Timing of Fetal Meconium Absorption by Amnionic Macrophages

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ABSTRACT

This study sought to determine the time interval from fetal defecation to membrane staining and subsequent meconium uptake by amnionic macrophages. Pieces of external membranes were fastened to Teflon rings to create a well. The amnionic surface was exposed to meconium and amniotic fluid for 1 to 48 hours and analyzed microscopically for the presence of free meconium and meconium-laden amnionic macrophages. In each of the experiments, prior to the 12-hour time point, few meconium-laden macrophages were present in the membrane layers. A significant rise in the number of meconium-laden macrophages and a concomitant increase in staining intensity was noted in the membranes at 24 and 48 hours. Contrary to previous reports, our in vitro model of meconium incorporation into placental membranes demonstrated that significant numbers of meconium-laden macrophages were only observed after 24 and 48 hours.

KEYWORDS: Meconium, pigment-laden macrophages, placenta, transport kinetics

The mechanisms responsible for meconium passage are still not well understood. Meconium, which is intestinal content of the fetus that is usually not eliminated until after birth, consists of mucus, dead cells, and bile pigment, which gives meconium its characteristic coloration.[1] It remains controversial as to whether in utero passage of meconium results from hypoxic stress or the normal maturation of the gastrointestinal tract. Recent studies have found that postterm infants discharge meconium in the absence of fetal distress or hypoxia.[1]

Nevertheless, an association exists between the presence of meconium in the amniotic fluid and increased neonatal morbidity and mortality. It has been demonstrated that meconium passage is more than twice as likely to occur when biophysical profile scores are abnormal.[2] High concentrations and prolonged exposure to meconium are suggested to cause umbilical cord damage.[3,4] The ischemic conditions induced by chronic meconium passage and the toxic and diffusible meconium intermediaries may produce direct cellular damage to the fetal brain and lead to such conditions as hypoxic cerebral palsy and neonatal asphyxia in preterm births.[5] Although meconium has become the "red flag" for obstetricians and the legal profession, the details of meconium transit into the placental membranes has not been confirmed.

Determining if meconium passage is an antepartum versus an intrapartum event has significant clinical and medicolegal implications.[6] There is limited literature addressing the transport kinetics of fetal meconium. In the only in vitro study of meconium-transport kinetics, Miller et al proposed that meconium-containing macrophages are present in the amnion within 1 hour after

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meconium exposure and within the chorion after 3 hours.[7] They also reported that gross placental staining is a surface phenomenon proportional to the length of exposure and meconium concentration. Although these results have been generally accepted, the experimental model employed does not necessarily reflect in vivo conditions. For example, a diluted 10% solution of meconium was used, instead of starting with pure meconium as occurs at the time of defecation. In addition, membrane pieces were free-floating in the meconium mixture, allowing meconium absorption by both sides of the membrane simultaneously. This does not mimic in utero conditions, in which meconium is taken up only by the smooth amnionic surface. We sought to more closely replicate true antepartum conditions and address these experimental concerns.

MATERIALS AND METHODS

This study was approved by the Human Investigation Committee at Yale University. In each of the three experiments, a syringe filled with 10 mL of clear amniotic fluid was obtained from elective, repeat cesarean sections performed in the Section of Maternal-Fetal Medicine at Yale-New Haven Hospital. None of the subjects had any obstetric or medical complications. To minimize contamination with blood, the maternal myometrium was carefully dissected while leaving the membranes intact, so that fluid was obtained immediately upon rupture. The placenta was also collected under sterile conditions after being allowed to separate spontaneously. Fresh meconium was taken from a newborn's first defecation in the well-born nursery on the same day as membrane and placenta were collected.

Seven squares of external membrane measuring ~5 × 5 cm each were dissected from each placenta. To serve as a control and "0-hour" time point with no meconium exposure, one square of membrane was immediately placed in formalin fixative. Additionally, 10 mg of meconium and a section of placenta were also fixed in formalin. The remaining six squares of membrane were uniformly stretched tightly across the top of Teflon® (DuPont, Wilmington, DE) rings such that the amnion surface formed the base of the newly created well.[8] The membrane was fastened tightly in place by a rubber O-ring that snapped into a groove in the outer surface of the Teflon ring. The resulting Teflon-membrane assembly (TMA) was then oriented so that the well was facing up (Fig. 1).

