Investigation of Zona Pellucida Hardening with Atomic Force Microscopy and Nonlinear Optimization

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ABSTRACT

The Zona Pellucida (ZP) is the extracellular coat surrounding mammalian oocytes. The so called "zona hardening" has a key role in the fertilization process as it produces a block of polyspermy also through an increase of the stiffness of the membrane. The full comprehension of the mechanisms involved in the structural reorganization of the ZP leading to mechanical hardening as well as a correct estimation of its elastic properties is still lacking. In this study, mechanical properties of the ZP membranes extracted from mature and fertilized bovine oocytes were investigated with Atomic Force Microscopy nanoindentation measurements. Both inner and outer sides of the fertilized oocyte's ZP were characterized in order to investigate the propagation of the zona hardening through the thickness of the membrane. This work proposes the application of a hybrid procedure combining experimental measurements, Finite Element analysis and optimization algorithms to analyze the indentation curves.

Keywords: Zona Pellucida, Atomic Force Microscopy, nanoindentation, hyperelasticity, non linear optimization.

1 INTRODUCTION

The Zona Pellucida (ZP) is the extracellular coat surrounding mammalian oocytes. Penetration of this membrane by spermatozoa plays a crucial role in mammalian fertilization, and any inability of spermatozoa to penetrate the ZP as well as the physiologic polispermy will inevitably lead to infertility [1]. ZP is mainly composed of sulfated glycoproteins assembled into long fibrils constituting a three-dimensional network [2]. Upon fertilization, in order to prevent subsequent spermatozoon from penetrating, the ZP undergoes a so called "hardening" which involves the inactivation of the sperm receptors on the ZP surface, an increased resistance of the ZP to proteolytic digestion and also an increase in the mechanical stiffness of the membrane [3,4]

In this study, mechanical properties of the ZP membrane extracted from mature and fertilized bovine oocytes were investigated with Atomic Force Microscopy (AFM) nanoindentation measurements. Both inner and outer sides of the ZP isolated from the fertilized oocyte were characterized in order to gain a deeper comprehension of the mechanisms involved in the structural re-organization of the ZP membrane leading to mechanical hardening. The full understanding of the "zona-hardening" mechanisms as well as a correct estimation of ZP elastic properties is of fundamental importance as alterations of ZP represent a potential cause of infertility.

This work proposes the application of a hybrid procedure combining experimental measurements, Finite Element analysis (FE) and optimization algorithms to analyze the indentation curves [5].

Traditional analysis of AFM indentation curves relies on an inappropriate application of the classical Hertz theory [6] with its hypotheses of linear elastic material properties, infinitesimal strains and infinite sample thickness and dimensions. None of these assumptions is likely to be valid when a biological membrane is indented with an AFM. Most biological materials exhibit non linear constitutive behavior, the AFM probe induces large deformations during the indentation process and the half-space assumption cannot be adapted to the thin biological membranes. The novel methodology presented in this study allows a more realistic description of the mechanical behavior of the ZP membrane and a more reliable derivation of its elastic properties. It is attempted to find the hyperelastic constitutive model which better describes the structural behavior of the ZP membrane as well as to analyze the changes in the distribution of material properties induced by the structural rearrangement of the membrane after fertilization.

2 MATERIALS AND METHODS

Oocytes collection, their maturation in vitro and isolation of Zona Pellucidae were performed according to the protocol reported in [3].

2.1 Atomic Force Microscopy

Mechanical measurements on ZP were carried out using an SPMagic SX AFM (Elbatech, Italy). Samples were maintained in an aqueous environment (Dulbecco's phosphate-buffered saline, PBS; Sigma, USA), and at a constant temperature of 37°C, throughout the measurement acquisition phase. The microscope probe consisted of an ultrasharp silicon nitride cantilever of calibrated force constant, with a tip radius of 10nm (MikroMash). The sample indentation as a function of the loading force applied by the tip was derived by means of an *ad hoc* MATLAB routine coded by the authors.

2.2 Finite Element Analysis

The AFM nanoindentation experiments conducted on the ZP membrane were simulated with the ABAQUS® Version 6.7 commercial finite element software [7]. For that purpose, an axisymmetric FE model was developed: the model includes a rigid blunt-conical indenter (tip radius of 10nm and half-open angle of 20°) pressing against a soft layer adherent on a rigid substrate. The Young's modulus of the silicon-nitride AFM tip is 300 GPa. The ZP membrane was modeled as an incompressible hyperelastic slab with diameter 60 µm and thickness 10 µm. Figure 1 shows the finite element model with the rigid indenter and the ZP membrane: the deformation field corresponding to 200nm indentation also is presented.

