6

Weakening and Rupture of Human Fetal Membranes – Biochemistry and Biomechanics

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1. Introduction

In spite of advances in the quality of prenatal care, management of high-risk pregnancies and treatment strategies targeting prevention of preterm births, the rate of preterm births in the US has continued to rise over the last two decades and is associated with high mortality and morbidity. Care of preterm infants also poses a significant financial burden on limited health care resources. Infants born at less than 37 weeks gestation account for 69% of all US infant deaths and their care has been modestly estimated at 26 billion dollars per year (Behrman et al., 2006; MacDorman et al., 2008). Preterm Premature Rupture of Membranes (PPROM) causes 30 - 40% of all preterm births and is disproportionately distributed such that African Americans suffer twice the rate of PPROM than Caucasians (Goldenberg et al., 1996a). Although some limited success has been achieved in the treatment of iatrogenic fetal membrane (FM) rupture resulting from amniocentesis or fetal surgery (Quintero et al., 1999; Young et al., 2000; O'Brien et al., 2001; Bilic et al., 2010), there has been no success in the repair of spontaneously ruptured FM, as occurs with PPROM (Quintero et al., 1998; Sciscione et al., 2001; Young et al., 2004b; Devlieger et al., 2006). Recent progress in the understanding of the biochemically-mediated processes which lead to FM weakening and rupture suggests that inhibition of these processes may be possible. This generates hope that many cases of PPROM may ultimately be preventable (R.M. Moore et al., 2009b, 2010; Kumar et al., 2011).

2. New techniques used in the studies on FM rupture

2.1 Mapping procedure

We have developed and utilized a systematic procedure to map the rupture strength of FM over its entire topographic surface, thereby identifying relatively weak areas for further biochemical, proteomic, and sophisticated biomechanical testing.

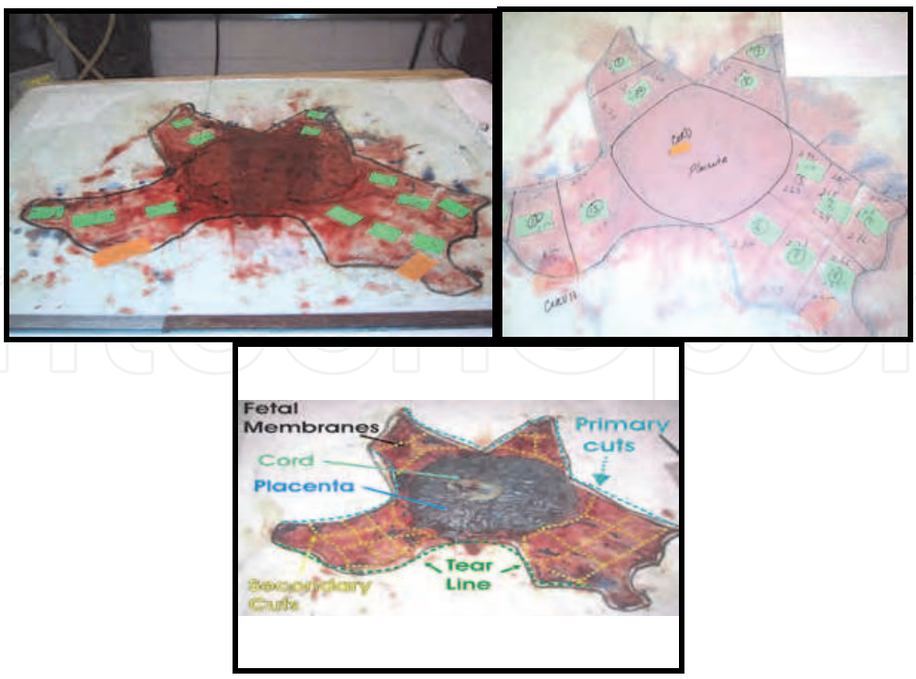


Fig. 1. Fetal Membrane cutting procedure**.** (Top Left) Marked membranes showing location of primary cuts required to lay the membranes flat. (Top Right) Membrane fragments removed with outlines of pieces on lab table paper. (Bottom) Paper tracing reproduction showing cuts, orientation and location of all pieces, and strength results. (Reproduced with permission from El-Khwad et al. Biol Reprod 72:720-726, 2005.)

Although animal data document homogeneous weakening of the FM over the entire surface with increasing gestation (Lei et al., 1995, 1997; Paavola et al., 1995; Parry et al., 1998), attempts to demonstrate similar gestational changes in human FM were unsuccessful (Manabe et al., 1991). Studies in which whole FM or randomly selected pieces were sampled showed high variability and no definite pattern of remodeling and apoptosis. In response to the challenge of interpreting variable data, we developed a systematic method of cutting the membranes (El-Khwad et al., 2005; Fig. 1.). Fetal membranes to be tested were cut along specific grids. We identified the region of the membranes overlying the cervix by marking it in utero with Gentian Violet. As a result, the exact location and orientation of each piece cut from the membranes relative to both the placental disc and to the region that formerly overlay the cervix was determined. A paper model (tracing) mapping the location and the rupture strength data of each piece is ultimately constructed. This two dimensional map can be folded to show the original three-dimensional physical configuration of the amniotic sac with superimposed Rupture Strength results. This approach confirmed an inherent inhomogeneity in the biomechanics of the gestational sac and clearly demonstrated the para-cervical weak zone. These features had been camouflaged in many previous studies where parts of weak and strong regions were tested together with the assumption of FM homogeneity over its entire surface.

2.2 Rupture test equipment

Specialized equipment is necessary to determine FM physical properties. Our testing apparatus, developed in collaboration with Com-Ten industries (a national supplier of

tensile testing equipment) (Moore et al., 2006), has a number of advantages over that used in previous biomechanical studies of FM: 1. It applies a bi-axial (two dimensional) stretch on the membranes similar to normal physiology; 2. It uses the puncture mode with any size probe and tissue clamp – it is thus possible to perform a detailed survey of physical properties over the entire fetal membrane surface in a reasonable time period; 3. It will hold preset displacements or forces for viscoelastic studies; 4. Probes may be fit with ultrasound sensors for simultaneous measurement of membrane thickness; 5. High precision - calibrated accuracy is 0.05 Newton force and 0.01 cm displacement over appropriate intervals; 6. Tissue specimens are kept moist at all times. 7. Data is captured in digital form, displayed graphically, and analyzed by the software. A force-displacement curve (Fig. 2.) is computer generated during each experiment and the strength characteristics (membrane Rupture Strength, Work to Rupture, Stiffness and other physical properties) are derived and calculated from the data. Data is also exportable for independent analysis.

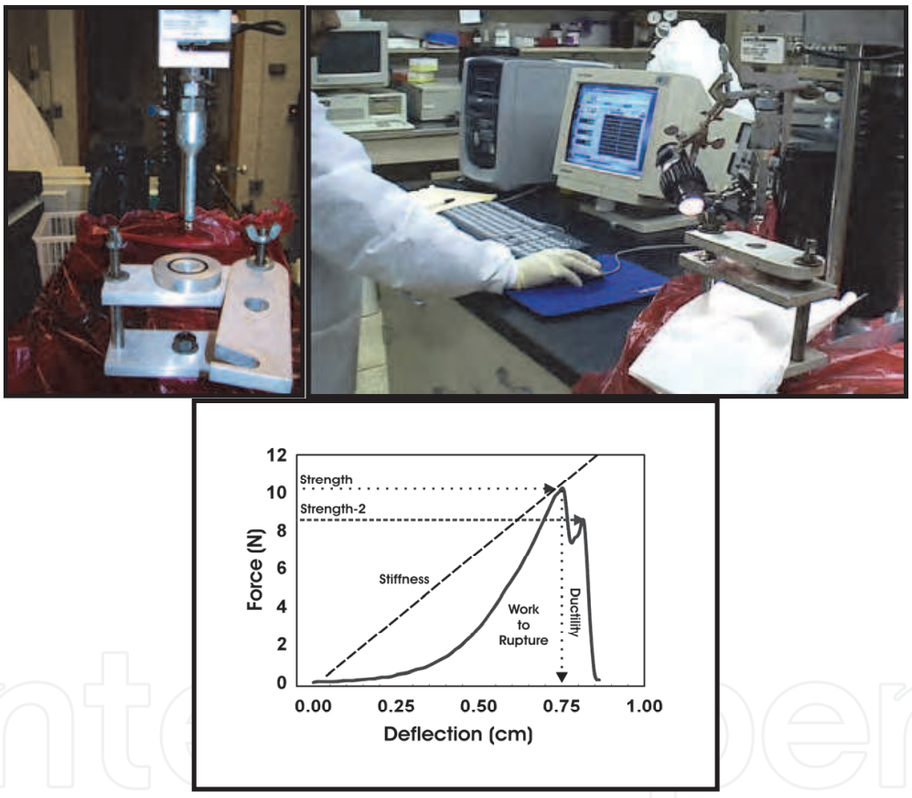


Fig. 2. Strength testing equipment. (Top Left) Close-up view of fetal membrane holding apparatus (from top to bottom – load cell force sensor, rupture probe with spherical tip, membrane holding assembly). (Top Right) Com-Ten Ball-Burst Compression Testing Equipment (from left to right – computer controller, analog-digital converter, membrane rupture assembly). (Bottom) Typical force displacement curve. (Reproduced with permission from El-Khwad et al. Biol Reprod 72:720-726, 2005.)

