

# Microscopic changes induced by the intratracheal inoculation of amniotic fluid and meconium in the lung of neonatal rats

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**Summary.** Meconium aspiration syndrome is a major contributor to neonatal respiratory distress in infants and it has been sporadically recognized in neonatal animals. This investigation was designed to study the short and long term effects of meconium and amniotic fluid in the lungs of neonatal rats. Seven-day-old rats (n=123) divided in three groups were intratracheally inoculated with saline solution, amniotic fluid or meconium. Rats were euthanatized on 1, 3, 7, 14, 28, 56 and 112 postinoculation days (PID) and the lungs were examined by light microscopy. Saline solution did not induce any change while amniotic fluid elicited only a mild foreign body response which disappeared by PID 14. In contrast, meconium induced an exudative alveolitis characterized by recruitment of neutrophils in the bronchoalveolar spaces. Meconium also induced atelectasis, hyperinflation and thickening of alveolar septa all of which had disappeared by PID 14. Starting at PID 7, neutrophils were progressively replaced by macrophages, giant cells, and some fibroblasts. There were sporadic foci of mineralization starting at PID 14 and lasting up to PID 112. Some mineralized foci became lined with cuboidal epithelial cells at PID 28. Meconium was slowly degraded but still evident by PID 112. It was concluded that inoculation of meconium in neonatal rats induces acute microscopic changes typical of meconium aspiration syndrome. The long term lesions induced by meconium consisted of persistent multifocal histiocytic alveolitis and bronchiolitis reaction with occasional foci of calcification.

**Key words:** Lung, Neonatal rats, Amniotic fluid, Meconium, Aspiration

## Introduction

Meconium aspiration syndrome (MAS) is an important condition in human perinatology with a convoluted pathogenesis that causes severe respiratory distress, lung inflammation and occasional death in newborn babies (Cleary and Wiswell, 1998; Srinivasan and Vidyasagar, 1999; Wiswell, 2001). A quantum of work has been published on the prevention and clinical management of MAS (Katz and Bowes, 1992; Wiswell and Bent, 1993; Wiswell, 2001) however, little attention has been paid to the long term morphologic changes that may develop in the lungs of babies surviving this syndrome (Jovanovic and Nguyen, 1989).

During fetal stress and hypoxia, redistribution of blood away from the abdominal viscera increases intestinal peristalsis and causes relaxation of the anal sphincter eventually culminating with defecation of meconium into the amniotic sac (Wiswell and Bent, 1993). If severe anoxia persists, the fetus gasps for "air" *in utero* aspirating amniotic fluid contaminated with meconium into the lung. Another form of MAS occurs when babies are born with meconium lodged in the hypopharynx that is subsequently aspirated with the first breaths of air during birth. In either case, meconium reaches the lung causing pulmonary irritation, inflammation, airway obstruction and aeration problems (Co and Vidyasagar, 1990; Cleary and Wiswell, 1998; Srinivasan and Vidyasagar, 1999).

Autopsy reports from babies succumbing to MAS during the first few days of life typically show meconium in the lungs, which in most cases, can be easily detected in hematoxylin eosin stained sections because of its bright yellow appearance. In the lung, meconium induces a moderate inflammatory reaction centered around the terminal bronchiolar and alveolar regions causing a pathological condition commonly referred to as chemical pneumonitis (Tyler et al., 1978; al Mateen et al., 1994). However, the long term effects of meconium aspiration on lung morphology remain

unknown.

Although many investigators have described the detailed microscopic appearance of the lungs of babies succumbing to an acute episode of MAS, there is lack of information regarding the long term effect that aspirated meconium has in the lung (Brown and Gleicher, 1981; Wiswell and Bent, 1993). This paucity of information is particularly intriguing since delayed clinical signs such as persistent pulmonary hypertension, airway hyperresponsiveness, obstructive airway disease and high risk for pulmonary infection have all been reported in neonates with history of MAS (Swaminathan et al., 1989; Wiswell and Bent 1993; Srinivasan and Vidyasagar 1999). It is important to point out that researchers in many experimental studies on the pathology of MAS have utilized adult laboratory animals disregarding the fact that MAS is a disease of the newborn and without considering the differences in the inflammatory response amongst fetus, neonate and adult animals (Cruickshank, 1949; Sugimoto et al., 1980). Also, the significance of using heterologous rather than homologous meconium in experimental models of MAS has not been properly evaluated. The objective of this investigation was to study the acute and chronic histologic changes in lungs of neonatal rats intratracheally inoculated with homologous murine amniotic fluid and meconium.

## Material and methods

### Animals

Fischer-344 female and male rats obtained from commercial sources (Charles River Inc., St Constant, Quebec, Canada) were kept at the laboratory animal facility of the Atlantic Veterinary College (AVC). Adult rats were housed individually and provided with commercial rat food and water *ad libitum*. Female rats were bred and put in a time pregnancy program based on vaginal cytology as reported by others (Waynfirth, 1980). At the time of birth, neonates were separated by sex. Male pups were assigned to experimental groups for inoculation studies while female pups were euthanatized and used as a source of meconium (Martínez-Burnes et al., 2001a). During the experiments, rat pups were maintained with the dam and kept on a 12/12-hour light/dark cycle at 22 °C and 50% relative humidity. The Animal Care Committee, AVC, approved experimental protocols and experiments were conducted following official guidelines (Canadian Council on Animal Care, 1993).

### Collection and preparation of inocula

Amniotic fluid aseptically collected from the placenta of full-term pregnant rats was placed in sterile plastic vials and stored at 4-6 °C (Martínez-Burnes et al., 2001a). The inoculum consisted of 100% undiluted amniotic fluid. Female rat pups were euthanatized with

an anaesthetic overdose of halothane and meconium was aseptically obtained from the intestine immediately after birth and before the intake of the colostrum. Prior to inoculation, samples of meconium and amniotic fluid were submitted for bacteriological analyses and the results confirmed that the inoculum was sterile. Meconium was stored at -80 °C until preparation of the inoculum. The final concentration of unfiltered meconium was adjusted to 200 mg of meconium (wet weight) in 1 ml of saline (Martínez-Burnes et al., 2001a,b). This volume of inoculum was chosen based on a dose range-finding study which showed that 200 mg of meconium in 1ml of saline was the maximum tolerated dose that could be inoculated in neonatal rats without causing significant mortality.

