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Deformation mechanisms of human amnion: Quantitative studies based on second harmonic generation microscopy

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ABSTRACT

Multiphoton microscopy has proven to be a versatile tool to analyze the three-dimensional microstructure of the fetal membrane and the mechanisms of deformation on the length scale of cells and the collagen network. In the present contribution, dedicated microscopic tools for in situ mechanical characterization of tissue under applied mechanical loads and the related methods for data interpretation are presented with emphasis on new stepwise monotonic uniaxial experiments. The resulting microscopic parameters are consistent with previous ones quantified for cyclic and relaxation tests, underlining the reliability of these techniques. The thickness reduction and the substantial alignment of collagen fiber bundles in the compact and fibroblast layer starting at very small loads are highlighted, which challenges the definition of a reference configuration in terms of a force threshold. The findings presented in this paper intend to inform the development of models towards a better understanding of fetal membrane deformation and failure, and thus of related problems in obstetrics and other clinical conditions.

1. Introduction

Preterm birth concerns 11.1% of all pregnancies worldwide, can result in severe consequences for the newborns and is one of the major causes of neonatal death (Blencowe et al., 2012, 2013). Approximately one third of the preterm births occur due to preterm premature rupture of membrane (PPROM), which denotes the rupture of the fetal membrane (FM) prior to the completed 37th week of gestation (Mercer et al., 1999). PPROM can have manifold reasons (Devlieger et al., 2006) and it is distinguished between "spontaneous" and "iatrogenic" causes, where the latter refers to rupture initiated by invasive intrauterine procedures (Deprest and Gratacos, 1999), including amniocentesis (Devlieger et al., 2006) or minimally invasive fetal surgery (Beck et al., 2012).

The FM is composed of two gross layers, amnion and chorion, fusing early in gestation and generating an interface layer (Bourne, 1962; Ilancheran et al., 2009). The chorion is a thick (120–400 μm) mainly cellular layer connecting the FM to the maternal tissue (decidua), whereas the amnion represents a thin biological membrane of 60–100 μm thickness that is in contact with the amniotic fluid (Fawthrop and Ockleford, 1994; Jabareen et al., 2009). The amniotic surface is covered by a monolayer of epithelial cells firmly attached to a thin basement membrane. The latter connects to the dense collagenous compact layer lying on top of a thick fibroblast layer and the spongy layer, which forms the interface with chorion (Bourne, 1962).

The higher stiffness and strength of amnion compared to chorion suggest that the mechanical properties of amnion play a critical role for the incidence of PPROM (Oyen et al., 2006). Mechanical characterization of amnion has therefore been the objective of several experimental studies (e.g. Oyen et al., 2004, 2005; Xu et al., 2013), in addition to investigations dedicated to the behavior of the intact FM (e.g. Moore et al., 2006; Calvin and Oyen, 2007; Joyce et al., 2009; Bürzle et al., 2013).

As typical for soft collagenous tissues, the mechanical response of amnion is largely determined by the organization of its microstructure, predominantly by the density and orientation of collagen fibers and the cross-linking density leading to an intricate collagen network. Towards a better understanding of the microstructural mechanisms underlying the macroscopically observed non-linear mechanical behavior of amnion, histological and

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biochemical studies have been combined with mechanical tests. For example, mechanical parameters were shown to correlate with elastin and collagen content in uniaxial tensile tests (Jabareen et al., 2009), as well as with the amount of pyridinium cross-links PYD and DPD in inflation tests (Bürzle et al., 2013). Moreover, small angle light scattering technique was applied to intact and separated FM samples to extract the orientation of collagen fibers in the unloaded state. A moderate alignment of collagen was found in the amnion, probably inducing the "modest" degree of anisotropy of the intact FM observed in planar biaxial tests (Joyce et al., 2009).

The sample preparation required for conventional histological sections and electron microscopy, such as fixation, dehydration, embedding and cutting of the samples, poses the risk of preparation artifacts. Laser scanning microscopy on the other hand allows analyzing the fresh, hydrated and unfixed microstructure of biological tissues. Second harmonic generation (SHG) multiphoton microscopy visualizes the collagen based on its specific emission with a sharp signal at half of the excitation wavelength (Zoumi et al., 2002). This signal, caused by the non-centrosymmetric geometry of the collagen molecule (Zipfel et al., 2003) can be collected by an appropriate filter in addition to the fluorescence emission caused by two-photon excitation. In recent years, multiphoton microscopy has successfully been applied to study the microstructure of collagenous tissues (Cox, 2011).