Development of rupture test equipment and use of this mapping methodology helped us establish the following:

2.2.1. Both term and preterm FM are biomechanically and biochemically heterogeneous over their surfaces. Only by use of this mapping procedure and equipment have we been able to

identify and topologically map weak areas so that characterization can lead to direct investigation of the mechanism of weakening (El-Khwad et al., 2005, 2006; Rangaswamy et al., 2011).

2.2.2. Sequence of FM rupture – amnion and choriodecidua first stretch together followed by their separation, then choriodecidua ruptures prior to reaching its elastic limit and amnion stretches further in a non-elastic fashion, then ruptures (Arikat et al., 2006).

2.2.3. FM strain hardens with acute cyclical stretch. Like metal pulled into wire it becomes stronger and less elastic. It does not weaken (Pandey et al., 2007).

2.2.4. Determine which physiological agents or processes cause sufficient FM weakening to precipitate rupture, and under what conditions (dose, duration, etc.) this occurs.

2.3 Equipment to measure adhesion of amnion to choriodecidua

Discovery that FM (amnion and choriodecidua) separate during the process of rupture led to development of equipment to measure their adherence and allowed us demonstrate that FM peel apart and become less adherent with increasing gestation (Kumar et al., 2009; Strohl et al., 2010). Commercial industrial tensile testing equipment (Com-Ten) was adapted to perform a standard engineering T-peel test to measure the adhesive force of FM (Fig. 3.). Use of this equipment allowed us to determine that FM components become less adherent and separate with increasing gestational age (Strohl et al., 2010).

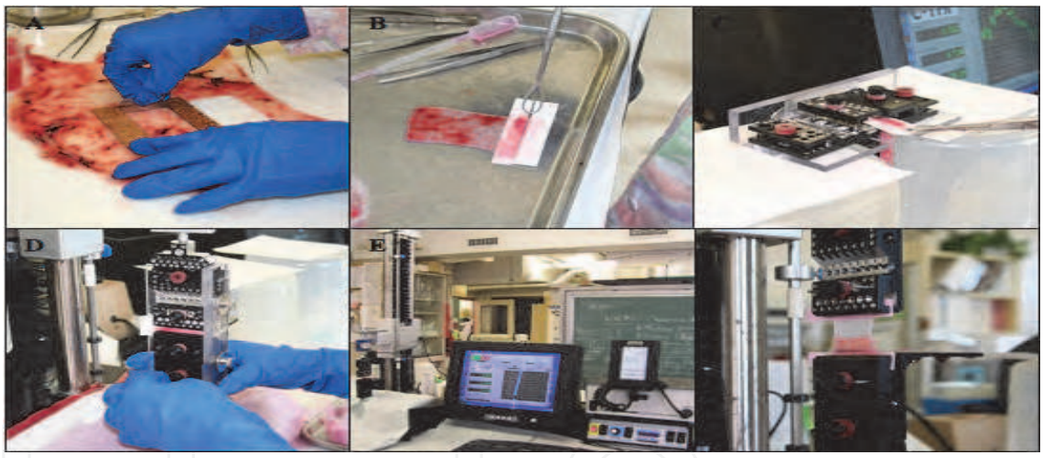


Fig. 3. Peel testing procedures**.** Tissue samples are cut out from FM kept moist and laid flat and kept moist (A). Stiff filter paper stints assist in holding and mounting the partially manually peeled amnion and choriodecidua by maintaining shape (B). Membrane piece mounted on custom clamps which are maintained in fixed position by a plexiglass mount support to assist sample loading (C). Sample loaded clamps with supporting Plexiglas mount as a single unit attached to the peel testing device (D). The peel tester with the FM piece loaded and the plexiglass mount support removed before beginning the test (E). FM components, amnion and choriodecidua, being peeled apart between the clamp jaws (F).

3. Para-cervical weak zone in FM – A result of programmed biochemical weakening processes

Rupture of FM (ROM) is an integral event in the onset and development of labor. Even though ROM usually follows uterine contractions, it precedes the onset of contractions in at

least 10% of term labor and 40% of premature labor. This implies that the stretch force alone is not the cause of FM weakening.

3.1 Term FM develops a para-cervical "weak zone" where rupture initiates

Rat model studies suggest that amnion, the strongest component of FM undergoes collagen remodelling as gestation progresses (Paavola et al., 1995; Lockwood et al., 1999). As a result of these phenomena, FM is postulated to weaken and become more susceptible to rupture near the end of gestation. Apoptosis has also been suggested to occur near term in human amnion and chorion (McLaren et al., 2000a). Malak and Bell first identified an area of so-called "high morphological change" in the zone of FM overlying the cervix (Malak & Bell, 1994). This distinct para-cervical region has been demonstrated in the FM from both term vaginal and caesarean section deliveries without labor and comprises approximately 2-10% of the total FM surface area (McLaren et al., 1999a; McParland et al., 2003). Bell's group further characterized this area of FM as having increased matrix metalloproteinase (MMP) -9, increased trophoblast apoptosis, differences in the thickness of membrane sub layers, and increased myofibroblasts in this area (McLaren et al., 1999b, 2000a, 2000b; McParland et al., 2003). Lappas and colleagues have confirmed an increase in apoptotic markers, and have reported increased NF-kB activity and acetylated-forkhead box O1 protein expression in the para-cervical FM region (Reti et al., 2007; Lappas et al., 2009). All these studies have presumed that remodelling in this para-cervical zone leads to FM weakening and subsequent rupture.

We have identified a focal "weak zone" with biochemical characteristics equivalent to the previously described "zone of high morphological change" in the para-cervical region of FM. Using FM from repeat cesarean section performed prior to any labor, we showed that this region ruptures with only 20 - 50% of the force required to rupture other areas of the FM (Fig. 4.), and that it exhibits a biochemical signature of increased collagen remodeling and apoptosis (El-Khwad et al., 2005). Western Blot analysis indicated increases in matrix metalloproteinase 9 (MMP-9) and cleaved PARP, and decreases in fibulins 1, 3, 5 and TIMP-3 in the Weak Zone. MMP-2 did not show differences. TIMP-1 was barely detectable; TIMP-2 and TIMP-4 protein were detectable in all specimens but did not change with Strength. Other groups have also confirmed the increase in markers of collagen remodeling and apoptosis (Malak & Bell et al., 1994; El-Khwad et al., 2005, 2006; Meinert et al., 2007, Reti et al., 2007; Han et al., 2008; Lappas et al, 2008, 2009, 2010a, 2010b).

Using FM from patients with AROM (artificial rupture of membranes) and SROM (spontaneous rupture of membranes), we have further demonstrated that the para-cervical weak zone persists with the same biochemical signature as that identified in pre-labor cesarean section membranes. As the rupture tear line usually extends through the weak zone, we have postulated that it contains the site of initiation of FM rupture (El-Khwad et al., 2005, 2006). We have also demonstrated that acute, repetitive stretch does not weaken, but paradoxically strengthens FM (Pandey V et al., 2007). Our studies, those of the Bell group, and now those of four other groups (as indicated above) thus strongly support the concept of regional differences in FM properties, most specifically, that the area over the cervix is significantly weaker than the rest of the FM with concomitant differences in biochemical and histological properties. Taken together, the data indicate that term FM normally weakens in late gestation as a result of programmed biochemically mediated processes. Presumably, spontaneous FM rupture at term usually results from the mechanical stresses of labor upon biochemically pre-weakened membranes.

