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Transoral Intratracheal Inoculation Method for Use with Neonatal Rats

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Background and Purpose: **Studying the effects of toxic and infective compounds on the respiratory system requires a reliable method for delivering inoculum into the distal region of the lung. Although transoral intratracheal inoculation methods have been well documented for adult rats, to the authors' knowledge, a reliable method has not been validated for neonatal rats. The purpose of the study reported here was to develop a simple method for transoral inoculation in rat neonates.**

Methods: **Seven-day-old Fischer 344 rats were anesthetized with halothane, and a spinal needle was inserted in the tracheal lumen, by use of illumination and a modified otoscope. Meconium was injected into the lungs as a marker, and the neonates were kept under close observation. After euthanasia at 24 h, lungs were removed and fixed in formalin, and the microscopic distribution of the inoculum was assessed in the left, right cranial, middle, median, and caudal lung lobes.**

Results: **Microscopic examination of lungs indicated that intratracheal inoculation was achieved in 100% of neonatal lungs and the inoculum was consistently distributed in the alveoli of all pulmonary lobes. Important complications or mortality were not observed in the neonates.**

Conclusions: **Intratracheal inoculation of neonatal rats is possible by use of a modified otoscope for transoral illumination. This technique is simple and reproducible and ensures, without complications, widespread distribution of inoculum in the lungs of neonatal rats.**

Rats are one of the preferred animal species for experimental lung research because they are reasonably priced, easy to raise in disease-free environments, available from commercial sources, and genetically homogeneous (1, 2). As a result of the widespread use of rats in lung research, there is abundant information about methods for lung fixation, pulmonary morphology, lung pathology, study of ultrastructure, and morphometry (3, 4).

To experimentally assess the effects of noxious compounds on pulmonary structure and defense mechanisms, it is necessary to deliver foreign material into the distal region of the lung. The delivery methods most commonly used for non-gaseous materials are aerosol exposure (5, 6), transoral intratracheal inoculation (5, 7), and transtracheal inoculation with or without tracheotomy (8, 9). Each delivery route has its inherent advantages and disadvantages, and selection of a method is based principally on study objectives and nature of the inoculum. The main advantages of aerosol exposure are uniform distribution of inhaled particles and the fact that large numbers of rodents can be inoculated at the same time (5). However, the physicochemical properties of some types of inoculum often preclude use of aerosols (7, 10). To get around this problem, researchers use intratracheal inoculation (ITI), which delivers the inoculum directly into the lungs regardless of particle size and viscosity (11, 12). The ITI method is fairly simple, and a wide range of treatment doses can be administered to the distal region of the lung of laboratory animals (10, 13).

Transoral ITI is routinely used in adult rats, but not in neonates, in which more invasive techniques, such as surgical incision and transtracheal intubation, are required (8). The complexity of neonatal ITI has compelled many investigators to use adult rats in the study of pulmonary diseases known to develop in the neonate, despite the fact that the lung response in the neonate differs from that of adult rats (14). Several factors explain why researchers have avoided use of neonatal rodents in experimental models. The small size of the neonatal rodent makes ITI a difficult task since it requires direct visualization of the laryngeal lumen of these rodents under general anesthesia (8, 10, 13). Also, the metal laryngoscope routinely used in adult rats is unsuitable for rat pups, not only because of the small size of the oral cavity, but also because of the fragility of neonatal mucosal tissues (13). Finally, susceptibility to neonatal hypothermia, maternal rejection, and cannibalism, create additional problems for ITI in rat neonates (15-18).

The objective of the study reported here was to develop and standardize a transoral, non-surgical method for ITI in neonatal rats that could be used in murine models of neonatal respiratory tract diseases.

Materials and Methods

Animals. Fischer 344 pregnant rats were obtained from a commercial source (Charles River, St. Constant, Quebec, Canada) 14 to 16 days before parturition and were kept as original breeding stock at the Atlantic Veterinary College (AVC). Once pups were born, neonates were selected to be part of a timed pregnancy program that followed standard reproductive protocols described by others (19, 20). Rats were housed individually, provided with commercial food (Rodent laboratory Chow 5001, Ralston Purina Co., St. Louis, Mo.) and water ad libitum, and maintained on a 12/12-h light/dark cycle at 22°C and 50% relative humidity. Experimental protocols were approved by the AVC Animal Care Committee and experiments were conducted following the guidelines of the Canadian Council on Animal Care (21).

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Breeding programs. Cell patterns during the estrous cycle and mating were determined daily by use of vaginal cytologic examination (19, 20). Presence of spermatozoa in vaginal samples was considered an indication that mating had taken place (day zero). All births were carefully monitored, and the age and sex of rat pups was recorded. Only male pups were used for inoculation studies, and female pups were removed from the dam and euthanized. Male pups were kept with their dams in groups of five to six and were only removed from the cage during the time of inoculation.

