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Second harmonic generation microscopy of fetal membranes under deformation: Normal and altered morphology

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ABSTRACT

Introduction: Insight into the microstructure of fetal membrane and its response to deformation is important for understanding causes of preterm premature rupture of the membrane. However, the microstructure of fetal membranes under deformation has not been visualized yet. Second harmonic generation microscopy, combined with an in-situ stretching device, can provide this valuable information.

Methods: Eight fetal membranes were marked over the cervix with methylene blue during elective caesarean section. One sample per membrane of reflected tissue, between the placenta and the cervical region, was cyclically stretched with a custom built inflation device. Samples were mounted on an in-situ stretching device and imaged with a multiphoton microscope at different deformation levels. Microstructural parameters such as thickness and collagen orientation were determined. Image entropy was evaluated for the spongy layer.

Results: The spongy layer consistently shows an altered collagen structure in the cervical and cycled tissue compared with the reflected membrane, corresponding to a significantly higher image entropy. An increased thickness of collagenous layers was found in cervical and stretched samples in comparison to the reflected tissue. Significant collagen fibre alignment was found to occur already at moderate deformation in all samples.

Conclusions: For the first time, second harmonic generation microscopy has been used to visualize the microstructure of fetal membranes. Repeated mechanical loading was shown to affect the integrity of the amnion–chorion interface which might indicate an increased risk of premature rupture of fetal membrane. Moreover, mechanical loading might contribute to morphological alterations of the fetal membrane over the cervical region.

1. Introduction

The integrity of the fetal membrane (FM) is essential during pregnancy in order to avoid preterm delivery. The preterm premature rupture of membrane affects about 3% of pregnancies and causes around 25–30% of all preterm deliveries [1]. A better understanding of the microstructure of FM, its alterations and its behaviour under deformation broaden the understanding of the mechanisms leading to premature mechanical rupture [2]. In addition, the increasing interest in the amniotic membrane as scaffold for tissue-engineered implants [3–6] and as in vitro biological models [7,8] further motivates the present investigation.

The microstructure of amnion and chorion was previously studied by using confocal light microscopy [9–12,15] and electron microscopy [11,13–15] to identify membrane's sub-layers, collagen types, cellular and extracellular components. A zone of altered morphology (ZAM) was identified over the cervix in term FM both pre labour [16] and post labour [17,18]. The ZAM tissue shows swelling of the amnion and reduced thickness of the choriodecidua compared to the reflected tissue (between the placental border and ZAM) [16,21]. These microstructural alterations were considered to be a consequence of biochemical factors, such as a local increase of

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matrix metalloproteinases [19,20] and poly(ADP-ribose) polymerase cleavage [21], which influence collagen remodelling and cellular apoptosis, respectively.

The microstructure of FM could not be visualized under deformation due to limitations of the used methods. Nonlinear microscopy techniques, such as second harmonic generation (SHG) and multiphoton microscopy, allow the visualization of collagen and elastin structures without fixation and staining of the tissue [22]. Moreover, the two photon excitation can penetrate deeper into the tissue allowing imaging of relatively thick samples. Thus, this new technique lends itself to investigate the response of extracellular components under deformation, e.g. for porcine arterial tissue [23] or rat-tail tendons [24].

Acute in-vitro stretching of amniotic epithelial cells and FM has been shown to enhance expression of cytokine (interleukin8 and Visfatin) that are usually up-regulated in association with labour [25–27]. Repeated physiological mechanical stimuli affect mechanical properties of FM [28,29], but their effect on the microstructure has not been investigated yet.

In this study, for the first time SHG microscopy was used to investigate the normal and altered morphology of FM. This technique was combined with an in-situ stretching device to visualize and quantify collagen alignment under uniaxial tension. Comparison of reflected, ZAM and cyclically inflated tissue addressed the hypothesis that repeated stretching of FM over the cervix might contribute to the morphological alterations observed in the ZAM.

2. Methods

2.1. Samples procurement and preparation

Fetal membranes ($n = 8$) were collected from patients who underwent elective caesarean sections at 38 gestational weeks. Patients were recruited with informed written consent using a protocol approved by the Ethical Committee of the District of Zürich (study Stv22/2006). The selected pregnancies had no labour contractions prior delivery, no preterm rupture of the membrane, no diabetes mellitus and were negative for streptococcus B, HIV, hepatitis A and B, chlamydia and cytomegaly. Membranes were perioperatively marked over the internal cervical os with a cotton-tipped applicator dipped in methylene blue. One sample from the marked area and two samples of reflected membrane were harvested, stored in Ringer's lactate solution, tested and imaged within 5 h from the delivery (Fig. 1a).