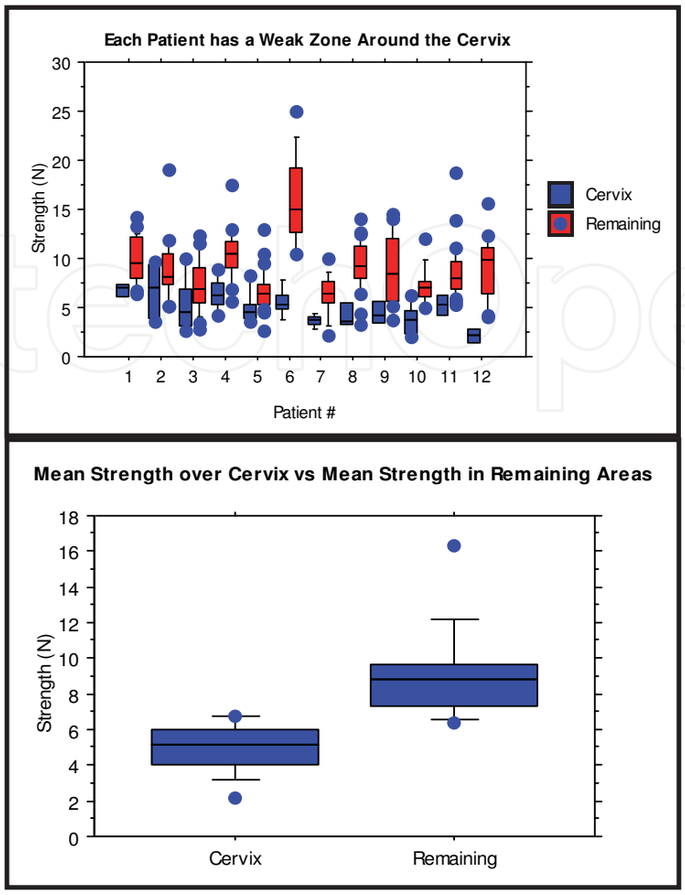


Fig. 4. Fetal membrane strength. [Bottom] Mean strength of cervical zones for each patient is less than the mean strength of the remaining areas ($P < 0.001$). [Top] Individual patient data showing a weaker cervical zone for each of the 12 patients relative to the remaining areas. (Reproduced with permission from El-Khwad et al. Biol Reprod 72:720-726, 2005.)

3.2 Role of MMP activation and apoptosis in the weak zone formation

Fortunato and Menon (2004) have extensively reviewed the roles played by MMPs and apoptosis in FM rupture. The strength of amnion and chorion is largely due to collagen. Collagens I, III, IV, V and VI have been described in various layers of amniochorion. The major strength in amnion is derived from collagen I (seen extensively in the compact layer and adjacent mesoderm) and collagen IV (a major component of the basement membrane and of the bundles connecting the mesenchymal layer and the epithelium) (Bachmaier et al., 1999). Degradation of collagen is controlled by specific MMPs as modulated by tissue inhibitors of matrix metalloproteinases (TIMPs). Thus, the ratio of MMPs and TIMPs is a good indicator of collagen degradation, which along with the deposition rate of new collagen by fibroblasts, determines the ultimate tissue strength. Although MMP types 1, 2, 3, 8 and 9 have been well described in amniochorion; major investigative work in FM has been done with MMP types 2 and 9. MMP-1 predominates prior to the onset of contractions as described by Bryant-Greenwood and Yamamoto. FM MMP-2 is constitutive and reportedly does not respond to cytokines or change with PPROM or labor (term or preterm) [Fortunato et al., 1999; Maymon et al., 2001; Xu et al., 2002]. In contrast, both active and latent forms of

MMP-9 have been shown to increase in human amniotic fluid with PPROM and in amniotic fluid of rhesus monkeys after inducing labor with cytokines (Bryant-Greenwood et al., 1995; Osmers et al., 1995; Athayde et al., 1999; Vadillo-Ortega et al., 2002). MMP-9 can also be induced in FM tissue with PGE_2, $PGF_{2\alpha}$, TNF_α and ROS (Buhimschi et al., 2000; Sciscione et al., 2001; Ulug et al., 2001; Arechavaleta-Velasco et al., 2002; Zaga et al., 2004). TIMP-1, which controls the activity of MMP-9, has been extensively studied in FM and found to decrease with PROM and labor (Ulug et al., 2001; Arechavaleta-Velasco et al., 2002; Buhimschi et al., 2000; Zaga et al., 2004; McLaren et al., 2000b). Thus we conclude that MMP-9 plays a major role in FM remodeling, weakening and rupture. Our work also demonstrates that MMP-9 levels are an excellent marker for FM strength (El-Khwad et al., 2005, 2006; Kumar et al., 2006; R.M. Moore et al., 2009b) [Fig. 5, 7, 8, 10]. TIMP-3 has a direct correlation with FM rupture strength (unlike TIMP types 1 and 2) and was significantly decreased in the weak zone (El-Khwad et al., 2005, 2006). Unlike other TIMPs, TIMP-3 inhibits all the gelatinases, TNF-α, and also is stored within ECM (Mannello et al., 2001).

MMP activation and apoptosis are often interrelated. The ECM acts a major stabilizing factor in many tissue systems. This stability is compromised when MMP activation leads to the breakdown of ECM leading to apoptosis (Boudreau et al., 1995; Chintala e al., 2002). MMPs may also induce apoptosis by cleaving membrane bound cytokines, including TNFα and FasL (Gearing et al., 1994; Kayagaki et al., 1995). Apoptosis can also induce activation of MMPs. In addition, the same agents which have been reported to cause apoptosis in FM tissue also activate and increase transcription of MMPs, specifically, MMP-1, MMP-9 (So et al., 1992; Fortunato et al., 2002). We have reported a parallel increase in prostaglandins with induction of apoptosis in amnion epithelial, mesenchymal and WISH cells with several non-physiological (actinomycin D, cycloheximide, staurosporin) and physiological (ceramide, lactosylceramide, PGJ2 metabolites) apoptotic agents (Moore et al., 1988, 1993).

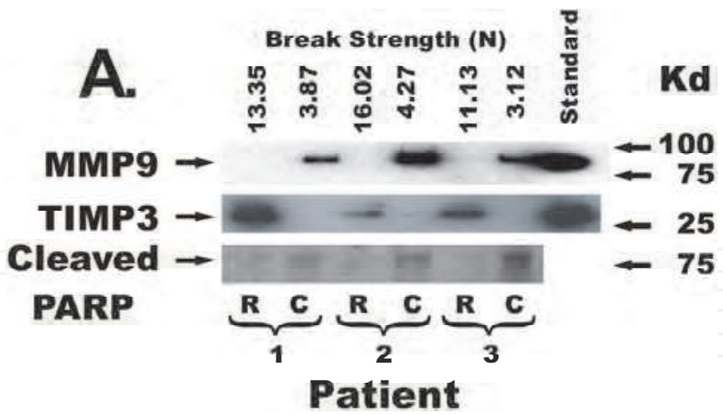


Fig. 5. Biochemical characteristics of fetal membrane regions: Western blot showing differences in MMP-9, TIMP-3 and PARP cleavage between weak, paracervical regions (C) and relatively strong remaining areas (R). Typical Western blot showing parallel strength differences in cervical (C) and remaining (R) areas of the fetal membranes from three patients. (Reproduced with permission from El-Khwad et al. Biol Reprod 72:720-726, 2005.)