### Intratracheal inoculation and euthanasia

Rat neonates were intratracheally inoculated following a novel methodology recently reported (Martínez-Burnes et al., 2001b). In short, pups were anesthetized with halothane and a spinal needle with rounded edges was transorally inserted into the trachea with the aid of a modified otoscope. After inoculation, pups were maintained on a heating pad (37 °C) and kept under direct observation until fully recovered from the anesthesia. At the end of the experiment, rats were euthanatized with an overdose of halothane and exsanguination (Martínez-Burnes et al., 2001a).

### Fixation and processing of lung samples

Following euthanasia, the lungs were fixed *in situ* using procedures described by others (Tyler et al., 1985). Briefly, the thorax was opened, the trachea was exposed and through a transverse section between the first and second tracheal rings a plastic catheter (Cathlon IVJ Critikon Canada Inc, Ontario) was inserted into the tracheal lumen. The diameter of the catheter was selected based on the age of the rat as follows: 20-gauge and 32 mm length for neonates killed at postinoculation day (PID) 1, 3 and 7; 18-gauge and 32 mm length for rats killed at PID14; 16-gauge and 57 mm for PID 28; and 14-gauge and 57 mm for PID 56 to 112. Neutral 10% buffered formalin was intratracheally perfused at a constant pressure of 20 cm of fixative using modified "Marriott bottles system" (Tyler et al., 1985). After 30 minutes of fixation *in situ*, a tight ligature was placed around the trachea to maintain the intrapulmonary pressure of the fixative once the catheter was removed. The lungs were dissected free from the thorax and placed in the same fixative for at least 24 hours before further manipulation (Tyler et al., 1985).

Longitudinal slices of the entire left lung and transverse sections of the right cranial (anterior or apical), middle (cardiac), median (azygous or accessory) and caudal (posterior) lobes were obtained from each rat for histological examination (Tyler et al., 1985). Lung samples were processed, embedded in paraffin,

sectioned at 3  $\mu\text{m}$ -thickness and stained with hematoxylin and eosin (HE) following routine procedures. For the meconium identification in some sections, lungs were also stained with periodic acid-Schiff (PAS) counterstained with light green SF yellowish, PAS counterstained with hematoxylin, Alcian Blue-PAS and Hall's stain (Luna, 1968). The von Kossa stain was also used to confirm pulmonary deposits of calcium (Luna, 1968). Identification of keratin was done by morphological appearance, tinctorial characteristics and birefringence under polarized light (López and Bildfell, 1992).

#### Experimental design

Seven-day-old male rats ( $n=123$ ) with an average weight of  $14.4 \pm 0.5$  g, were randomly assigned to three experimental groups (amniotic fluid, meconium and saline control). Neonates from the amniotic fluid group ( $n=37$ ) received 0.05 ml of undiluted amniotic fluid. Neonates from the meconium group ( $n=45$ ) were inoculated with 0.05 ml of a suspension of 20% meconium and control animals ( $n=41$ ) received 0.05 ml of sterile saline solution. Histologic studies were conducted in two independent experiments. Neonates were euthanatized at 1, 3, 7, and 14 postinoculation days (PID) for the acute study, while for the chronic study rats were killed at 28, 56 and 112 PID. The lungs were examined microscopically and the following changes were evaluated: 1) presence of meconium or amniotic fluid in the lungs; 2) types of lesions; and 3) distribution of lesions in the lobes, bronchioles, alveoli and interstitium.

The frequency and severity of histologic changes in the lungs were scored as follow: absent (-) when meconium or microscopic changes were not observed; equivocal ( $\pm$ ) for questionable or sporadic evidence of meconium or of changes; mild (+) when meconium and microscopic changes were present but with low frequency; moderate (++) when meconium and changes were pronounced but not in all pulmonary lobes; and severe (+++) when meconium and microscopic changes were pronounced and commonly observed in all pulmonary lobes.

#### Results

Saline solution inoculated intratracheally did not produce atelectasis or any other gross lesion. Similarly, intratracheal saline did not induce any influx of inflammatory cells or any other microscopic change. Amniotic fluid did not induce any gross lesions but microscopic examination of the lungs revealed squamous epithelial cells, keratin, and a few neutrophils and macrophages in proximal alveolar regions. The inflammatory response was most obvious at PID 1 and 3, diminished by PID 7 and was no longer observed on subsequent days.

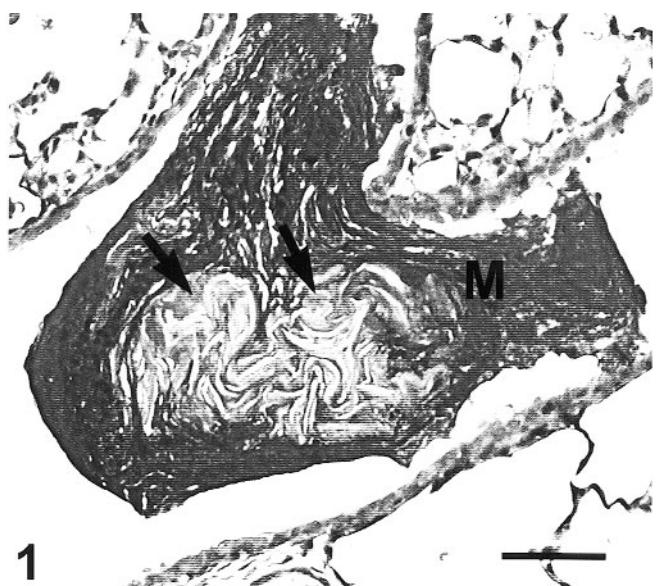
Intratracheal inoculation of meconium caused a

moderate but transient dyspnea in neonatal rats with no mortality. Morphologically, meconium induced gross multifocal areas of consolidation and caused a generalized but discreet green discoloration of the lungs. In addition, the lungs of rats in the meconium group did not distend well during perfusion of fixative.