Preconditioning program. To avoid infant rejection and cannibalism, pregnant rats were preconditioned 7 to 10 days before parturition following the protocol described by Park and coworkers (16). During that period, dams were exposed in their cages to the smell of materials to be used for anesthesia and inoculation. Briefly, a perforated stainless steel oval container was attached to the cage's wire lid and suspended freely into the cage. A piece of cotton was introduced into the container and several drops (0.39 ml) of anesthetic, saline solution, and tattoo ink, along with small pieces (2×2 cm) of latex gloves, were placed daily on the cotton inside of the oval container. Twelve litters with an average of eight pups per litter were used. Six of them were exposed to the preconditioning program, and the remaining six were kept as controls without the procedure. To assess rejection and cannibalism, pups were manipulated after delivery including daily weighing during the first week, as well as, tattooing, anesthesia, and inoculation procedures. For pup identification, animal tattoo ink (Ketchum Manufacturing Inc., Ottawa, Ontario, Canada) was injected subcutaneously, using a 0.5-ml disposable plastic syringe with 28-gauge needle (Becton Dickinson, Franklin Lakes, N.J.), into the base of the tail and the dorsal middle aspect of the tail.

Anesthesia. Three pilot studies were conducted in 1-day-old (n = 15), 5-day-old (n = 15) and 7-day-old (n = 42) rat pups. In a plexiglass chamber, neonates were anesthetized with 3% halothane in 100% oxygen administered at a rate of 100 to 200 ml/min via a gas anesthetic machine, using a modified delivery system adapted for small rat neonates (16). The loss of pedal reflex was used as an indicator of the proper depth of anesthesia for ITI (22). Induction and recovery times were recorded.

Intratracheal inoculation. In pilot studies using euthanized neonatal rats, various methods of ITI were tested, including percutaneous-transtracheal inoculation, with and without cervical transillumination, and transoral ITI (13). To ensure successful delivery into the lung, a liquid marker (methylene blue) was used as inoculum.

Once the ITI technique was standardized in dead pups, the procedure was subsequently tested in anesthetized pups. Saline solution was inoculated at various ages, starting with 15-day-old neonates and reducing the age until 7-day-old rats were successfully inoculated. The neonate, under deep halothane anesthesia (loss of pedal reflex), was placed in a supine position in the left hand of the operator. The mouth of the neonate was opened, and the tongue was gently pulled to one side. The oral cavity was moved toward the tip of an operating otoscope (Welch Allyn, Skaneateles Falls, N.Y.) attached to a support stand with clamps. The larynx was visualized, using a small sterile speculum and otoscope/throat illuminator system (power source handle model 70500, 2.5 V and an illuminator model 21600 Welch Allyn). Details of the otoscope are illustrated in Fig. 1. A sterile spinal needle 25-gauge, 8.8-cm length (Becton Dickinson), without the bevel and with the edge rounded, was inserted into the proximal part of the trachea, and the inoculum was instilled into the trachea and lungs. Fig. 2 illustrates the inoculation procedure.

After inoculation, pups were placed on a heating pad (38°C) and maintained in direct observation until respiratory movements were regular and pups had completely recovered from anesthesia. The pups continued to be monitored for at least 30 min after ITI before they were reunited with the dam. The efficacy of the ITI was tested in two experimental groups. Neonates from the control group were inoculated with 0.05 ml of sterile saline solution. Pups of the second group received as a marker 0.05 ml of a 20% solution of meconium in sterile saline. Animals that developed severe respiratory distress (dyspnea, stertor, or cyanosis) were humanely euthanized by administration of an overdose of halothane. Twenty four hours after the inoculation, rat pups under halothane anesthesia (5% halothane in 1,000 ml of oxygen) were euthanized by exsanguination. The lungs were removed and fixed in buffered 10% formalin solution. Sections of lung lobes were processed, embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and eosin in routine manner. Microscopic distribution of the inoculum was evaluated by use of light microscopy.

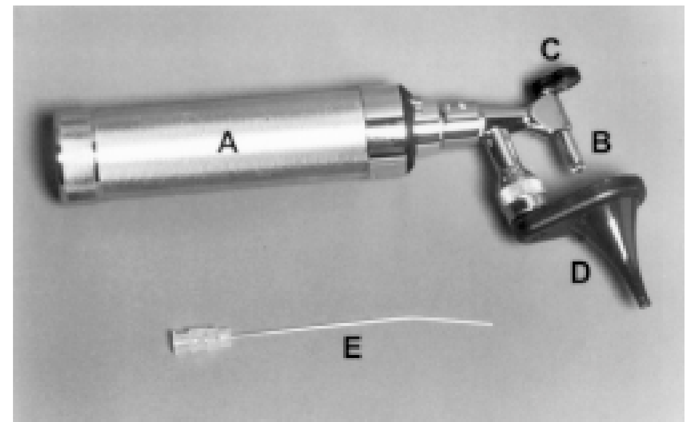

Figure 1. Otoscope/throat illuminator system components. (A) power source 2.5 V; (B) transilluminator; (C) magnifying lens; (D) speculum; E. spinal needle 25-gauge, 8.8-cm length, with the edge rounded and slightly bent.