Prostaglandins also induce transcription and activate MMPs in most tissues (Lyons et al., 2002; Yoshida et al., 2002). The apoptotic process, thus, potentially weakens FM by eliminating fibroblastic cells, which lay down new collagen, and simultaneously activates

enzyme systems, which break down existing collagen. Activation of MMPs feeds forward to increase apoptosis, which subsequently feeds back to further increase MMP activation.
The physiological mechanisms that initiate MMP activation and apoptosis in FM are not understood. Many constituents of amniotic fluid whose concentrations increase with gestation, with infection, or even with rupture of membranes (TNFα, IL-1β, lactosylceramide, etc) have been shown to cause apoptosis in cells derived from FM and intact membranes (Moore et al., 1988, 1993). Most of these "apoptotic" agents also increase transcription or activation of MMPs, especially MMP-9 (Kumar et al., 2006; R.M. Moore et al., 2009b; Menon et al., 2004; Zaga et al., 2004). Several groups have proposed the synergistic effects of MMP activation and apoptosis leading to rupture of the FM (McLaren et al., 2000a; Fortunato et al., 2000; Bowen et al., 2002; Lei et al., 1996). The promoter polymorphisms in some cytokines (TNFα and IL-1β) and MMPs (-1,-8,-9) have been identified to be associated with PPROM (Ferrand et al., 2002; Fujimoto et al., 2002; Hernandez-Guerrero et al., 2003; Roberts et al., 1999). Patients with these polymorphisms may have earlier initiation of the programmed weakening process resulting in premature rupture of FM and earlier deliveries.

3.3 Structural protein changes in the extracellular matrix of the "weak zone" in FM

As part of the remodeling processes in the weak zone, there must be changes in the structural proteins that could contribute to the weakening of FM. Changes in MMPs and TIMPs on their own do not result in weakening and rupture of FM. The collagen structural units or other components of extracellular matrix (ECM) may undergo degradation resulting in weakening of FM. At least half of the published reports found no decrease in fibrillar collagen in the area of the rupture site in human FM, however (Manabe et al., 1991; Al-Zaid et al., 1980; Evaldson et al., 1987; Halaburt et al., 1989). This is in contrast to animal studies in which collagen degradation is the major factor in remodeling of FM (Lei et al., 1996). There is some evidence that other processes play a crucial role in human FM weakening: 1. Collagen fiber bundles are more dispersed and less well organized in the area of FM rupture site as demonstrated through X-ray diffraction and microscopy (Connon et al., 2007). This disorganization is also supported by evidence that decorin and biglycan are decreased and increased respectively at the site of FM rupture (Meinert et al., 2001 & 2007). Decorin is a protein that promotes protein fiber organization and biglycan has the opposite effect. 2. FM mesenchymal cells undergo "phenotypic switching" from strength promoting myofibroblasts to macrophages that are capable of producing cytokines which weaken the FM (Kim et al., 2008). 3. Numerous studies have demonstrated increased apoptotic activity near the site of SROM (Runic et al., 1998; Yuan et al., 2009; Sagol et al., 2002). 4. We have reported that three members of the fibulin protein family are decreased in the para-cervical weak zone (R.M. Moore et al., 2009a). These proteins are involved in making bridges in the microfibrillar component of the ECM, thus this protein family is clearly of potential interest in the remodeling process that weakens FM prior to rupture. There have been no previous reports describing fibulin family proteins in amnion. We also demonstrated that amnion epithelial and mesenchymal cells produced all three fibulins and their abundance was inhibited by TNF-α, a cytokine involved in weakening of FM (Ossovskaya et al., 2004). We speculate that the amnion microfibrillar layer undergoes significant remodeling with the development of FM weak zone.

3.4 Stretch forces increase rupture strength of the FM rather than decreasing it

It was previously thought that during labor, the FM weakens progressively as a result of repetitive stretching due to contractions. Toppozada (Toppozada et al., 1970) showed that the specific contraction causing rupture of membranes was rarely the most forceful contraction that had been experienced up to that time. They reasoned that prior contractions weakened the membrane so that it subsequently gave way with less force. Lavery described stress relaxation, creep and thinning in FM as part of their study of viscoelastic properties (Lavery et al., 1977, 1982). They assumed, but did not demonstrate, that membranes were weaker (had a lower Rupture Strength) after experiencing viscoelastic, non-recoverable deformation. To test this hypothesis, we collected term vaginally delivered, FM, which were strength tested after being cyclically stretched (Pandey et al., 2007). Rupture Strength and Work to rupture were determined for intact unstretched FM (control) and compared with topographically adjacent FM pieces which were stretched to 75 % of the Rupture Strength of controls, for 5, 10 or more cycles of 10 seconds each. Repeated stretching caused non-elastic deformation of the FM as reported by Lavery et al but the Rupture Strength after repeated stretching paradoxically increased rather than decreased. Stretched FM Rupture Strength increased 20-40% with respect to controls after 5 or 10 cycles. In contrast, Work to Rupture decreased. After a large number of cycles (20-30), Rupture Strength ultimately decreased. The number of cycles of stretch required to promote weakening of the FM was highly variable and may be related to the cycles that the membrane had gone through prior to delivery. When separated FM components were tested, amnion alone showed the same altered pattern of Rupture Strength and Work to Rupture after stretch cycling as the intact tissue - Rupture Strength initially increased while Work to Rupture decreased. Choriodecidua did not exhibit properties of stretch induced deformation. FM thus undergo deformation with stretch cycling. The initial cycles of high force stretching causes an increase in FM Rupture Strength, but decreased Work to Rupture (likely due to the amnion component alone). With continued cycling, Rupture Strength also ultimately decreased. We speculate that collagen fibers in FM may realign during initial stretch cycles as they do in stretched skin and cartilage. This may effectively strain harden the FM, increasing Rupture Strength and thus protecting the FM from precipitous rupture. This protective mechanism may fail after many cycles of stretch allowing FM to rupture at lower Rupture Strength and lower Work to Rupture.

4. Preterm Premature Rupture of FM

The precipitant causes of preterm FM weakening leading to PPROM remain uncertain. Limited data is available on FM physical properties in preterm patients. The studies in our laboratory utilizing our methodology confirm that premature FM are stronger than term with a significant drop-off after 37 to 38 weeks gestation (Rangaswamy et al., 2011; fig. 6.). Other researchers (Pressman et al., 2002; Lavery et al., 1979; Chua et al., 2009) have studied the effects of gestational age on FM strength and noted a statistically significant decrease in FM tensile strength after 39 weeks gestation which is consistent with our findings. Our study is the first to demonstrate heterogeneity in biomechanical properties across preterm FM as we have previously shown in term FM (El-Khwad et al., 2005). This mandates that a greater degree of remodeling and/or degradation would be necessary for preterm FM to rupture than occurs in term FM. Although a definitive para-cervical Weak Zone cannot usually be located in PPROM FM because the para-cervical region cannot be marked prior to delivery, a relative weak zone along the rupture tear line is generally present.

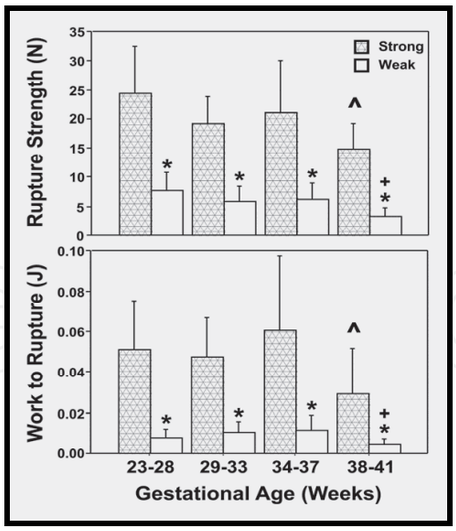


Fig. 6. FM rupture strength (upper panel) and work to rupture (lower panel) comparing the weak zone vs. strong areas across gestational groups: A decreasing trend in both rupture strength and work to rupture is shown from preterm to term gestation (Data are presented as the mean ± SD, +^* indicate $p < 0.001$). Reproduced with permission from Gynecol Obstet Fertil. 2011 ; 39(6):373-377 (Rangaswamy et.al., 2011).