2.2. Inflation cyclic tests

For each FM, one sample of reflected membrane was first cyclically stretched with a custom-built inflation device [30] (Fig. 1b). Circular specimens (70 mm diameter) were clamped between a cover ring and an aluminium cylinder and inflated with Ringer's lactate solution using a peristaltic pump, controlled with LabView. A chamber was placed on the cover ring and filled with the solution to keep samples moist during experiments (Fig. 1b). Samples were stretched by application of pressure ranging between 10 and 40 mmHg for 60 cycles lasting 1 min each, using a protocol representative of contractions during early labour [29].

2.3. In situ imaging with the multiphoton microscope

Samples from reflected (R), ZAM and cycled (C) tissues were collected, cut in rectangular shape (50 mm × 10 mm), stained with a nuclear staining (DAPI, 4′, 6-diamidino-2-phenylindole, dihydrochloride, Invitrogen) and mounted on a custom-made stretching device with the amnion upwards (Fig. 1c). Samples were tested at room temperature in Ringer's lactate solution to ensure a physiological hydration and imaged with a multiphoton microscope (Fluoview 1000 MPE, Olympus) using a water objective (XLPlan N 25x, NA 1.05) (Fig. 1c). 3D stacks were acquired starting from the amniotic epithelium through the whole thickness using a Ti:Sapphire Laser (wavelength = 820 nm) with an in-plane resolution of about 363 nm [31]. Second harmonic generation for collagen was detected with a specific filter (Olympus FV10-MRROPT, BA397-412), whereas the fluorescence of the nuclei (DAPI) was simultaneously detected in a second channel (BA455-490).

2.4. Histological sections

Samples of tissue near to the imaged specimens were fixed in 4% paraformaldehyde for 12 h at 4 °C and routinely embedded in paraffin. From all tissue blocks, sections of 4 μm were cut and stained with either hematoxyline and eosine or resorcin fuchsin for elastin according to routine procedures. Sections were coverslipped and analysed by bright-field microscopy (Zeiss Axiovert 200M).

2.5. Processing of images

Image analysis was performed to extract the entropy, the thickness and the collagen orientation. 2D images were exported from the microscope data and post processed in Matlab (MathWorks, Natick, Massachusetts, U.S.A.) using custom written algorithms.

The image entropy is a statistical measure of the randomness based on the intensity histogram and is used to characterize the texture of images [32]. This parameter was calculated for representative images of the spongy layer with the Matlab function "entropy".

The thickness of collagenous layers (compact, fibroblast, spongy and reticular layers) was measured from the intensity of grayscale images in the stack. An intensity factor was defined for each image as the sum of pixels intensity normalized for its dimensions and grey scale. An intensity threshold was used to define the beginning and the end of the sample. The thickness was computed for each sample 16 times over quadratic 32 × 32 μm^2 regions and averaged.

The collagen orientation was identified by using the principal direction analysis [33]. Images containing only the SHG signal of collagen were first converted to grayscale and filtered with an average filter; successively the angular orientation for each sub-region was extracted (2 × 2 μm^2) and fitted with a truncated normal distribution. The standard deviation (σ) of this distribution is representative of the

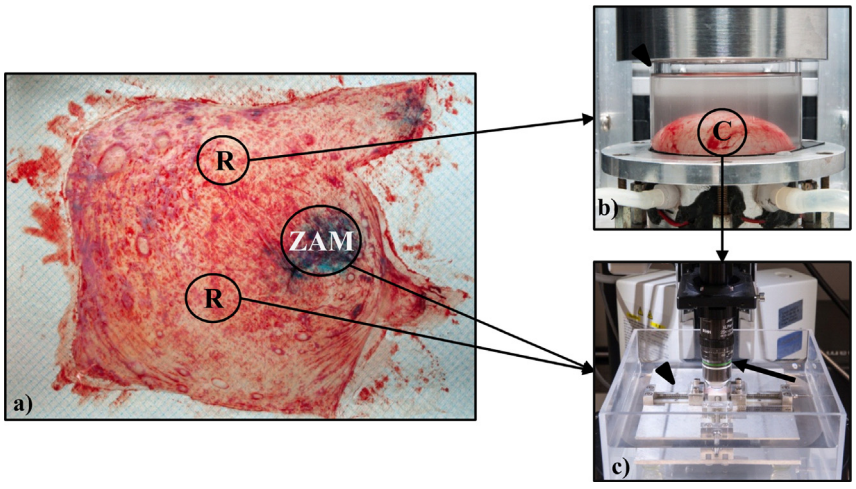

Fig. 1. Samples procurement and testing: a) fetal membrane with harvested samples (R: reflected membrane, ZAM: zone of altered morphology); b) inflation device with a cyclically stretched sample (C) (arrow: inflating cylinder, arrow head: upper chamber filled with Ringer's solution); c) multiphoton microscope (arrow: objective) with in-situ stretching device (arrow head).