Microscopically, meconium appeared in HE stained sections as a pale, amorphous, gold-yellow granular material frequently plugging airways and alveoli. Periodic acid-Schiff counterstained with light green proved to be the best stain to identify meconium in the lung (Fig. 1) while Hall's stain did not enhance the visualization of meconium in lung sections. At a higher magnification, squamous epithelial cells, squames, cellular debris and keratin were observed in the meconium plugs. Keratin generally appeared as tightly arranged spicules embedded in the core of the meconium plug (Fig. 1). Under polarizing light, some spicules appeared birefringent.

The microscopic appearance of meconium notably changed with time since keratin, squames and cellular debris were no longer evident by PID 28. Also, the tinctorial characteristics of meconium in HE stained sections began to change with time and by PID 56 the original bright gold color of the meconium matrix turned a pale pink-grey. Sequestered meconium became frequently mineralized at PID 14 and lasted up to PID 112 (Table 1).

All rats of the meconium group had microscopic evidence of meconium in the conducting system and alveoli at PID 1, 3 and 7 (Table 1). After PID 7, the percentage of rats with meconium in conductive airways



**Fig. 1.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 1. Bronchiole contains a large plug of meconium (M). Mucopolysaccharides stain dark and irregular arrangements of epithelial squames and keratin (arrows) are evident in the core of the meconium. PAS. Bar: 50  $\mu\text{m}$ .

decreased from 80% at PID 14 to 50% at PID 112. In contrast, meconium remained visible in the alveoli of all rats (40/40) even in those killed at PID 112 (Table 1).

The distribution of lesions in the lungs following intratracheal inoculation of meconium was not uniform. Focal atelectasis associated with intraalveolar accumulation of meconium was often alternating with well-aerated areas that were free of meconium. The frequency and lobar distribution of atelectasis are summarized in Table 2. Peripheral displacement of meconium toward the subpleural regions of the lung was evident as early as PID 1.

Atelectasis or alveolar collapse was present at PID 1 and 3 in all rats (100%) inoculated with meconium (Fig.

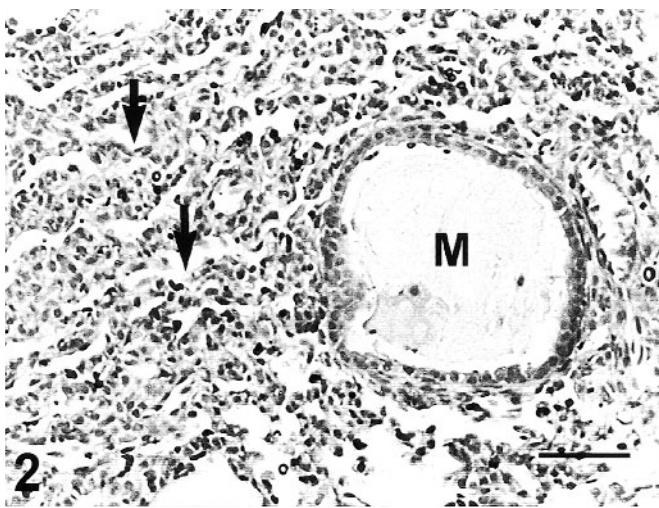
2, Table 2). Hyperinflation with over distension of alveoli was present in all rats (100%) inoculated with meconium at PID 1, 3 and 7 but the severity decreased over time (Table 1). In contrast, the lungs of rats inoculated with saline solution were normal and without evidence of atelectasis or alveolar collapse (Fig. 3).

Pulmonary inflammation was a conspicuous change in the rats inoculated with meconium. At PID 1 and 3 bronchioles and alveoli contained many polymorphonuclear leukocytes (PMNs) and macrophages (Fig. 4; Table 1). Saline solution did not induce any influx of inflammatory cells into the airways or alveoli (Fig. 5). At PID 7, meconium was generally surrounded by polymorphonuclear neutrophils but in

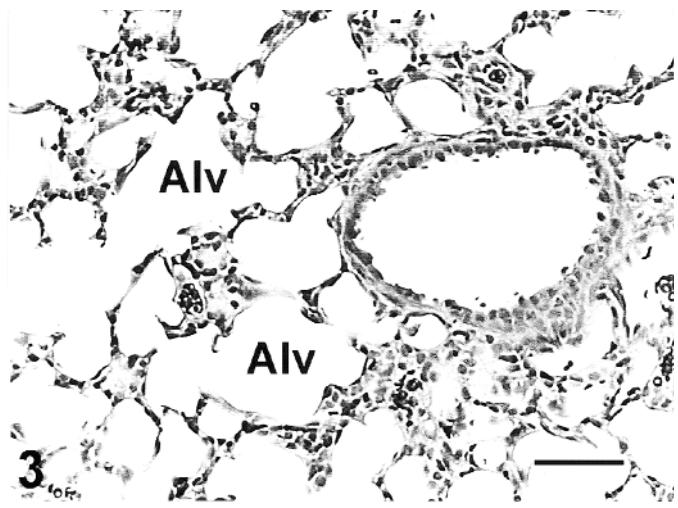
**Table 1.** Histologic scoring and frequency of pulmonary changes induced by intratracheal inoculation of meconium in neonatal rats.

MICROSCOPIC CHANGES	POSTINOCULATION DAY						
	1	3	7	14	28	56	112
Meconium-bronchioles	+++	+++	++	+	+	+	+
Meconium-alveoli	+++	+++	++	++	++	++	++
Neutrophils-bronchioles	+++	+	-	-	-	-	-
Neutrophils-alveoli	+++	++	+	-	-	-	-
PAM-bronchioles	+	+	+	+	+	+	+
PAM-alveoli	++	++	+	+	+	+	+
Edema	±	-	-	-	-	-	-
Atelectasis	+++	++	-	"	-	-	-
Hyperinflation	+++	+++	++	+	±	±	±
Alveolar thickening	+++	++	-	-	-	-	-
Granulomas	-	+	+	++	++	++	++
Giant Cells	-	"	+	++	++	++	++
Mineralization	-	-	-	+	+	++	++

- : absent; ±: equivocal; +: mild; ++ : moderate; +++: severe. PAM: pulmonary alveolar macrophages.