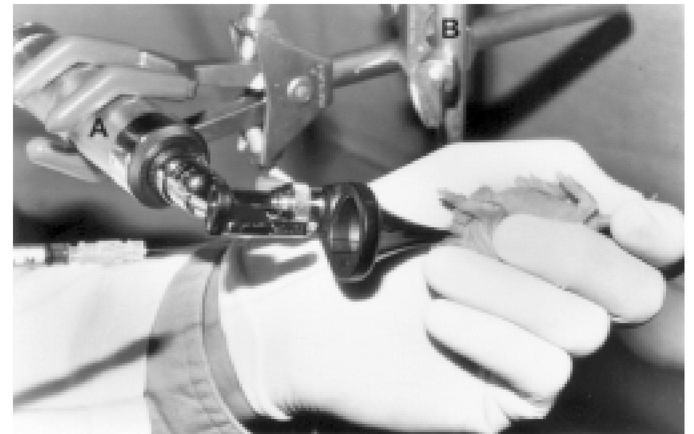

Figure 2. Intratracheal inoculation. The otoscope/throat illuminator system (A) is attached to a support stand (B) to achieve the proper height to the operator's view.

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Results

Breeding programs. Using daily vaginal cytologic examination, it was possible to recognize the characteristic cellular patterns of the estrous cycle. Mating occurred in late estrus, and day zero of gestation was considered when vaginal spermatozoa were first detected. From the spermatozoa-positive females, 90% delivered pups in 21.8 ± 0.47 days of gestation.

Preconditioning programs. Handling rat neonates after birth did not elicit abnormal behavior in the dams. Infant rejection or cannibalism was not detected after weighing, tattooing, anesthesia, or inoculation. Interestingly, rejection or cannibalism was not detected in the six litters that were not preconditioned prior to the ITI.

Anesthesia. The number of neonates, body weight, and induction and recovery times are summarized in Table 1. Anesthesia with halothane was associated with better induction and recovery times in 7-day-old neonates.

Intratracheal inoculation. The ITI was achieved by visualizing the small laryngeal opening with the help of an otoscope and inserting a spinal needle into the tracheal lumen. This procedure was well tolerated by rat pups and the whole maneuver took less than a minute. The minimal age at which the ITI could be consistently and safely achieved was seven days. The small magnifying lens in the transilluminator permitted direct observation of the larynx, leaving sufficient free space in the oral cavity to insert the spinal needle in the speculum. This magnifying lens also made the placing of the needle into the tracheal lumen much easier. Bending of the last portion of the spinal needle at a 30° angle facilitated introduction of the needle through the cone shape of the speculum and placement of the inoculum into the larynx. After intratracheal injections of inocula, some neonates developed transient apnea, but all regained normal respiratory movements within 2 min.

In the pilot studies, intratracheal inoculation of methylene blue stain revealed multifocal distribution of inoculum involving all pulmonary lobes. Inoculation of saline solution did not cause noticeable gross changes in the lungs. The lungs of neonates inoculated with meconium consistently contained multifocal areas of green discoloration of the pulmonary parenchyma.

Light microscopy of the lungs of neonates inoculated with saline solution did not reveal any detectable morphologic changes such as hemorrhage. All neonates inoculated with meconium had this material in the lungs. The distribution of this marker was widespread in all areas of the lung, as indicated by the percentage of pulmonary lobes containing this material. The marker was present in 100% of the conductive airways of the inoculated pups and involved 97.5% of the pulmonary lobes (Table 2). It was microscopically apparent in conducting airways of all pulmonary lobes of all pups except for the right caudal (diaphragmatic) lobe where it was only detected in 87.5% of the total caudal lobes. Meconium was present in alveoli of all pulmonary lobes (Table 2).

Discussion

It is well known that the percentage of conception in laboratory rats following mating is high (20). Results of this study indicated that the probability of pregnancy in young Fischer 344 rats with a spermatozoa-positive vaginal test was 90%. The time pregnancy program played a pivotal role in obtaining neonates of the same age and body size as experimental animal for ITI.