The six TMAs were then placed in a sterile 15-cm petri dish with 20 mL of Dulbecco's modified Eagle's medium containing 1 μg/mL Fungizone® (Bristol-Myers Squibb, Princeton, NJ) and 100 units/mL penicillin and 0.1 mg/mL streptomycin. Meconium (10 mg) and 1 mL of amniotic fluid were added to each well. This concentration correlates with what clinicians describe as "thin" meconium.[9] To mimic maternal ambulation, each petri dish was then placed on a battery-operated shaker and gently stirred inside of a humid incubator set at 37°C in a 5% CO_2 atmosphere for 1, 3, 6, 12, 24, and 48 hours, at which times one TMA was carefully removed with a spatula and placed in a cup of formalin and refrigerated for later analysis.

After fixation for at least 1 day, the amniotic fluid-meconium mixture was filtered to collect the residual meconium in each well and processed for histological examination. The excess membrane around the rubber O-ring was cut into strips with a sharp scalpel blade and processed for histological examination. The rubber O-ring was then removed from each TMA, and the resultant membrane section was similarly cut into thin strips and processed for histological examination. All histological sections were stained with hematoxylin and eosin (H&E) to visualize the meconium-laden

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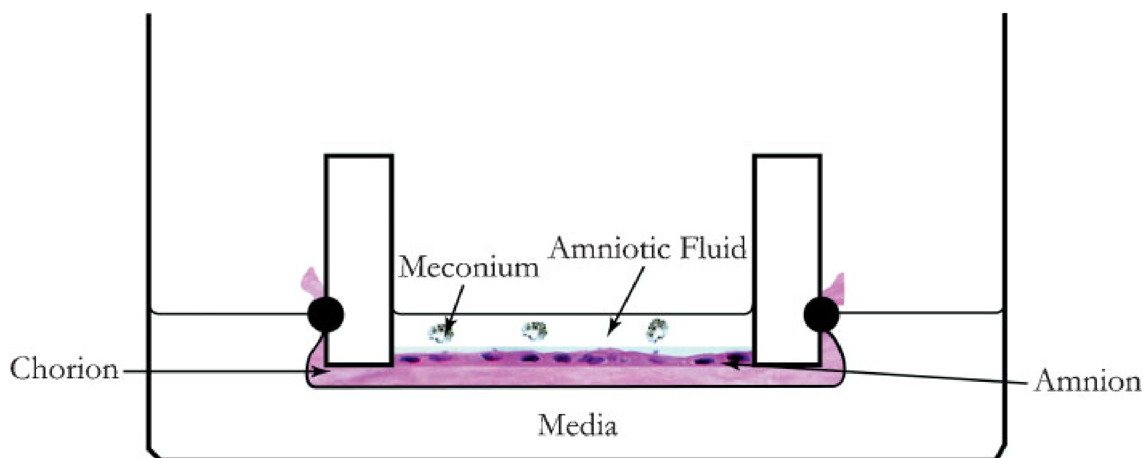

Figure 1 Teflon-membrane assembly. A piece of external membrane is placed over a Teflon® (DuPont, Wilmington, DE) ring and held in place by a rubber O-ring so that the amnion epithelium forms the base of the formed well. Amniotic fluid and meconium are placed into the well and the assembly is placed in culture medium in a petri dish and incubated with agitation at 37°C.

Table 1 Summary of Experimental Observations

Hours	Gross Membrane Color (Ex. 1, Ex. 2, Ex. 3)	Amnionic Epithelium (Ex. 1, Ex. 2, Ex. 3)	Free Meconium in Amnionic Epithelium (Ex. 1, Ex. 2, Ex. 3)	Free Meconium in Amnion (Ex. 1, Ex. 2, Ex. 3)	Free Meconium in Chorion/Decidua (Ex. 1, Ex. 2, Ex. 3)
0	Pink, pink, pink	I, I, I	−, −, −	−, −, −	−, −, −
1	Pink, pink, pink	I, IV, I	−, −, −	−, +, −	−, −, −
3	Pink, pink, pink	I, IV, I	−, +, −	−, +, −	−, −, −
6	Pink, pink, pink	I, IV, I	−, +, −	−, +, +	−, −, −
12	Pink, pink, FGT	I, IV, I	+, −, −	−, +, −	−, −, −
24	LGT, LGT, LGT	NI, IV, I	+, −, +	+, +, −	−, −, −
48	LGT, DGT, DGT	NI, IV, I	+, −, +	+, +, +	−, +, −

