S1 C-E*), which would result in ion flux per cell of $7.8 \times 10^6/\text{min}$, estimating 1.7×10^3 active ion transporters per cell. In addition to estimating the average number of active Na/K-ATPases operating in TE cells, we also looked at how embryos responded to rapid changes in osmolarity, which revealed water transfer kinetics reaching rates of $400 \mu\text{m}^3/\text{s}$, as 252 mOsm of sucrose was added to embryos developing in channels (*SI Appendix, Fig. S4 A and B*). Water flux during transient response is over 20 times the normal water transport rates seen during normal development (Table 1), suggesting that cavity expansion is limited and regulated by the rate of ion rather than water transport.

Mechanical Model of Zona Pellucida. Although liquid inflow and sealed epithelial layer are the prerequisites for cavity formation, pressure ultimately arises due to the constricting nature of the external polysaccharide shell zona pellucida. Since shell thickness can be measured by bright-field microscopy to be less than 1/10 of the embryo diameter, the blastocyst can be modeled as a thin-walled

Table 1. Summary of embryonic microphysiology

Parameter	Mean	SD
Mean pressure	1,400 Pa	300 Pa
Maximum pressure	1,500 Pa	300 Pa
Mean volume	$6.6 \times 10^5 \mu\text{m}^3$	$1.8 \times 10^5 \mu\text{m}^3$
Rate of volume increase	$22 \mu\text{m}^3$	$12 \mu\text{m}^3$
Osmolyte flux	$10.1 \times 10^6 \text{s}^{-1}$	$6.72 \times 10^6 \text{s}^{-1}$
Na-K ATPase density	$1.7 \times 10^3/\text{cell}$	$1.2 \times 10^3/\text{cell}$
Water permeability	$1.50 \mu\text{m}^3 \cdot \text{Pa}^{-1} \cdot \text{s}^{-1}$	$1.01 \mu\text{m}^3 \cdot \text{Pa}^{-1} \cdot \text{s}^{-1}$
Zona pellucida stiffness	31.0 kPa	15.9 kPa
Zona pellucida thickness	4.3 μm	1.4 μm
Zona pellucida breaking stress	5.4 kPa	4.5 kPa

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open the hydrogel channel before injecting the embryos and letting the channels relax and deform the embryos.

Imaging Setup and Image Analysis. Imaging was performed on a Zeiss Axiovert spinning-disk confocal microscope equipped with a Hamamatsu emCCD camera and a 37 °C and 5% CO₂ incubator. Embryos were cultured for periods of 2 h between 3.0 and 4.0 dpc stages, recording images every 6 min. Embryos were analyzed using a combination of manual and automatic image analysis approaches. Manual analysis was used to ensure no algorithmic bias, while the high-throughput analysis relied on computational methods. The main measured parameters were channel diameter and embryo width and length, which made it possible to calculate microphysiological embryo properties. Zona thickness was measured only manually, at either of the unconfined embryo ends. Blastocyst cavity volume was determined from the total embryo volume by subtracting a constant volume of the inner cell mass cells assumed to be $2.1 \times 10^{-5} \text{ } \mu\text{m}^3$. Average volumes and pressures were calculated by taking mean values of an embryo's time lapse, summarizing them, and taking means of the means (data seen in histograms in Fig. 2).

Thin-Walled Vessel Pressure Derivation. As is familiar to anyone who has inflated a party balloon, the inflation is difficult at first, but once the balloon is modestly inflated, it becomes much easier. This occurs because the pressure in the balloon first increases with inflation, and then, past a critical volume, the pressure in the balloon falls. This “balloon instability” ultimately has a geometric origin: As the balloon inflates, its walls become thinner and flatter and thus able to contain less pressure. We can understand this phenomenon (28) by considering a spherical rubber balloon, with natural radius R_0 and thickness t . If the balloon is inflated such that its radius dilates to $R = \lambda R_0$, then circumferences of the balloon dilate by λ , while to conserve volume of the rubber, the thickness of the balloon must fall to $t\lambda^2$. Therefore, the deformation gradient in the rubber will be as follows:

$$F = \begin{pmatrix} \lambda & 0 & 0 \\ 0 & \lambda & 0 \\ 0 & 0 & 1/\lambda^2 \end{pmatrix}, \quad [4]$$

and assuming the rubber is well described by a neo-Hookean energy, the total elastic energy will be as follows:

$$W_{\text{tot}} = \frac{E}{6} (Tr(F \cdot F^T) - 3) 4\pi R_0^2 t = \frac{E}{6} \left(2\lambda^2 + \frac{1}{\lambda^4} - 3 \right) 4\pi R_0^2 t. \quad [5]$$

Since in this inflation the volume of the balloon changes from its undeformed volume V_0 to $V = \lambda^3 V_0$, we can express this elastic energy as follows:

$$W_{\text{tot}} = \frac{E}{6} \left(2 \left(\frac{V}{V_0} \right)^{\frac{2}{3}} + \left(\frac{V}{V_0} \right)^{-\frac{4}{3}} - 3 \right) 4\pi R_0^2 t, \quad [6]$$

and we can calculate the pressure in the balloon as follows:

$$P = \frac{\partial W_{\text{tot}}}{\partial V} = \frac{E}{6} \left(\frac{4}{3} \left(\frac{V}{V_0} \right)^{-\frac{1}{3}} - \frac{4}{3} \left(\frac{V}{V_0} \right)^{-\frac{7}{3}} \right) \frac{4\pi R_0^2 t}{V_0}. \quad [7]$$

This pressure can be recast in terms of R and R_0 as follows:

$$P = \frac{\partial W_{\text{tot}}}{\partial V} = \frac{2Et}{3R_0} \left(\left(\frac{R}{R_0} \right)^{-1} - \left(\frac{R}{R_0} \right)^{-7} \right). \quad [8]$$

This form for the pressure does indeed rise from zero for modest inflations (R a little above R_0), but then reaches a maximum pressure when

$$\frac{\partial P}{\partial R} = \frac{2Et}{3R_0} \left(-\left(\frac{R}{R_0} \right)^{-2} + 7 \left(\frac{R}{R_0} \right)^{-8} \right) = 0, \quad [9]$$

which occurs when $R = 7^{1/6} R_0 \sim 1.38 R_0$, and the pressure falls with subsequent additional inflation. The form of pressure-inflation curve is shown in Fig. 3A, the essential point being that if the embryo can exert enough pressure to inflate the zona (balloon) radius by 38%, then it does not require additional pressure to expand the shell further until it breaks. The exact value of 38% is particular to the neo-Hookean rubber model, but calculations for other material models, and experiments with real balloons, indicate that the critical dilation reliably lies between $7^{1/6}$ and 1.5 (29). Also, for thin-walled cylindrical pressure vessels, the longitudinal stress can be expressed as a combination of pressure, wall thickness, and the radius of the spherical part: $\rho = Pr/2t$, which makes it possible to estimate the mean breaking stress of the zona pellucida.

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