Table 1. Number of neonates, age, body weight, and induction and recovery times in the anesthesia protocol

Age & body weight	Halothane[1] Induction Time (min)	Halothane[1] Recovery Time (min)
1 day (n = 15) 4.95 ± 0.16g	10.95 ± 1.45	1.95 ± 1.10
5 days (n = 15) 7.85 ± 0.53g	10.65 ±1.45	0.95 ± 0.23
7 days (n = 42) 13.9 ± 1.81g	7.81 ± 0.69	0.88 ± 0.32

[1]Induction: halothane 3% and oxygen 200 ml/min; maintenance: halothane 2% and oxygen 200 ml/min.
Weights and times are given as mean ± SD.

Table 2. Transoral intratracheal inoculation: microscopic distribution of the marker in conductive airways and alveoli in the various pulmonary lobes of neonatal rats

Anatomic region	Lungs Total	Lobes Total	Left lung lobe	Right Cranial	Right Middle	Right Median	Right Caudal
Conductive airways	8/8	39/40	8/8	8/8	8/8	8/8	7/8
Alveoli	8/8	40/40	8/8	8/8	8/8	8/8	8/8

Under the experimental conditions of this study, difference in maternal rejection or cannibalism was not observed between the preconditioned and non-preconditioned litters of Fischer 344 rats. This was in agreement with observations by other researchers who reported that cannibalism in rats, is restricted to stillborn pups, dead pups, or pups severely weakened by experimental procedures (15). It is then concluded from the results of our study that transoral ITI does not cause important weakening or any other adverse problems in 7-day-old rats. Nonetheless, preconditioning is still recommended to avoid problems in experimental work involving neonatal surgery.

Halothane, dispensed by use of a modified delivery system in a plexiglass chamber, proved to be an effective anesthesia technique for ITI in neonatal rats. The modified apparatus can be easily constructed and provides a quick solution to the scarcity of commercial devices for anesthesia in rat neonates. An interesting finding was the long induction time for halothane in rat pups, compared with what is reported in mature or adult rats. According to some reports (16, 22), anesthesia in adult rats is achieved within three minutes, whereas neonates required at least 7.8 min of halothane anesthesia. This age effect has been observed by others, and is presumably due to physiologic differences between newborn and adult rats (16).

Some authors have proposed use of methoxyfluorane and isofluorane as a neonatal anesthetic (22, 23). However, to the authors' knowledge, the effect of these anesthetics on experimental lung research has not been properly investigated in neonates (16). Additionally, isofluorane is more pungent than halothane and causes direct irritation of the airways, which may lead to coughing, laryngospasm, and bronchospasm (24). Although hypothermic anesthesia has been recommended for neonatal rodents (25), its use is controversial (16), and therefore, was not tested as an alternative in the ITI of this study.

Although the technique for intratracheal (transoral) inoculation in neonatal rats required a meticulous approach, it provided consistent results and allowed inoculation of a large number of neonates in a short time. Once standardized, this technique is as effective and as rapid as are the intratracheal techniques recommended for mature and adult rats (10, 20). The apnea observed in some pups immediately after inoculation

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was only transient, and respiratory movements spontaneously returned without the need of manual resuscitation. This complication following ITI also has been reported in adult rats (13).

Use of the modified otoscope illuminator system was essential to perform laryngeal intubation since the metal laryngoscope routinely used in adult rats is inappropriate because of the small oral cavity of the neonatal rat (13). Attaching the illuminator to a fixed metal support proved to be a useful approach as it frees one hand of the operator for intubation and injection of the inoculum. Most other techniques generally require two operators, one to administer the anesthetic and hold the rat and illuminator, and another to inject the inoculum (10).

One important attribute of ITI is that large quantities of inoculum, regardless of particle size and viscosity, can be rapidly introduced into the airways and alveoli of the various lung lobes (7, 8). Results of this study clearly indicated that a high-viscocity material, such as meconium, could be consistently delivered to the lungs of neonatal rats. The distribution within individual lobes (lobar distribution) also was widespread, as indicated by the high percentage (100%) of left, right cranial, right middle, and right median lobes containing meconium in conducting airways and alveoli. The only exception was the right caudal lobe, in which the inoculum was always present in the alveoli, but only in 87.5% of conducting airways. The reason for differences is unclear, but may be related to the anatomic branching as suggested by other researchers (5, 7).

This study indicated that it is possible to deliver high-viscosity materials into all pulmonary lobes of 7-day-old rats. This method will be of important value in experimental studies in which neonatal rats are required to reproduce pulmonary pediatric conditions as it is the case of meconium aspiration syndrome (26).

It was concluded from results of this investigation that it is possible to intratracheally inoculate anaesthetized neonatal rats by use of a modified illuminator (otoscope). The procedure is well tolerated, causes minimal discomfort, and allows inoculated particles to consistently reach all pulmonary lobes of the rat neonate. To our knowledge, this is the first description of transoral ITI in neonatal rats. This technique offers a simple and reproducible method to study the effect of particles in the lungs of neonatal rats.

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