Although the mechanism of FM rupture at term or with PPROM is not well understood, we believe that PPROM occurs due to an abnormal early activation of the processes that weaken FM at term. Supportive findings of this theory are the marked upregulation of MMPs, pro-inflammatory cytokines and chemokines in amniotic fluid and FM in PPROM and chorioamnionitis (Cox et al., 1997; Maymon et al., 1999; Thomakos et al., 2010; Bryant-Greenwood et al., 1995; Fortunato et al., 2003; Riley et al., 1999; Locksmith et al., 2001; Xu et al., 2002). TNF and IL-1β specifically have been shown to induce apoptosis, induce MMP 9 and increase PGE_2 production in chorioamnion and cultured primary amnion cells (R.M. Moore et al., 2009b; Runic et al., 1998; Fortunato et al., 2001; Furuta et al., 2000; Lundin-Schiller et al., 1991; Garcia-Lloret et al., 1996; Lockwood et al., 2008). We previously reported that several physiological agents and reactive oxygen species (hydrogen peroxide) induce apoptosis with concomitant PGE_2 release in amnion derived WISH cells, primary amnion cells and intact amnion (Kumar et al., 2004 a, 2004 b).

Inflammation due to an ascending bacterial infection from the female genital tract remains the most widely speculated cause as up to 55 percent of patients with PPROM had culture or PCR evidence of infection (Jones et al., 2009). Inflammatory processes at sites remote from the female genital tract (e.g.: periodontal infections) may also increase the levels of proinflammatory cytokines at the materno-fetal, interface (Offenbacher et al., 2006; Carta et al., 2004). Decidual hemorrhage/abruption is also highly associated with PPROM (Harger et al., 1990; Salafia et al., 1995). Additional risk factors include advanced maternal age, primiparity, (Ladfors et al., 2000) smoking, (Burguet et al., 2004) short cervical length (Iams et al., 1996) structural abnormality of chorioamniotic membranes (Stuart et al., 2005), and PPROM with preterm birth in a previous pregnancy (Ladfors et al., 2000; Lee et al., 2003; Shen et al., 2008). African American women are at higher risk of PPROM than Caucasian women (Goldenberg et al., 1996a), which might be due to racial differences in the regulation of promoter activity of pro-inflammatory cytokines and MMPs (Simhan et al., 2003; Ferrand et al., 2002).

Two major processes that have been associated with PPROM are inflammation (due to infection and other etiologies) and decidual hemorrhage/abruption. We have developed and utilized a unique model system to investigate FM weakening due to each of these etiologies and identified a pharmacological agent with potential role in preventing this weakening process, which will be discussed in the next two sections.

5. Investigations to understand mechanism(s) of FM weakening and rupture

5.1 Development of an in vitro model system to study the mechanisms of FM rupture

There are no animal models for the study of human FM weakening and rupture. We have used FM strength testing equipment and methods that allow us to systematically measure and map human FM biomechanical properties over the entire FM surface and correlate these with local biochemical properties (as discussed above) in conjunction with an adaptation of the explant culture system developed by Fortunato and Menon (Fortunato et al., 1994) to produce a model system to study the process of human FM weakening (R.M. Moore et al., 2006, 2009b, 2010; Kumar et al., 2011; Mercer et al., 2010). Full thickness FM explants are cut from regions of the FM distant from the Weak Zone of FM from unlabored Cesarean deliveries. The explants are then incubated for 2 to 4 days with agents postulated to affect the weakening process. Biomechanical testing is then performed and correlated with local induced biochemical changes in the immediately adjacent tissue. This model system is novel and unique in its ability to quantitatively measure FM weakening, the major parameter of clinical interest, as well as the associated biochemical changes. We are using this model system to explore the mechanisms by which FM weakening occurs as the result of two processes, *inflammation/infection and decidual hemorrhage/abruption,* which are both highly associated with preterm birth due to PPROM.

5.2 Inflammation/Infection induced FM weakening

Under normal circumstances, pregnancy is regulated by a balanced activity of anti-inflammatory and pro-inflammatory cytokines/chemokines (Ugwumadu et al., 2002; Diehl et al., 2002). They play an important role in placental growth and development (Croy et al., 2002). Progesterone may play an important role in continuation of pregnancy by promoting anti-inflammatory cytokines over pro-inflammatory cytokines. During the end of pregnancy at term, the activity of pro-inflammatory cytokines dominates resulting in the weakening and rupture of FM. At term, the amniotic fluid contains anti-inflammatory cytokines IL-10, IL-4, IL-1 receptor antagonist (Il-1RA) and transforming growth factor -β (TGF-β) [Jones et al., 1997; Dudley et al., 1996; Baergen et al., 1994; Romero et al., 1992b; A.G. Moore et al., 2000; Heikkinen et al., 2001]. The functions of these cytokines include suppressing pro-inflammatory cytokines (by IL-4), modulation of IL-1 mediated inflammatory effects (by IL-1RA) and inhibition of production of pro-inflammatory cytokines and suppression of the activity of antigen presenting cells (by IL-10).

The role of cytokines is not just limited to normal childbirth but also occurs in births (including preterm births) associated with infection. However, there are likely some differences (Bryant-Greenwood et al., 2007; Osman et al., 2006; Haddad et al., 2006; Beutler et al., 2004). The severity of inflammatory response that occurs in normal labor is relatively less compared to that seen in infections (Rusterholz et al., 2007). Relaxins, nuclear factor (NF)-kB and toll-like receptor (TLR)-2 are all part of the inflammatory cascade that result in

term/preterm labor or weakening and rupture of FM (Bryant-Greenwood et al., 2009; Millar et al., 1998; Werner et al., 2005; Hoebe et al., 2004). The amniotic fluid in normal uncomplicated pregnancy shows elevated pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) with increasing gestation (Opsjln et al., 1993; Romero et al., 1990b; Laham et al., 1994 & 1996), which is further increased during labor at term (Opsjln et al., 1993; Romero et al., 1990b; Laham et al., 1994; Cox et al., 1997; Maymon et al., 1999; Gunn et al., 1996; Romero et al., 1990a & 1992a). Infection during pregnancy further elevates the levels of these cytokines in amniotic fluid, FM and decidua (Goldenberg et al., 2000). This fact holds well even in preterm deliveries (compared to term) especially with infection and PPROM (Cox et al., 1997; Maymon et al., 1999; Romero et al., 1988 & 1993). The source of these cytokines is the infiltrating leucocytes as demonstrated by immunolocalization studies of gestational tissues (Young et al., 2002). Both amniotic fluid IL-16 and IL-18 are increased with infection. IL-18 is a pleiotropic cytokine that plays a role in host defense against infection whereas IL-16 is unique, functioning as a chemoattractant, modulator of lymphocytes, monocytes, eosinophils and dendritic cells, and in cell cycle control (Jones et al., 2009). On the other hand, the anti-inflammatory cytokines also increase with term and preterm labor, the most marked elevation occurring in preterm delivery with infection.

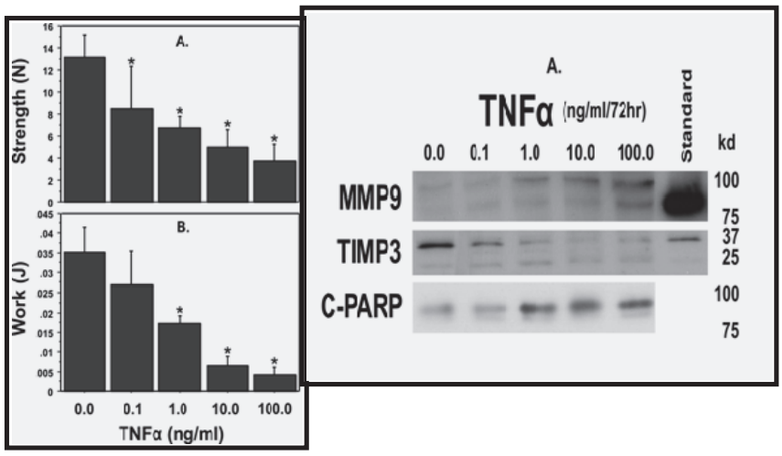


Fig. 7. TNF weakens full thickness FM. [Left] TNF-induced dose-dependent decreases in FM strength (A) and work to rupture (B). Incubations were for 72 h. [Right] TNF (0–100 ng/ml for 72 h) induced increases in MMP9 protein (upper panel) and decreased TIMP3 protein (middle panel) in FM explants in a dose-dependent manner. TNF also induced apoptosis in FM explants as evidenced by cleavage of PARP1 (113 kD) into 85 kD fragments (lower panel). Reproduced with permission from Biol Reprod 74:29-34, 2006 (Kumar D et al., 2006).