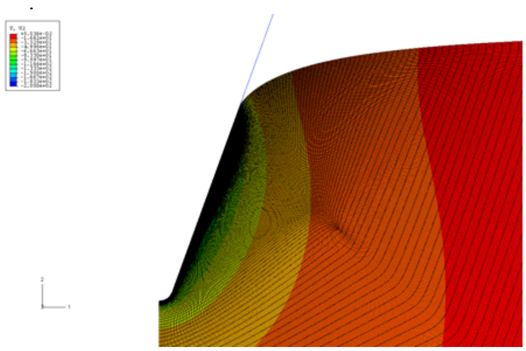

Figure 1: Finite element model simulating the nanoindentation process.

2.3 Hyperelastic constitutive models

Three different hyperelastic constitutive models were considered in this study in order to describe the structural behavior the ZP membrane in the mature oocyte and after the fertilization process: (i) Two-parameter Mooney-Rivlin (MR); (ii) Neo-Hookean (NH); (iii) Arruda-Boyce Eight-chain (8chain) model (AB).

The two-parameter MR constitutive law [8,9] is a very classical phenomenological model described by the following strain energy density function:

$$W = C_{10}\left(\overline{I}_1 - 3\right) + C_{01}\left(\overline{I}_2 - 3\right) \tag{1}$$

where C_{10} and C_{01} are the MR constants given in input to ABAQUS as material properties. Strain invariants are defined, respectively, as $\overline{I}_1$ =tr[C] and $\overline{I}_2$ ={tr^2[C]- tr[C]^2} where [C] is the Cauchy-Green strain tensor. The corresponding uniaxial stress (σ) – stretch (λ) equation can be derived as:

$$\sigma = 2C_{10}\left(\lambda - \frac{1}{\lambda^2}\right) + 2C_{01}\left(1 - \frac{1}{\lambda^3}\right) \tag{2}$$

The shear modulus μ_{MR} is defined as $\mu_{MR}=2(C_{10}+C_{01})$ while the Young modulus is equal to $E_{MR}=4(1+\nu)(C_{10}+C_{01})$

The NH model [10] was selected in this study because it is based on the statistical thermodynamics of cross-linked polymer chains. Although this model is not phenomenological, it can be however derived from the two-parameter MR model by setting $C_{01}=0$. Consequently, only one material parameter must be given in input to ABAQUS. The shear modulus μ_{NH} is defined as $\mu_{NH}=2C_{10}$ while the Young's modulus is equal to $E_{NH}=4(1+\nu)C_{10}$.

The AB model [11] relies on the statistical mechanics of a material with a cubic representative volume element containing eight chains along the diagonal directions. The strain hardening behavior of an incompressible material is described by using two constants: the shear modulus μ_{8chain} and the distensibility λ_L where the latter parameter corresponds to the limiting network stretch. The strain energy function can be expressed as:

$$W = \mu_{8chain}\left[\begin{array}{l} \frac{1}{2}\left(\overline{I}_1 - 3\right) + \frac{2}{20\lambda_L^2}\left(\overline{I}_1^{\,2} - 9\right) + \frac{33}{1050\lambda_L^4}\left(\overline{I}_1^{\,3} - 27\right) + \\ \frac{76}{7000\lambda_L^6}\left(\overline{I}_1^{\,4} - 81\right) + \frac{519}{673{,}750\lambda_L^8}\left(\overline{I}_1^{\,4} - 243\right) \end{array}\right] \tag{3}$$

The corresponding uniaxial stress (σ) – stretch (λ) equation is:

$$\sigma = 2\mu_{8chain}\left(\lambda^2 - \frac{1}{\lambda}\right)\left(\frac{1}{2} + \frac{2\overline{I}_1}{20\lambda_L^2} + \frac{33\overline{I}_1^{\,2}}{1050\lambda_L^4} + \frac{76\overline{I}_1^{\,3}}{7000\lambda_L^6} + \frac{519\overline{I}_1^{\,4}}{673{,}750\lambda_L^8}\right) \tag{4}$$

The Arruda-Boyce model is activated in ABAQUS by giving in input the values of μ_{8chain} and λ_L as material parameters. The Young's modulus is defined as $E_{8chain}=2(1+\nu)\,\mu_{8chain}$.