**Fig. 2.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 1. Bronchiole contains pale meconium (M) and shows peribronchiolar atelectasis; alveolar walls appear parallel and the alveolar lumens are poorly distended (arrows). HE. Bar: 50  $\mu$ m.



**Fig. 3.** Lung; neonatal rat inoculated with saline solution and euthanatized at PID 1. Bronchiole and alveoli (Alv) are clean and normally distended. HE. Bar : 50  $\mu$ m.

some instances, large pulmonary alveolar macrophages containing golden pigment in the cytoplasm were already present at this time (Fig. 6). These meconium-laden macrophages were particularly conspicuous at PID 1 and 3, and their number decreased after PID 7, but remained visible up to PID 112. The frequency and lobar distribution of inflammation are summarized in Table 3.

The number of neutrophils in airways and alveoli started to decrease at PID 3 and by PID 14 the

inflammatory reaction became predominantly histiocytic. This histiocytic reaction was typically localized and closely associated with the presence of meconium in the bronchioles and proximal alveolar regions (Table 1). At PID 28, the multifocal granulomatous reaction was characterized by calcification and presence of multinucleated giant cells (Table 1). A discontinuous layer of columnar epithelial cells sometimes lined the meconium lodged in

**Table 2.** Lobar distribution of atelectasis in the lungs of neonatal rats intratracheally inoculated with meconium.

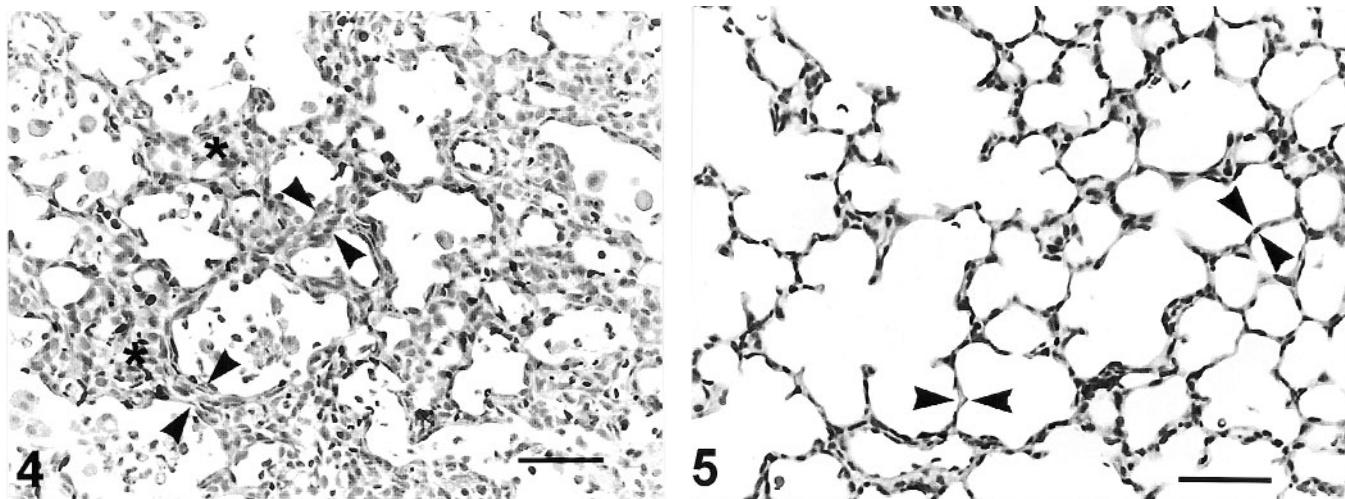
PID	ATELECTASIS						
	Rats	Total lobes	Left	Right cranial	Right middle	Right median	Right caudal
1	4/4	10/20	4/4	2/4	1/4	3/4	0/4
3	4/4	7/20	2/4	1/4	1/4	3/4	0/4
7	0/4	0/20	0/4	0/4	0/4	0/4	0/4
14	1/5	1/25	1/5	1/5	0/5	0/5	0/5

PID: Postinoculation day; Number of rats with atelectasis/total number of rats in the group.

**Table 3.** Lobar distribution of the neutrophilic inflammatory response in the lungs of neonatal rats intratracheally inoculated with meconium.

PID	NEUTROPHILS						
	Rats	Total lobes	Left	Right cranial	Right middle	Right median	Right caudal
1	4/4	18/20	4/4	4/4	4/4	3/4	3/4
3	4/4	13/10	2/4	3/4	1/4	4/4	3/4
7	1/4	2/20	0/4	0/4	1/4	1/4	0/4
14	0/5	0/25	0/5	0/5	0/5	0/5	0/5

PID: Postinoculation day. Number of rats with neutrophilic inflammation in the lung / total number of rats in the group.