The tissue and cellular pathways involved in cytokine induced FM weakening are not fully understood. Although it has been generally assumed that collagen remodeling and apoptosis lead to FM weakening and rupture, this had not been directly demonstrated prior to our recent studies. Using our *in vitro* model system we have shown that inflammatory cytokines (TNF and IL-1β) weaken full thickness FM explants in a dose dependent manner concomitant with biochemical changes that mimic the signature of the natural Weak Zone (Fig. 7.) Cytokines act on FM in a complex manner. As the amnion is the strongest component of the FM, it must be significantly weakened in any mechanistic schema leading

to rupture (Arikat et al., 2006). Our published data show that cytokines do not weaken the isolated amnion, but readily weaken amnion when it is adherent to the choriodecidua as part of the full thickness FM (Kumar et al., 2011) [Fig. 8.]. In contrast, conditioned media produced by incubating isolated choriodecidua with cytokines readily weaken the amnion. It is thus clear that a critical cytokine target for FM weakening is in the choriodecidua, where one or more soluble products are produced which are necessary for TNF or IL-1β induced weakening of the amnion.

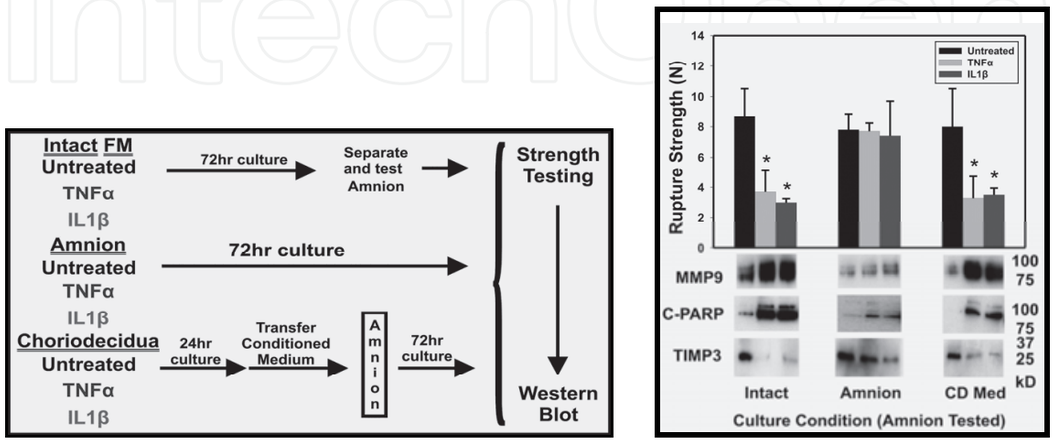


Fig. 8. TNF and IL-1β cannot weaken isolated amnion directly: Experiment description [left] and results [right]. Isolated amnion when incubated with cytokines (center group of results) showed no decrease in strength and minimal changes in MMP9, TIMP3 and cleaved PARP. Amnion exposed to conditioned media from choriodecidua incubated with cytokines showed weakening and biochemical changes (right group of results). Control study results show amnion weakening with intact FM stimulation (left group of results). Reproduced with permission from Placenta 32(3):206-213, 2011 (Kumar D et al., 2011).

5.3 Decidual hemorrhage/abruption induced FM weakening

Thrombin may be produced in FM by decidual hemorrhage/abruption and activation of the extrinsic coagulation cascade. Decidual cells have been shown to have abundant tissue factor thus facilitating the production of Thrombin after hemorrhage (Lockwood et al., 1993 & 2001). PPROM patients have increased tissue factor (Erez et al., 2008). Thrombin is known to have many actions not related to coagulation. Thrombin can act directly on the extracellular matrix (ECM) by converting MMPs 1, 2, 3 and 9 from zymogen to active forms (Fang et al., 2006; Lafleur et al., 2001). Alternatively, it can act indirectly, through cells, by activating Protease Activated Receptors (PAR) (Hollenberg et al., 2005; Ossovskaya et al., 2004) and thereby inducing MMPs and other proteins, which may remodel the ECM. Thrombin has been shown to induce MMP9 in monocytes (Chi-Jen Chang et al., 2009), oral squamous cell carcinoma (Liu et al., 2001), human dermal fibroblast (Wang et al., 2007) and mesangial cells (Liu et al., 2000), microvascular endothelial cells from human brain (Kolev et al., 2003) and rabbit aortic smooth muscle cells (Fabunmi et al., 1996). Four PARs (1 – 4) have been described but none have yet been reported in FM. PARs are activated by the proteolytic activity of thrombin and other serine proteases (thrombin acts on PARs 1, 3 and 4 and trypsin acts upon PAR 2). These enzymes cleave the N-terminus of the receptor,

which thereafter permanently acts as a tethered ligand causing a physiological response. Synthetic PAR activating peptides (PAR-AP) composed of the initial amino acid string of the new N-terminus generated when thrombin cleaves PAR, can activate their specific PAR independent of thrombin or receptor cleavage (Refer table below).

Receptor	Activating Peptide	Control Peptide
PAR 1	TFLLR-NH2	FTLLR-NH2
PAR 2	SLIGRL-NH2; 2furoyl-LIGRLO-NH2	LSIGRL-NH2; 2furoyl-OLRGIL-NH2
PAR 3	NOT activated by PAR-APs	---------
PAR 4	AYPGKF-NH2	YAPGKF-NH2

Table 1.

These PAR-APs have been used extensively as pharmacological tools for probing the effects of specific PAR activation in cells and tissues independent of the other effects of thrombin. PAR-APs are commercially available for most of the PAR receptors (table). Inactive control peptides with a reversal of two AA's are also available (Ramachandran & Hollenberg, 2008). We have evidence that the PAR-AP for PAR 1 weakens isolated amnion (fig 10 – sub figure 2). The PAR receptors act through G-proteins families including: I (cAMP inhibitory), 12/13 (Raf/Ras activation) or q (calcium signaling) which in turn activate a number of signaling cascades including MAP kinases, Akt/PKB, P13 kinase and NFkB (101).

Our recent report demonstrates that Thrombin weakens full thickness FM in a dose dependent fashion in our model system (Moore et al., 2010, fig. 9). We have also reported that Thrombin increases both the zymogen and active forms of MMP-9 and MMP-3 in the amnion (Furuta et al., 2000). The latter is particularly interesting as MMP-3 can activate other MMPs, especially MMP-2, which is prevalent in the amnion but is not further induced by either cytokines or Thrombin.

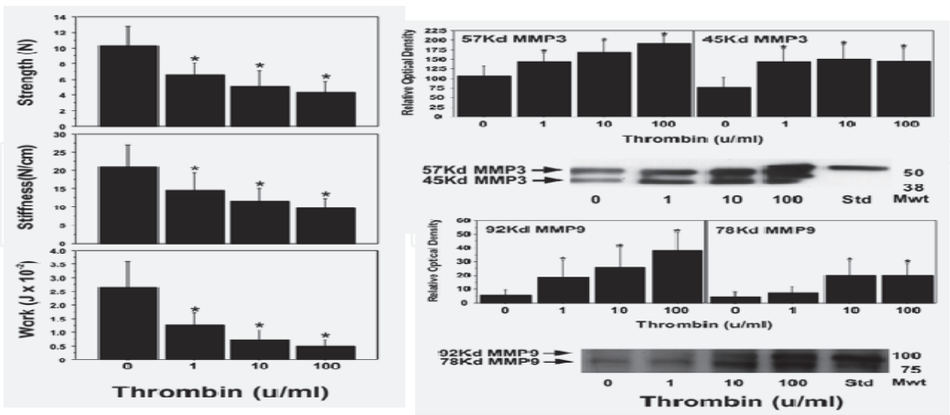
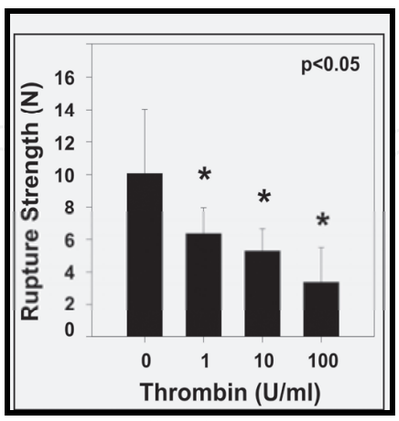
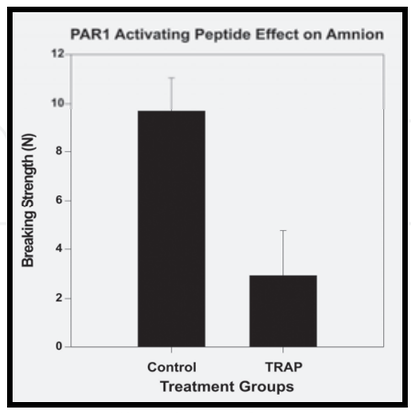


Fig. 9. Thrombin weakens full thickness FM. [Left] Thrombin-induced dose-dependent decreases in FM strength, stiffness and work to rupture. All incubations were for 48 h. (* $P < 0.01$). [Right] Thrombin induced increases in MMP3 protein (upper panel) and MMP9 protein (lower panel) in FM explants in a dose-dependent manner. (Reproduced with permission from Moore et al. Placenta 2010.)