2.4 Non linear optimization

In order to extract more realistically the hyperelastic properties of the ZP membrane from the above described FE model which accounts for non-linearity of finite indentation process and material non-linearity, a hybrid procedure combining experimental measurements, FE analysis and nonlinear optimization was utilized. Displacement values measured experimentally are compared with the corresponding results of FE analysis. This leads to formulate an optimization problem including the unknown material properties as design variables. The optimization problem describing the inverse problem of material characterization can be stated as follows:

$$\begin{cases} \text{Min}\left[\Omega(X_1,X_2,\ldots,X_{NMP}) = \sqrt{\dfrac{1}{N_{CNT}}\displaystyle\sum_{j=1}^{N_{CNT}}\left(\dfrac{\delta_{FEM}{}^{j}-\overline{\delta^{j}}}{\overline{\delta^{j}}}\right)^{2}}\,\right] \\ X_1{}^{L} \le X_1 \le X_1{}^{U} \\ X_2{}^{L} \le X_2 \le X_2{}^{U} \\ \\ X_{NMP-1}{}^{L} \le X_{NMP-1} \le X_{NMP-1}{}^{U} \\ X_{NMP}{}^{L} \le X_{NMP} \le X_{NMP}{}^{U} \end{cases} \tag{5}$$

where Ω is error functional to be minimized, and the design vector $\mathbf{X}(X_1,X_2,\ldots,X_{NMP})$ includes the unknown number of material properties (NMP) to be determined that can vary between the lower and upper bounds.

In Eq. (5), δ^{j}_{FEM} and $\overline{\delta^{j}}$, respectively, are the displacement values for the j-th load step computed with FE analysis and those measured experimentally with AFM. The number of control locations N_{CNT} is equal to the number of load steps to complete nonlinear FE analysis. Nanoindentation values measured experimentally can be taken as target values in the identification problem because the execution of AFM measurements does not require any *a priori* knowledge of material properties. Conversely, the “correct material properties”, i.e. the actual material properties, must be given in input to the FE model to obtain the force-indentation curve that matches the F-δ curve determined experimentally. Theoretically, the error functional Ω computed at the optimum design (i.e. the target material properties) will be equal to 0. However, since target values are measured experimentally, there will be a residual deviation between the force-indentation curve reconstructed numerically and the actual F-δ curve measured with AFM.

The suitability of the optimization-based for mechanical characterization problems of nonlinear materials is well documented in literature [5].

The inverse problem (5) was solved with the Sequential Quadratic Programming (SQP) method, a gradient-based optimization algorithm that has the property of global convergence. The powerful SQP optimization routine implemented in the commercial general mathematics software MATLAB® Version 7.0 was utilized.

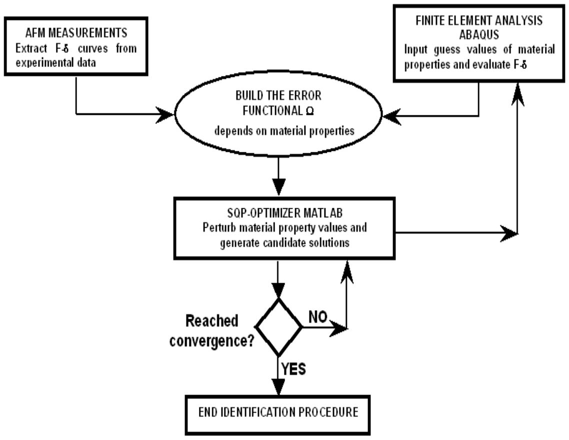

Figure 2: Flow-chart of the hybrid identification procedure

3 RESULTS AND DISCUSSION

Elastic properties of the ZP membranes isolated from mature and fertilized oocytes were evaluated by examining the indentation curves with the hybrid procedure described in the Material and Methods section. An indentation depth of 0-200nm was analyzed. Three different constitutive models for the membrane were considered (NH, MR, AB) and it was hypothesized a heterogeneous distribution of elastic properties of the ZP through its thickness. In particular, the membrane was divided into two layers of thicknesses respectively d_1 and d_2, and the Young modulus was supposed to change linearly inside both layers. In the hybrid characterization procedure, the material parameters optimized were the elastic constants and the layer thicknesses. d_1 and d_2 could vary across the entire thickness of the membrane with the unique constraint that $d_1 < d_2$.

The best fit of the experimental curves with the FE model was obtained with the AB model both for mature and fertilized ZP (outer and inner layer).

In Figure 3 it is shown the comparison between the force-indentation curves acquired experimentally on mature and fertilized ZP and those simulated numerically with the optimization algorithm for the AB constitutive law.