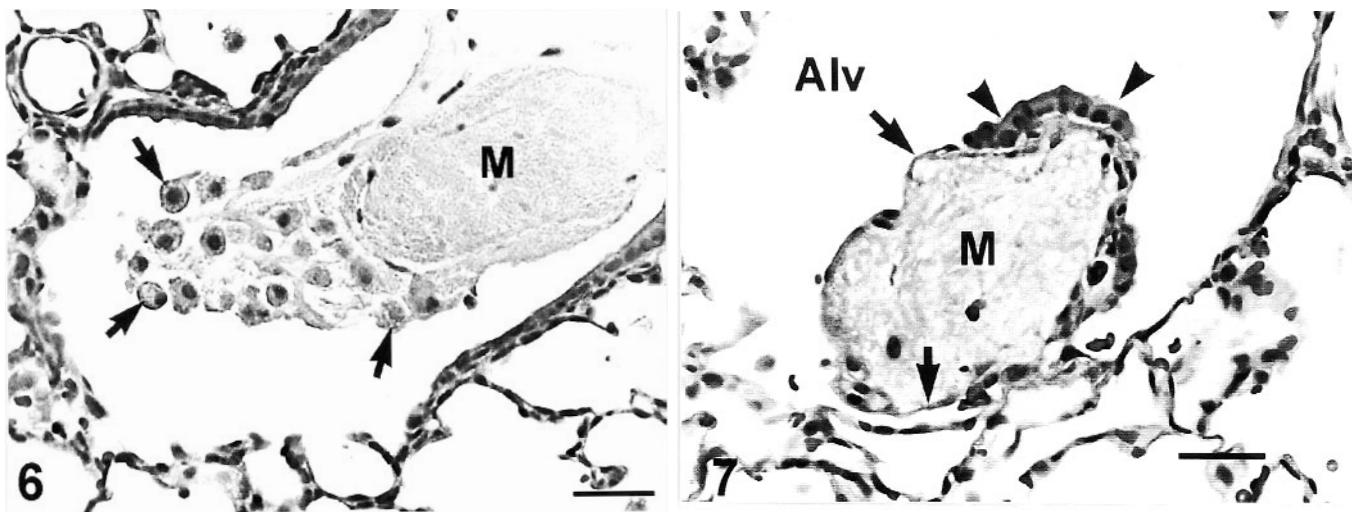
**Fig. 4.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 1. There is alveolar infiltration of neutrophils and macrophages and expansion of the interstitium (asterisks). The alveolar walls are thickened (arrow heads) HE. Bar: 50  $\mu$ m.

**Fig. 5.** Lung; neonatal rat inoculated with saline solution and euthanatized at PID 1. The normal alveolar walls are thin (arrow heads) HE. Bar: 50  $\mu$ m.

**Table 4.** Lobar distribution of alveolar thickening in the lungs of neonatal rats intratracheally inoculated with meconium.

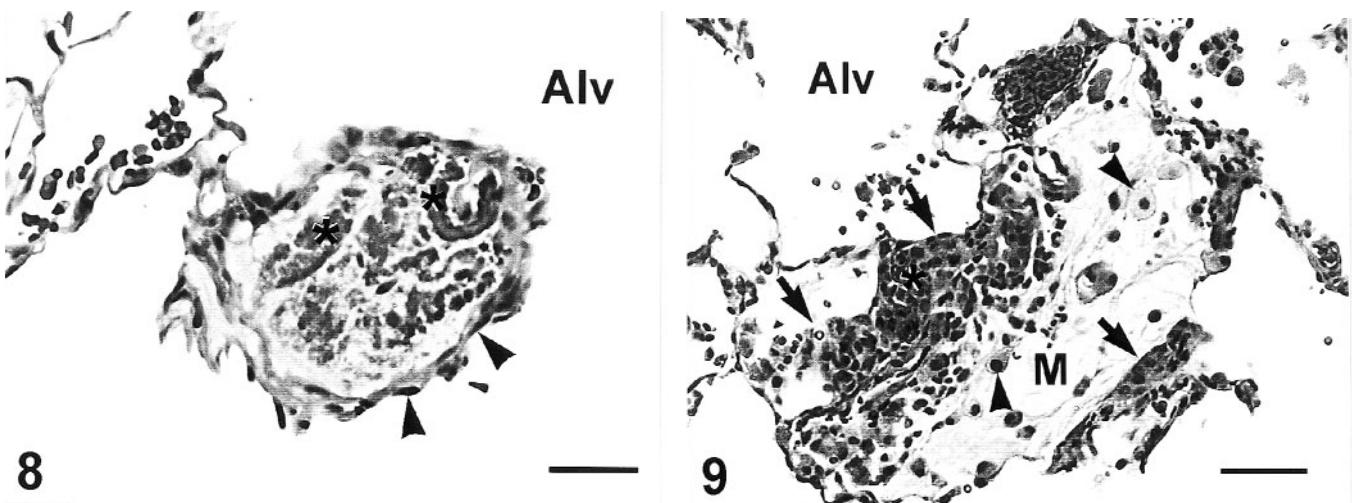
PID	ALVEOLAR THICKENING						
	Rats	Total lobes	Left	Right cranial	Right middle	Right median	Right caudal
1	4/4	20/20	4/4	4/4	4/4	3/4	4/4
3	4/4	19/10	4/4	4/4	3/4	4/4	4/4
7	0/4	0/20	0/4	0/4	0/4	0/4	0/4
14	0/5	0/25	0/5	0/5	0/5	0/5	0/5

PID: Postinoculation day. Number of rats with alveolar thickening in the lung/total number of rats in the group.



**Fig. 6.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 7. Terminal bronchiole contains a large piece of meconium (M) surrounded by macrophages (arrows). HE. Bar : 50  $\mu$ m.

**Fig. 7.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 28. Meconium (M) impacted in the alveoli (Alv) and surrounded by fibroblastic cells (arrows) and epithelial cells (arrow heads). HE. Bar: 50  $\mu$ m.



**Fig. 8.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 56. Meconium trapped in the alveolar region (Alv) is partially calcified (asterisks) and lined by fibroblastic cells (arrow heads) HE. Bar : 50  $\mu$ m.

**Fig. 9.** Lung; neonatal rat inoculated with meconium and euthanatized at PID 112. Persistent multifocal granulomatous inflammation in alveolar region (Alv) of the lung. Macrophages (arrow heads), fibroblastic cells (arrows). Meconium (m) is still evident. HE. Bar: 50  $\mu$ m.

bronchioles and alveoli (Fig. 7) and by PID 28 fibroblastic cells were present in affected areas. Calcification was conspicuous at PID 56 (Fig. 8) and remained evident up to PID 112. At this last postinoculation time, meconium was still present in bronchioles and alveoli associated to a sparse multifocal granulomatous inflammation with giant cells (Fig. 9).