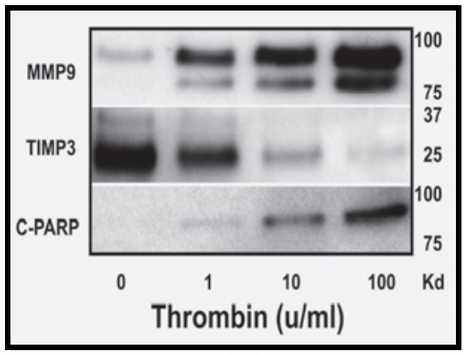
In further work we have found that, unlike cytokines, thrombin can directly weaken the isolated amnion. The critical targets for thrombin are thus in amnion rather than the choriodecidua. [Fig. 10 – subfigures 1, 3 & 4]



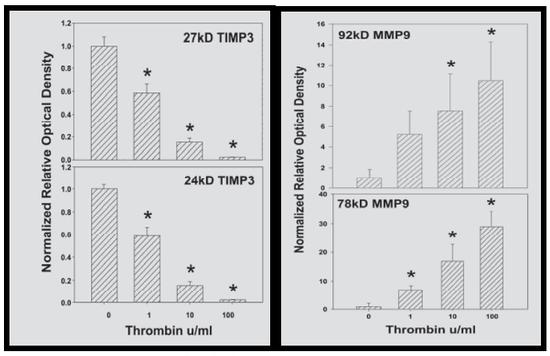
Subfigure 1



Subfigure 2



Subfigure 3



Subfigure 4

Fig. 10. [Top Left] Thrombin weakens isolated amnion. (* p < .01 vs. control),
[Top Right] PAR 1 (1 uM) activating peptide (TRAP) weakens isolated amnion (p<.001),
[Below] Thrombin increases MMP9, decreases TIMP3 and increases PARP cleavage in isolated amnion (all *p<.001). . (Reproduced with permission from Kumar et al. Placenta 2011.)

5.4 Inflammatory cytokines and thrombin have different mechanisms of FM weakening

As the amnion is the strongest component of the FM, it must be significantly weakened in any mechanistic schema leading to rupture. For this reason agents that weaken FM were tested for their effect upon isolated amnion membranes. Cytokines (TNF or IL-1β) do not weaken the isolated amnion, but readily weaken amnion when it is adherent to the choriodecidua as part of the full thickness FM. In contrast, conditioned media produced by incubating isolated choriodecidua with these cytokines readily weaken the amnion. It is thus clear that a critical cytokine target for FM weakening is in the choriodecidua, where one or more soluble products are produced which are necessary for TNF or IL-1β induced weakening of the amnion (Kumar et al., 2011). On the other hand Thrombin can directly

weaken the isolated amnion. The critical targets for Thrombin induced FM weakening are thus in amnion rather than the choriodecidua (Kumar et al., 2011).

6. Investigations targeting the potential prevention of FM weakening and rupture

6.1 Tissue repair and prevention of weakness

A major objective of research into the physiology of FM rupture is the development of possible strategies for membrane repair or prevention of premature weakening. Studies of tissue repair after physically induced injury have been limited. Artal (Artal et al., 1976) demonstrated that membranes placed in pseudo-amniotic fluid become weaker over 24 hours but remain unchanged if a mixture of pharmacological enzyme inhibitors is included. The tissue surrounding a stab wound in FM has been shown to be viable for up to 12 days but the wound remained unhealed (Devlieger et al., 2000a). Millar et al (2003) demonstrated increased growth of amnion epithelium with IGF-II. Using a rabbit model, Devlieger et al (2000b & 2003) have shown some success in incorporating plugs into iatrogenic ruptured sites. Growth factors, TGFβ and FGFβ, which normally accelerate repair in other tissues, were shown by the same group to improve the rate of FM repair, although the concomitant increased inflammatory response was of concern because of its possible exacerbation of events leading to premature delivery (Devlieger et al., 2003). FM injured *in vitro*, thus seem to have minimal tendency for self-repair. Clinical studies of FM sealants and sealing techniques have been undertaken in hopes of restoring amniotic fluid volume and averting the risks of prolonged oligohydramnios after extremely early PPROM. An *in vitro* study has revealed that platelets aggregate with exposure to amnion, but not to chorion or amniotic fluid, and that platelet adhesion and activation occur with exposure to the connective tissues underlying amnion and chorion but not amniotic epithelium (Louis-Sylvestre et al., 1998). Similarly, Harmali et al have demonstrated improved membrane tensile strength (Rupture Tension, Strain to Rupture, and Work to Rupture) with iatrogenic defects after application of a fibrin sealant, but that the membrane strength remains less than that of unruptured membrane segments (Harmanli et al., 1998). Fibrin/thrombin based sealants, but not platelet infusions, have been shown to cause temporary cessation of leakage (Reddy et al., 2001). Regarding non-biologic barriers, O'Brien et al (2001) evaluated gelatin sponge (Gelfoam, TM) applied over larger defects and found it to effectively dam linear defects of up to 7 mm in length, but not complex defects. The ability to fuse ("weld") FM with Nd: YAG Laser applied to polytetrafluoroethylene has also been demonstrated *in-vitro*, though the ability of such intervention to seal a membrane defect has not (Mendoza et al., 1999).

There has been some success in the treatment of iatrogenic membrane rupture. In 1999, Quintero and co-workers (Quintero et al., 1998 & 1999) demonstrated successful membrane sealing for persistent oligohydramnios after amniocentesis and fetoscopy, using intra-amniotic injection of platelets and cryoprecipitate ("amniopatch") through a 22-gauge needle. Successful membrane sealing has been achieved with intrauterine injection of gelatin sponge (O'Brien et al., 2002). Sciscione and co-workers (2001) were able to achieve cessation of leakage and restoration of normal amniotic fluid volume in some women with intracervical instillation of fibrin sealants. However, the need for concurrent cervical cerclage placement to decrease fluid leakage has been described (Baumgarten & Moser, 1986). Young and colleagues (Young et al., 2000, 2004a) demonstrated successful endoscopically guided sealing of an iatrogenic membrane defect using maternal platelets

and fibrinogen/thrombin and subsequently demonstrated success with fibrin glue and powdered collagen slurry applied to puncture sites. While these studies, and other small case series, have raised the potential that membrane sealing can be performed effectively when the defect occurs in otherwise normal FM, further study regarding the optimal timing and method of intervention after iatrogenic PROM is needed.

Ultimately, because of the presence of antecedent membrane degradation and sub-clinical inflammation in the fetal membranes, membrane sealing may not be appropriate for those with spontaneous membrane rupture. It is not surprising that membranes that have ruptured as the result of having undergone extensive extracellular remodeling and cellular apoptosis may not readily reverse their physiological direction and repair themselves. Further study regarding the maternal/fetal risks and fetal benefits of invasive interventions for membrane rupture remote from term are warranted before such practices are incorporated into clinical practice. A detailed understanding of the process of physiological and pathological fetal membrane weakening may allow appropriate early intervention. Our models of in vitro tissue weakening with biochemical agents and stretch may prove useful in evaluating potential repair strategies.