When observations were performed in duplicate, means are reported. Summary of observations made at each time point between 0 and 48 hours for each of three experiments, including gross membrane color, state of amnionic membrane, presence of free meconium within the amnion epithelium, amnion connective tissue, and chorion/decidua layers, as well as the total number of meconium-laden macrophages counted in the amnion layer. Ex., experiment; FGT, faint green tint; LGT, light green tint; DGT, dark green tint; I, intact; V, vacuolated; NI, not intact; +, presence; −, absence.

macrophages. To quantitate the total number of macrophages present in each membrane strip, histological sections were also stained with mouse anti-human CD68 (anti-macrophage clone EBM11; Dako, Carpinteria, CA), CD15 (anti-neutrophil clone C3D-1; Dako), and NMA (normal mouse ascites negative control clone NS-1; Sigma-Aldrich, St. Louis, MO), as previously described.[10] These $\sim 0.2 \times 3$-cm sections were then examined to determine the presence of free meconium (meconium not within any cellular elements) in the amnion epithelial, amnion, and chorion/decidua layers; the total number of neutrophils and macrophages within the amnion and chorion/decidua layers; as well as the number of macrophages harboring meconium in the latter two layers. In total, 44 membrane cross sections were examined, derived from three placentas, and spanning a 0- to 48-hour time frame (Table 1). The free meconium and meconium contents of the macrophages were assessed on a scale from 1+ to 3+, in which 1+ represented weakly staining pigment and 3+ represented the strongest pigment staining. Thus, the visualization of meconium-laden macrophages determined the time course of meconium absorption by amnionic macrophages within placental tissue. The total number of meconium-containing macrophages divided by the total number of macrophages identified immunohistochemically in a serial section was calculated for each time point for each experiment. The mean percent ± standard error of the mean of the percent meconium-laden macrophages normalized to the total number of macrophages found in the amnion layer at each time point was calculated. The 0-hour placental membranes were closely examined microscopically to assess baseline pathologic findings.

RESULTS

The absence of neutrophils (as determined by H&E and CD15 staining) and free meconium in all three 0-hour time points confirmed that specimens were obtained from pregnancies without evidence of an intra-amniotic fluid infection or recent meconium passage. One specimen revealed a few meconium-laden macrophages, suggesting a remote exposure to meconium.

During the 48-hour time course, the amniotic fluid-suspended meconium clearly dissociated and became lighter in color in all three experiments. At 48 hours, there was little left of the original applied meconium. Upon gross examination, the fixed membrane squares prior to the 24-hour time point had a generally pink coloration. At the 24-hour time point, the membranes appeared to have a slight greenish tint, which become noticeably darker at the 48-hour time point. A summation of observations is provided in Table 1.

The amnionic epithelium in two of the three experiments remained intact prior to the 24-hour time point. No free meconium was found in the amnionic epithelium prior to the 3-hour time point. Two of the three trials exhibited no free meconium at the 3- and 6-hour time points. At the 12-hour time point, free meconium was found only in one trial. At the 24- and 48-hour time points, the amnionic epithelium of two of the three experiments displayed free meconium. At the 48-hour time points in all three experiments, there was significant free meconium present in the amnionic connective tissue layers. There was a general absence of free meconium in the chorion/decidua layer up until the 48-hour time point. In only one experiment was the presence of free meconium discerned at the 48-hour time point. No meconium-laden macrophages were detected in the chorion/decidua layer at any time point.

To normalize the number of observed meconium-laden macrophages between samples, we counted the total number of macrophages in each specimen using CD68 immunohistochemistry and expressed the meconium-laden macrophages as a percent of all the macrophages present (Fig. 2). The number of meconium-laden macrophages compared with total macrophages in these observations ranged from 0/126 at the 0-hour time point

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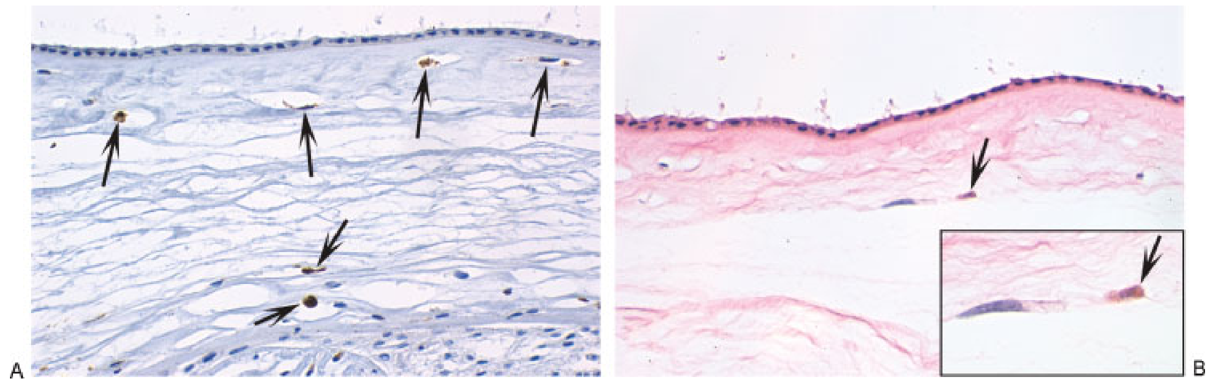