Thickening of the alveolar septum was another remarkable microscopic finding in the lungs of rat neonates inoculated with meconium. It was present at PID 1 and 7 (Fig 4) but was no longer observed at PID 14 or beyond (Table 1). This transient alveolar change was closely associated with areas of neutrophilic inflammation. The lobar distribution of alveolar thickening is summarized in Table 4. Intratracheal inoculation of meconium also provoked mild pleural thickening characterized by focal subserosal accumulations of neutrophils and macrophages at PID 1, 3, and 7. Loss of cilia (deciliation) and cell vacuolation was observed in the bronchiolar epithelium of rats inoculated with meconium. Inoculation of saline solution did not induce any changes in the alveolar interstitium (Fig. 5).

## Discussion

Inoculation with amniotic fluid elicited a mild inflammatory reaction presumably in response to the suspended cellular elements such as keratin and not to the fluid itself. This view is supported by another study with rabbits in which the typical foreign body reaction was abrogated when the suspended cells and keratin were removed from amniotic fluid (Cruickshank, 1949). The microscopic appearance of keratin in the lungs of neonatal rats was similar to that reported in other species (López and Bildfell, 1992).

It was noted during the preparation of inocula that the turbidity of the rat amniotic fluid was considerably lower than the bovine amniotic fluid used in a previous experiment (Carvajal-de la Fuente et al., 1998). This observation may in part explain why the inflammatory reaction in rat neonates was less intense than that reported with heterologous human or rabbit and bovine amniotic fluid in other experimental models (Cruickshank, 1949; Jovanovic and Nguyen, 1989; Carvajal-de la Fuente et al., 1998). For future studies it would be recommendable to assess turbidity, cellularity, and suspended matter of amniotic fluid to be used as inoculum. It is nonetheless unclear why a highly pro-inflammatory matter such as keratin, which was microscopically observed in the lungs of neonates, seems not to provoke severe inflammation as it does in other tissues (Davis et al., 1998).

Recent investigations have shown that human amniotic fluid contains high levels of an interleukin-1 receptor antagonist (IL-1-*ra*) that blocks interleukin-1 (IL-1), one of the most important chemical mediators of acute inflammation (Bry et al., 1995). Further, IL-10 another important cytokine that down-regulates local

inflammation has been reported in human amniotic fluid (Greig et al., 1995). This novel information supports the view of some researchers who speculate about a possible factor present in amniotic fluid that may be down-regulating the pulmonary inflammatory response in MAS (López et al., 1994; Carvajal-de la Fuente et al., 1998).

As recently reported in another study, intratracheal inoculation of meconium elicited a transient apnea in neonatal rats with normal respiratory movements becoming reestablished within two minutes of inoculation (Martínez-Burnes et al., 2001b). Pilot studies in our laboratory had previously shown that higher dose of meconium causes an irreversible apnea with unacceptable mortality. It seems that the dose and volume of meconium that can be safely inoculated in 7-day-old rats are comparatively lower than in mature rabbits (Tyler et al., 1978).

In contrast to amniotic fluid, meconium induced an acute inflammatory reaction characterized by a transient but remarkable increase of PMNs in the bronchoalveolar spaces. This finding agrees with a previous study where leukocytic cell counts were significantly elevated in the bronchoalveolar lavage fluid of neonatal rats inoculated with meconium (Martínez-Burnes et al., 2001a). The pulmonary recruitment of PMNs and macrophages induced by meconium is likely related to the local release of inflammatory mediators since tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1, and 8 (IL-1 $\beta$ , IL-8) have been linked to the pulmonary injury and inflammation in MAS (Jones et al., 1994). Further, *in vitro* studies have also confirmed that IL-8, a powerful chemoattractant for neutrophils is normally present in human meconium (de Beaufort et al., 1998) and that macrophages incubated with meconium generate oxygen radicals (Kojima et al., 1994).

Atelectasis was a consistent finding in the lungs of neonatal rats inoculated with meconium. According to the literature, atelectasis in MAS occurs not only because meconium causes airway obstruction and surfactant degradation, but also as a consequence of alveolar inflammation (Sun et al., 1993a). This view is further supported by a recent study which demonstrated that free radicals released by leukocytes at the site of inflammation cause lipid peroxidation of the surfactant (Bouhafs and Jarstrand, 1999). The coexistence of atelectasis and hyperinflation in the same section of the lung was an interesting, yet paradoxical finding in the neonatal rats of the meconium group. In human MAS, this paradox has been attributed to pulmonary interdependence (collateral ventilation) and aberrant movement of air in obstructed lungs (Vidyasagar et al., 1975). It has been suggested that complete obstruction of a terminal bronchiole leads to atelectasis while partial obstruction leads to hyperinflation. According to some investigators, this results from the "ball-valve" effect of meconium in which gas flowing into the airway during inspiration becomes trapped and cannot move out during expiration (Wiswell and Bent, 1993; al Mateen et al.,

1994; Srinivasan and Vidyasagar 1999).

Microscopic examination of lungs showed that meconium disperses from the terminal bronchioles into the alveolar acini and pleural tissue during the first hours following inoculation. This peripheral "migration" of meconium has been previously documented in rabbits, dogs and piglets but its pathogenesis remains poorly understood. It has been suggested that gasping during inspiration and the ball-valve effect may be incriminated in the movement and redistribution of meconium within the lung (Gooding and Gregory, 1971).