6.2 Prevention of weakening and rupture – Identification of at risk pregnancies

If the potential for FM repair after rupture is limited, perhaps premature weakening of the fetal membranes can be prevented. To be medically and ethically acceptable, any procedure done on pregnant women to prevent possible FM weakening must be either totally innocuous or highly targeted toward women at significant risk for PPROM. In order to target women at risk they must first be reliably identified.

Spontaneous preterm birth due to preterm labor and PROM is associated with a variety of clinical characteristics and also with abnormal ancillary test findings (e.g. short cervix on transvaginal ultrasound, a positive cervicovaginal fetal-fibronectin screen) (Rangaswamy et al., 2011; Iams et al., 1996; Mercer et al., 1996; Goldenberg et al., 1996b). However, such evaluations are neither sensitive nor specific when applied to asymptomatic women, and there is significant overlap in the characteristics of women who ultimately deliver due to preterm labor and PROM (Mercer et al., 2000). As such, it remains difficult to identify those who will develop PROM before the fact. Recently, several groups have demonstrated that African Americans, a group with a high incidence of PPROM with early delivery, have a higher incidence of certain more active polymorphisms of MMPs and pro-inflammatory cytokines (Ferrand et al., 2002; Fujimoto et al., 2002; Hernandez-Guerrero et al., 2003; Roberts et al., 1999). It is possible that a combination of genes and historical factors could eventually lead to prediction of risk of PPROM with adequate certainty that procedures designed to prevent FM rupture that carry some inherent risk would be justifiable.

In the presence of minimal or no identified risk, administration of vitamin C alone or in combination with vitamin E has been suggested for the prevention of PPROM, mainly for their antioxidant effect (Siega-Riz et al., 2003). However, there are concerns regarding the use of vitamin C as it can increase or decrease apoptosis (Orzechowski et al., 2002) depending on the cell system, dose, and co-effectors present. We have shown that vitamin C does not inhibit, and may exacerbate H_2O_2 induced apoptosis in amnion-derived WISH cells, amnion cell cultures, and amnion membrane explants (Kumar et al., 2004a, 2004b). In vivo studies have also shown mixed results with use of vitamin C. Although a single clinical trial of oral vitamin C demonstrated a decrease in PROM but not premature delivery, three much larger studies showed increased PROM and PPROM in the vitamin-supplemented

groups (Spinnato et al., 2008; Hauth et al., 2010; Xu et al., 2010). Warnings were issued in these publications about use of vitamins C/E, at least at the doses utilized. Using our *in vitro* model of TNF-α-induced FM weakening, we have demonstrated that vitamin C preincubation does not prevent TNF-α induced FM weakness. In fact, high doses of vitamin C actually increase MMP-9 activity and weaken the FM.

6.3 Alpha-Lipoic Acid (LA) inhibits cytokine and thrombin induced weakening

In contrast, we have shown that Lipoic acid, a naturally occurring dietary supplement [routinely used as part of prophylaxis for diabetic neuropathy (Ziegler et al., 2009)], with anti-oxidant and NFkB inhibitory properties prevented TNF-α induced FM weakening and also prevented concomitant TNF-α induced increases in MMP-9 and PGE2 (Fig. 11.) [R.M. Moore et al, 2009]. The mechanism of Lipoic acid action is unclear but this effect on FM is not isolated. It has been shown to inhibit TNF- α induced NF-κB transcriptional activity and MMP-9 expression in vascular smooth muscle cells, and attenuate LPS-induced NF-κB: DNA-binding in monocytes by activating the PI3K/AKT pathway (Kim et al., 2007; Zhang et al., 2007). It also metabolically regenerates glutathione and other antioxidants (Bilska & Wlodek et al., 2005). We have also demonstrated that LA inhibits FM weakening by Thrombin and inhibits Thrombin induced weakening of the isolated amnion (Kumar et al., 2011). As Lipoic acid inhibits both cytokine and thrombin induced FM weakening and remodeling, it is a promising candidate for possible clinical use to prevent PPROM. LA has been investigated in dozens of clinical trials (41 trials are now listed on clinicaltrials.gov) with no adverse effects reported. Unfortunately, all of the trials excluded pregnant women (Clinicaltrials.gov). There have also been promising toxicity studies in pregnant rats (Shirpoor A et al., 2008). A trial of LA in pregnant women at risk for PPROM awaits more definitive information of its mechanism of action in the prevention of FM weakening in addition to safety data.

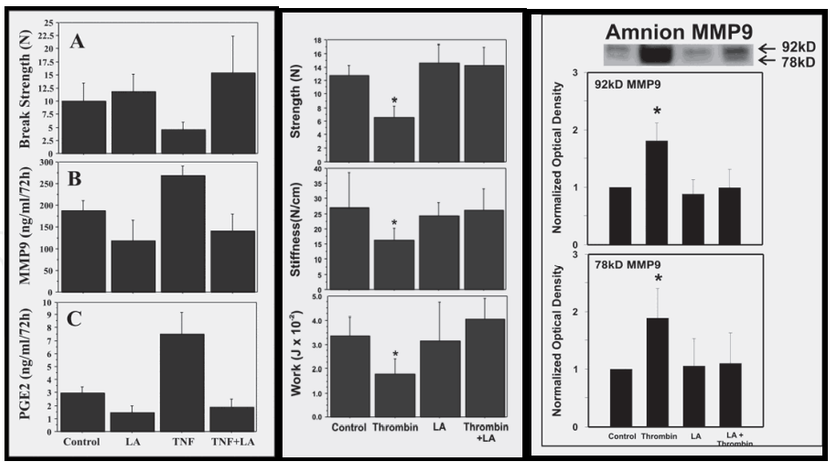


Fig. 11. Lipoic Acid inhibits TNF and Thrombin induced weakening and biochemical changes: [Left] TNF (50 ng/ml) induced weakening, increases in MMP9 and increases in PGE2 are inhibited by Lipoic Acid (0.5 mM); [Middle] Thrombin (10 U/ml) induced changes in biomechanics are inhibited by lipoic acid (.25 mM); [Right] Thrombin (10 U/ml) induced increases in MMP9 zymogen and active forms are inhibited by Lipoic Acid (.25 mM). Reproduced from Moore et al. Biol Reprod 80:781-87, 2009 and Placenta 31:886-892, 2010 with permission.

7. Conclusions

It had been previously thought that the mechanical force due to uterine contraction during labor weakens the FM. This theory has been negated by the clinical fact that the FM rupture prior to the onset of contractions and by our finding that the force required to rupture the membranes is increased rather than decreased by cyclical mechanical stretching of FM *in vitro*. We have demonstrated that a weak zone develops in the FM overlying the cervix at the end of gestation. This weak zone is associated with collagen remodeling and apoptosis as evidenced by elevated MMP-9, decreased TIMP-3 and elevated PARP cleavage. Even though the process of weakening in term FM has been described, detailed molecular mechanisms and the initiating signals have yet to be unraveled. Our in vitro model system has facilitated demonstration that the weakening effect of cytokines (TNF-α and IL-1β) and thrombin on FM results in collagen remodeling and apoptosis, similar to that observed in term FM delivered by unlabored cesarean and labored vaginal deliveries. Lipoic acid has been shown to inhibit the weakening effect of both these agents *in vitro*, suggesting that it could be a promising drug in the prevention of PPROM. However, studies are required to demonstrate its safety and efficacy in pregnant women.

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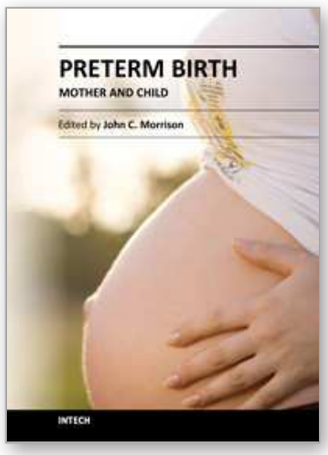

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While there are many studies and books regarding preterm birth, both the obstetric and in the neonatal/pediatric literature, what is missing is the integration of data from obstetrics through neonatal course and into pediatrics as the neonate transverses childhood. A continued dialogue between specialties is essential in the battle against preterm birth in an attempt to relieve the effects or after-effects of preterm birth. For all of our medical advances to date, preterm birth is still all too common, and its ramifications are significant for hospitals, families and society in general.

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