Figure 2 Normalization of meconium-laden macrophage counts with CD68 immunohistochemistry. (A) Membrane strips were immumonohistochemically stained with anti-CD68 antibodies to reveal macrophages (arrows), which were totaled for each sample. (B) Hematoxylin and eosin–stained membrane strips were examined to assess the total number of meconium-laden macrophages (arrow) present in each strip. Inset shows one macrophage without meconium and one containing meconium (arrow).

to a maximum of 80/120 at the 48-hour time point. The normalized percentage of meconium-laden macrophages at each time point from all three experiments illustrated a clear trend: very few meconium-laden macrophages were present prior to 12 hours, with a significant increase at 24 hours (Fig. 3). Not only was the frequency of meconium-laden macrophages greater at 24 and 48 hours compared with the first 12 hours, but these macrophages also exhibited increased amounts of intracellular meconium compared with earlier time points.

DISCUSSION

Our study endeavored to closely simulate in utero meconium absorption by amnionic membrane macrophages. Unlike the prior work by Miller et al,[7] who predissolved frozen meconium into saline and then applied this solution to both surfaces of their external membrane samples, we accounted for the fact that meconium dissolution into amniotic fluid is a crucial, time-consuming step in the entire process of meconium absorption by macrophages. In addition, meconium and amniotic fluid were only applied to the smooth amnionic surface normally exposed to the fetus. These results demonstrate that the depth of penetration by meconium pigment into the placental membranes, with subsequent uptake by amnionic macrophages, is directly related to length of exposure. More importantly, our results indicate that appreciable numbers of meconium-laden macrophages are not present in the amnion until the 24- and 48-hour time points. These findings suggest that other investigators[7] may have underestimated the timing of meconium absorption by amnionic membrane macrophages.

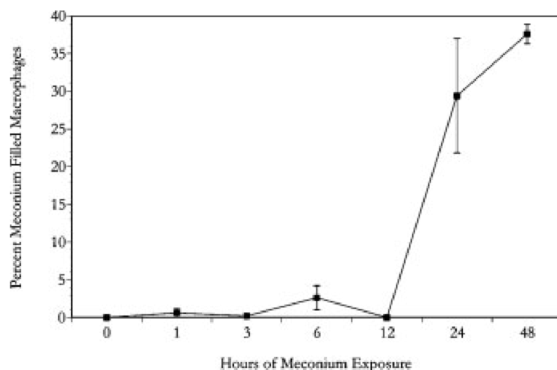

Figure 3 Time course of appearance of meconium-laden macrophages. A composite graph of all three trials showing the mean percent ± standard error of the mean of the percent meconium-laden macrophages normalized to the total number of macrophages found in the amnion layer at each time point.

Because the appearance of meconium-laden macrophages has been used to estimate the timing of in utero meconium passage, any experiment designed to replicate this in vivo process must include all the necessary steps. These steps include (1) meconium dissolution, (2) penetration of the amnionic epithelium and connective tissue, and (3) ingestion by the membrane macrophages. As we have shown, the minimum time for all three of these steps to be completed is at least 24 hours.

We acknowledge that our work may have limitations. Our in vitro model was able to neither replicate the in vivo proximity to the maternal decidua and its blood flow nor simulate the dynamic forces associated with uterine contractions. However, we believe our study is the closest approximation of meconium absorption kinetics published thus far.

These results may have clinical relevance beyond simple meconium kinetics. Aggressive oropharyngeal suctioning and amnioinfusion following meconium passage have been proven ineffective in decreasing the incidence and severity of meconium aspiration syndrome.[11,12] We posit that this is due to the fact that in many cases, meconium passage had taken place long before delivery. A new appreciation of the length of

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meconium exposure may help to more fully explain associated lung damage and our inability to mitigate meconium aspiration syndrome.[13] Finally, by more accurately determining the meconium passage-to-birth interval, we will be able to provide a better estimate of when the fetus may have been stressed in utero.

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