In the mature oocyte’s ZP it was found that the Young modulus varies almost linearly from E_o=6.46kPa to E_l=10.4kPa within a layer of total thickness d=200nm. In the outer side of the fertilized oocyte’s ZP the Young modulus increases linearly from E_o=6.43kPa to E_l=150.5 kPa within a layer of thickness d=200nm. It is present only a small deviation at 96.5nm with a local minimum (E=64.37kPa). For the inner side, it is observed a doubly-linear gradient of the Young modulus in the total indentation depth: E varies from E_o=6.4kPa to E_l=101.1kPa within a layer of thickness d_l=97.3nm and then increases up to E_2=532kPa in the subsequent layer of thickness d_2=101nm

It can be observed that the average value of Young modulus of the membrane increases considerably after fertilization: therefore, the expected mechanical hardening of the ZP actually occurs. The structural reorganization of the membrane after fertilization associated to the mechanical hardening is not described by a different constitutive law. Notably, the mature oocyte's ZP and the more superficial layers of the outer and inner sides of the fertilized oocyte's ZP have the same value of Young modulus (E~6.4kPa); then E increases across the depth range considered. Both in mature and fertilized ZP's outer layer the Young modulus increases almost linearly, but, in the fertilized case, the E gradient is 40 times steeper in the range examined. As a consequence the Young modulus heterogeneity estimated in the mature ZP can be considered negligible. In the inner layer of fertilized ZP it is observed a double gradient of the Young modulus where a dramatic change of the slope occurs at the distance of 100nm. Also in the outer layer of the fertilized ZP, it can be identified a local minimum of the Young modulus at a depth of ~100nm.

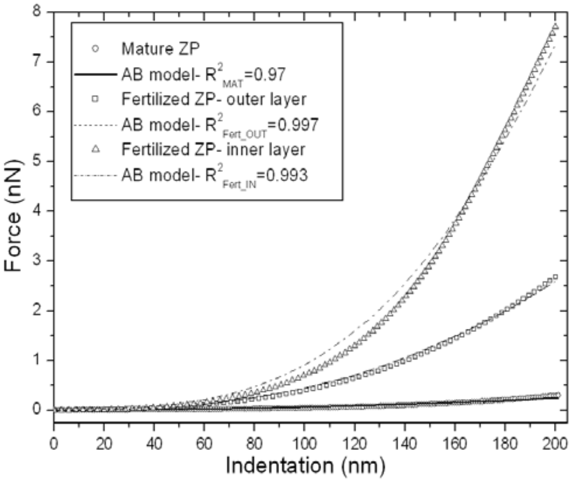

Figure 3: Force-indentation curves acquired experimentally on mature and fertilized ZP (symbol) and corresponding numerical curves obtained with the hybrid procedure considering the Arruda-Boyce constitutive model (line). R^2 values of the fit between experimental data ad FE model are indicated in the figure.

The findings of this study indicate that the structural reorganization of the membrane after fertilization involves a change from an almost homogeneous to a highly heterogeneous distribution of its elastic properties and a variation in stiffness of the fertilized oocyte's ZP occurs at a characteristic distance of 100nm.

Mechanical hardening of ZP after fertilization is attributed to an increase of the number of inter-filaments cross-links within the ZP and in mice the distance between two cross-link sites is estimated to be ~100nm [12].

Therefore, the characteristic distance of ~100nm found by FE analysis corresponding to the variation of the elastic properties may be correlated to the separation between two cross-link clusters. If we assume that during fertilization the number of cross-links increases, nanoindentation measurements should reveal the hardening of the membrane at a length correlated to the characteristic distance between two cross-link clusters. The higher gradient of Young modulus observed in the inner side of the fertilized oocyte's ZP with respect to the corresponding gradient found in the outer side can be consequent to the presence of a larger number of cross links per mass unity. This is in agreement with the hypothesis that the hardening of the membrane after fertilization should start in the inner side of the ZP and than extend toward the outer side.

4 CONCLUSIONS

In conclusion, mechanical properties of the ZP membrane isolated from mature and fertilized bovine oocytes were investigated in great detail with a hybrid procedure combining AFM nanoindentation measurements, finite element analysis and nonlinear optimization. The results obtained in this study confirm the hypothesis that the mechanical hardening of ZP after fertilization results from an increase of the number of inter-filaments cross-links within the ZP, whose density is expected to be higher in the inner side of the ZP.

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