In our group, both the intact FM and amnion have been investigated by a combination of this technique with mechanical in situ experiments (Mauri et al., 2013; Mauri et al., 2015; Perrini et al., 2015). By means of dedicated setups for uniaxial and multiaxial testing (Fig. 1) important insight could be gained from the SHG signal of collagen and the fluorescently labeled nuclei of the epithelial cells. It was shown that typical features of fiber architecture and reorientation observed in the zone of altered morphology (ZAM) overlying the cervix can be induced mechanically by repeated inflation of the membrane with a protocol representative of early labor contractions (Mauri et al., 2013). The tension–strain response during relaxation, creep and in cyclic loading were quantified for uniaxial and biaxial states of membrane tension (Mauri et al., 2015; Perrini et al., 2015), confirming the strong lateral in-plane contraction previously reported (Bürzle and Mazza, 2013). In the same studies, the MPM signals were used as a thickness gauge to measure the out-of-plane contraction of the amnion, which is generally not well accessible, during relaxation (Mauri et al., 2015) and after cyclic loading (Perrini et al., 2015). The thickness of unloaded and maximally loaded specimens was extracted and revealed that amnion does not only contract within the membrane plane upon uniaxial extension but also strongly reduces its thickness, entailing drastic volume changes in the order of 60% volume loss for 12% longitudinal strain in a uniaxial tension experiment (Perrini et al., 2015). However, the evolution of the amnion thickness during quasi-static uniaxial loading has not yet been quantified, leaving uncertainty in the evaluation of models describing its mechanical behavior.

The present paper describes the microscopic tools developed for MPM based investigations of amnion and the related methods for data interpretation. Corresponding new stepwise monotonic data are presented, providing detailed information on the change of amnion thickness and collagen distribution as a consequence of quasi-static loading. Microscopic data are also provided for tissue samples in an unloaded initial state and the reference configuration – defined by a threshold force – indicating large changes of thickness and collagen orientation in between.

2. Material and methods

2.1. Specimen collection

The SHG microscopy studies used FMs from elective cesarean sections (38–39 gestational weeks). FMs were collected with informed written consent of the patients (Ethical Committee of the District of Zürich Stv22/2006 and Stv07/07), who had no labor contractions prior to delivery, no preterm rupture of the membrane, no diabetes mellitus and were negative for HIV, hepatitis A and B, chlamydia and cytomegaly. After gentle separation from the chorion, the amnion was stored in physiological solution (NaCl 0.9%) at room temperature. Specimens were cut with a razorblade and tested within 4–5 h after delivery.

2.2. Monotonic uniaxial tensile testing

As described in detail in Mauri et al. (2015), specimens were mounted on our custom built in situ testing machine (Fig. 1a) with the help of a sacrificial plastic jig. The tissue was imaged with a multiphoton microscope (Fluoview 1000 MPE, Olympus; Facility: Center for Microscopy and Image Analysis, University of Zurich) with an excitation wavelength of 820 nm. Similarly to Mauri et al. (2015), a 3D stack was acquired throughout the amnion thickness (each 3 μm) to extract the SHG signal of collagen and the fluorescence of the stained nuclei (Hoechst 33342). Specimens with free length $L=60$ mm and width $b=15$ mm were stretched uniaxially in several steps and images of the same volume element were collected at each step: initial, reference (ref; defined with a force threshold of 0.01 N), 3%, 6%, 9%, 12%, 15%, 18%, 21% and 24% of nominal strain $\varepsilon_1 = L/L_{ref} - 1$. The initial configuration represents the specimen just after clamping. It includes both biological inhomogeneity of the tissue and the spatial variability of the deformation history due to sample preparation and mounting. To minimize viscoelastic effects, a very low loading rate (0.1 mm s^{-1}) was applied and stacks were acquired two minutes after a load step was completed. Force and displacement were continuously recorded at 10 Hz. Specimens with the same free dimensions were also tested on our custom built experimental setup (outside the microscope), as described in (Bürzle and Mazza, 2013; Mauri et al., 2015), with the same constant loading velocity and force threshold of 0.01 N. Top images of the central region of the specimen, force and displacement were recorded at 5 Hz. These tests will be referred to as "macroscopic". Two in situ monotonic uniaxial tests and two macroscopic experiments were performed on testpieces randomly chosen from the same membrane.

2.3. Microscopic parameters extraction

Microstructural parameters, such as collagen orientation and image entropy, were quantified from the imaged volume. The thickness of amnion was extracted manually by averaging the length of 10 lines traced perpendicular to the sample's

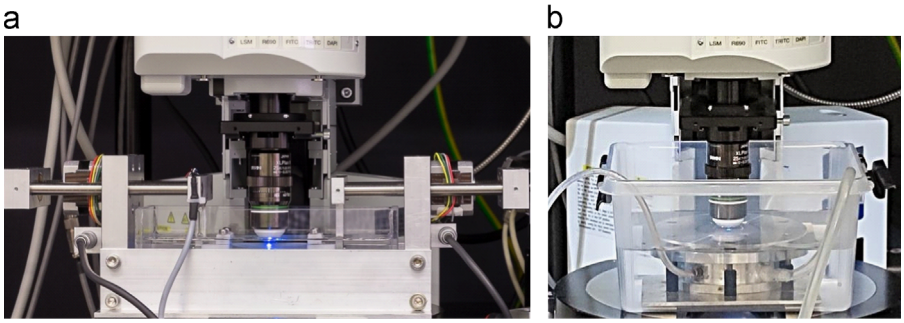

Fig. 1. Uniaxial (a) and inflation (b) in situ experimental setups allow simultaneous mechanical loading of the specimen and imaging of the amnion through the whole thickness with multiphoton microscopy.