As previously reported by others (Davis et al., 1985; López and Bildfell 1992), meconium was easily detected in the neonatal rats killed during the first few weeks after inoculation. However, as time passed, the characteristic color of meconium faded away and by PID 28 the gold tinge had practically vanished due to chemical degradation. In addition to physical removal, this chemical degradation and loss of tinctorial features may explain why meconium was difficult to detect in older calves with MAS (López and Bildfell 1992). Hall's stain for bile recommended for the diagnosis of MAS in pediatric pathology was not suitable in identifying degraded meconium in rat neonates. In contrast, Alcian blue-PAS and PAS were useful due to their ability to stain the mucopolysaccharides and mucins which remain detectable in meconium for a long time (Block et al., 1981; Brown and Gleicher 1981; Sun et al., 1993b). Loss of cilia, also referred to as deciliation, was one of the most important lesions caused by meconium in bronchioles of neonatal rats. This change was previously described in rabbits inoculated with human meconium suggesting that ciliary damage was produced by the chemical components present in meconium (Tyler et al., 1978; al Mateen et al., 1994). However, since deciliation occurred at the precise sites of inflammation in neonatal rats, it is also conceivable that enzymes locally released by leukocytes may have contributed to this change (Jones et al., 1994; Kojima et al., 1994).

The rapid influx of PMNs into air spaces indicates that meconium induces a robust inflammatory response in the lungs of rat neonates. This finding is consistent with autopsy findings in babies with MAS and with results obtained from experimental inoculations (Cleary and Wiswell, 1998). Microscopic examination of lungs failed to corroborate epithelial necrosis in neonatal rats as it has been previously reported in rabbits inoculated with meconium (Tyler et al., 1978). Electron microscopy and immunohistochemistry may be necessary to accurately resolve the pathogenesis of meconium-induced injury in the lungs of neonatal rats.

In contrast to other experimental studies, the pulmonary edema in neonatal rats inoculated with meconium was only mild and detected exclusively at PID 1. It is likely that the lack of an edematogenic effect of meconium was related to the age of the rat neonates. It may also be speculated that meconium-induced alveolar injury is dose-dependent and the concentration of meconium given to 7-day-old rats was insufficient to

cause injury. However, this is unlikely since the dose of meconium used in the neonatal rats was close to the maximum tolerated dose (Martínez-Burnes et al., 2001a).

The multifocal granulomatous response in the lungs of neonatal rats inoculated with meconium at PID 7 was consistent with the lesions found in adult rabbits inoculated with human meconium (Cruickshank, 1949). It seems that the initial chemical pneumonitis induced by meconium is followed by a foreign body response evoked by the keratin and other cellular components of meconium. It is not clear, however, if microscopic granulomas develop in the lungs of babies who have survived the acute episode of MAS. What is well documented in pediatric medicine is that spilling of meconium out of the fetal or neonatal intestine causes granulomas in the peritoneal cavity and serosa of genital organs (Herz et al., 1982; Ooi et al., 1995).

Some rats of the meconium group developed conspicuous nodules formed by inspissated meconium superficially covered by epithelium. According to the literature, similar lesions develop when focal epithelial injury caused by inhaled exogenous material is followed by compensatory hyperplasia of alveolar or bronchiolar epithelium (Boorman and Eustis, 1990). Mineralization first noted at PID 14 was interpreted as dystrophic mineralization of inflammatory foci. Although pulmonary mineralization has not been described in MAS, this change has been reported in abdominal tissues of fetuses or newborn babies with meconium peritonitis, as well as, in umbilical cords of babies born with meconium stained amniotic fluid (Forouhar, 1984; Benirschke and Pekarske, 1995).

The transient thickening of the alveolar septa during the acute stages of inflammation in neonatal rats inoculated with meconium was largely due to interstitial edema and accumulation of some leukocytes in the interstitial space. It was not possible however, to determine by light microscopy if meconium had induced reversible or irreversible changes in the air-blood barrier. Ultrastructural studies would be required to investigate if meconium causes alterations in endothelial and alveolar cells, particularly in type I pneumonocytes.

In conclusion, results of this study showed that homologous meconium induces a rapid but transient influx of neutrophils into the lungs of seven-day-old rats and that this neutrophilic inflammation is followed by a persistent multifocal histiocytic and granulomatous reaction which eventually becomes calcified and partially covered by cuboidal epithelium. Future work will study the ultrastructural changes in the air-blood barrier induced by meconium. Although the significance of MAS in veterinary medicine is unclear, this syndrome should be considered in the differential diagnosis of neonates with mild multifocal granulomatous alveolitis. Finally, special stains such as PAS should be done to investigate the presence of degraded meconium in the lung, particularly in cases in which suspected aspiration could have occurred several days or weeks

earlier.

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## References

al Mateen K.B., Dailey K., Grimes M.M. and Gutcher G.R. (1994). Improved oxygenation with exogenous surfactant administration in experimental meconium aspiration syndrome. *Pediatr. Pulmonol.* 17, 75-80.

Benirschke K. and Pekarske S.L. (1995). Placental pathology casebook. Extensive calcification of the umbilical cord and placenta. *J. Perinatol.* 15, 81-83.

Block M.F., Kallenberger D.A., Kern J.D. and Nepveux R.D. (1981). In utero meconium aspiration by the baboon fetus. *Obstet. Gynecol.* 57, 37-40.

Boorman G.A. and Eustis S.L. (1990). Lung. In: *Pathology of the Fischer rat: Reference and Atlas*. Boorman G.A., Eustis S.L., Elwell M.R., Montgomery C.A. and MacKenzie W.F. (eds). Academic Press, California. pp. 339-367.

Bouhafs R.K. and Jarstrand C. (1999). Phagocyte-induced lipid peroxidation of lung surfactant. *Pediatr. Pulmonol.* 27, 322-327.

Brown B.L. and Gleicher N. (1981). Intrauterine meconium aspiration. *Obstet. Gynecol.* 57, 26-29.

Bry K., Teramo K., Lappalainen U., Waffarn F. and Hallman M. (1995). Interleukin-1 receptor antagonist in the fetomaternal compartment. *Acta Paediatr.* 84, 233-236.

Canadian Council on Animal Care (1993). Guide to the care and use of experimental animals. C.C.A.C., Ottawa, Ont.

Carvajal-de la Fuente V., López-Mayagoitia A., Martínez-Burnes J., Barrón-Vargas C. and Loredo-Osti J.C. (1998). Efecto antiinflamatorio del líquido amniótico en el pulmón de ratas inoculadas intratraquealmente con sílice. *Vet. Mex.* 29, 147-153.

Cleary G.M. and Wiswell T.E. (1998). Meconium-stained amniotic fluid and the meconium aspiration syndrome. An update. *Pediatr. Clin. North Am.* 45, 511-529.

Co E. and Vidyasagar D. (1990). Meconium aspiration syndrome. *Compr. Ther.* 16, 34-39.

Cruickshank A.H. (1949). The effects of the introduction of amniotic fluid into the rabbits' lungs. *J. Pathol. Bact.* 61, 527-531.

Davis J.R., Miller H.S. and Feng J.D. (1998). Vernix caseosa peritonitis: report of two cases with antenatal onset. *Am. J. Clin. Pathol.* 109, 320-323.

Davis R.O., Philips, J.B., Harris B.A.J., Wilson E.R. and Huddleston J. F. (1985). Fatal meconium aspiration syndrome occurring despite airway management considered appropriate. *Am. J. Obstet. Gynecol.* 151, 731-736.

de Beaufort A.J., Pelikan D.M., Elferink J.G. and Berger H.M. (1998). Effect of interleukin 8 in meconium on in-vitro neutrophil chemotaxis. *Lancet* 352, 102-105.

Forouhar F. (1984). Origin of calcification in healed meconium peritonitis. *Med. Hypotheses* 14, 51-56.

Gooding C.A. and Gregory G.A. (1971). Roentgenographic analysis of meconium aspiration of the newborn. *Radiology* 100, 131-140.

Greig P.C., Herbert W.N., Robinette B.L. and Teot L. A. (1995). Amniotic fluid interleukin-10 concentrations increase through pregnancy and are elevated in patients with preterm labor associated with intrauterine infection. *Am. J. Obstet. Gynecol.* 173, 1223-1227.

Herz M.G., Stanley W.D., Toot P.J., Freedman S.I. and Ang E.P. (1982). Symptomatic maternal intraperitoneal meconium granulomata: report of two cases. *Diagn. Gynecol. Obstet.* 4, 147-149.

Jones C.A., Cayabyab R.G. and Hamdan H. (1994). Early production of proinflammatory cytokines in the pathogenesis of neonatal adult respiratory distress syndrome (ARDS) associated with meconium aspiration. *Pediatr. Res.* 35, 339A.

Jovanovic R. and Nguyen H.T. (1989). Experimental meconium aspiration in guinea pigs. *Obstet. Gynecol.* 73, 652-656.

Katz V.L. and Bowes W.A.J. (1992). Meconium aspiration syndrome: reflections on a murky subject. *Am. J. Obstet. Gynecol.* 166, 171-183.

Kojima T., Hattori K., Fujiwara T., Sasai T.M. and Kobayashi Y. (1994). Meconium-induced lung injury mediated by activation of alveolar macrophages. *Life Sci.* 54, 1559-1562.

López A. and Bildfell R. (1992). Pulmonary inflammation associated with aspirated meconium and epithelial cells in calves. *Vet. Pathol.* 29, 104-111.

López A., Löfstedt J., Bildfell R., Horney B. and Burton S. (1994). Pulmonary histopathologic findings, acid-base status, and absorption of colostral immunoglobulins in newborn calves. *Am. J. Vet. Res.* 55, 1303-1307.

Luna L. (1968). *Manual of histologic staining methods of the Armed Forces Institute of Pathology*. McGraw-Hill, Toronto, Canada.

Martínez-Burnes J., López A., Horney B., MacKenzie A. and Brimacombe M. (2001a). Cytologic and biochemical changes associated with inoculation of amniotic fluid and meconium into lungs of neonatal rats. *Am. J. Vet. Res.* 62, 1636-1641.

Martínez-Burnes J., López A., Lemke K. and Dobbin G. (2001b). Transoral intratracheal inoculation method for use with neonatal rats. *Comp. Med.* 51, 134-137.

Ooi K., Riley C., Billson V. and Ostör A.G. (1995). Ceroid granulomas in the female genital system. *J. Clin. Pathol.* 48, 1057-1059.

Srinivasan H.B. and Vidyasagar D. (1999). Meconium aspiration syndrome: current concepts and management. *Compr. Ther.* 25, 82-89.

Sugimoto M., Ando M., Senba H. and Tokuomi H. (1980). Lung defenses in neonates: effects of bronchial lavage fluids from adult and neonatal rabbits on superoxide production by their alveolar macrophages. *J. Reticuloendothel. Soc.* 27, 595-606.

Sun B., Curstedt T. and Robertson B. (1993a). Surfactant inhibition in experimental meconium aspiration. *Acta Paediatr.* 82, 182-189.

Sun B., Curstedt T., Song G.W. and Robertson B. (1993b). Surfactant improves lung function and morphology in newborn rabbits with meconium aspiration. *Biol. Neonate.* 63, 96-104.

Swaminathan S., Quinn J., Stabile M.W., Bader D., Platzker A.C. and Keens T.G. (1989). Long-term pulmonary sequelae of meconium aspiration syndrome. *J. Pediatr.* 114, 356-361.

Tyler D.C., Murphy J. and Cheney F.W. (1978). Mechanical and chemical damage to lung tissue caused by meconium aspiration. *Pediatrics* 62, 454-459.

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Tyler W.S., Dungworth D.L., Plopper C.G., Hyde D.M. and Tyler N.K. (1985). Structural evaluation of the respiratory system. *Fundam. Appl. Toxicol.* 5, 405-422.

Vidyasagar D., Harris V. and Pildes R.S. (1975). Assisted ventilation in infants with meconium aspiration syndrome. *Pediatrics* 56, 208-213.

Waynfirth H.B. (1980). Experimental and surgical technique in the rat. Academic Press, New York. pp. 247-248.

Wiswell T.E. (2001). Advances in the treatment of the meconium aspiration syndrome. *Acta Paediatr. Suppl* 90, 28-30.

Wiswell T.E. and Bent R.C. (1993). Meconium staining and the meconium aspiration syndrome. Unresolved issues. *Pediatr. Clin. North Am.* 40, 955-981.

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