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Ultrasound-guided Collection of Amniotic Fluid in Pregnant Rats

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Here we present an echographic method to withdraw amniotic fluid from pregnant rats. The method could be an alternative to the surgical amniotic fluid collection methods used currently. On day 18 of gestation, pregnant Sprague–Dawley rats underwent amniotic sac puncture by either surgical procedure or echographically guided method. This study evaluated the effect of the 2 collection procedures on parturition day, number of pups per litter, and weight of newborns compared with those of a control group without any fluid collection. These parameters did not differ statistically across groups. However, the echographically guided method did not require surgery or postsurgical recovery and was therefore advantageous from the perspective of animal use. Moreover ultrasound-guided collection allows experimental designs that require collection of multiple samples from the same animal during a single pregnancy.

Abbreviation: GD, gestational day

During pregnancy, amniotic fluid functions as a protective reservoir for the fetus against impact or temperature variation. Amniotic fluid is essential for normal fetal development.[8] Clinical observations emphasize the importance of normal amniotic fluid volume and composition as prerequisites for a favorable perinatal outcome.[5] Amniotic fluid also serves other diverse functions, including bactericidal and mechanical properties that facilitate fetal movement and development of the respiratory and digestive systems. Today, amniocentesis is often used in the 2nd trimester of pregnancy (usually 15 to 18 wk after a woman's last menstrual period) to diagnose or, far more likely, rule out chromosomal and genetic abnormalities or malformations. However, amniocentesis is not routinely offered to all pregnant women because it carries a small risk of miscarriage. Consequently, biochemical tests are most often used.[11] Amniotic fluid is also a potential biomarker of maternal and prenatal viral or bacterial infection exposure,[1,7,9] hypertensive disease such as preeclampsia,[4] and pesticide exposure.[3] Various animal models used to study amniotic fluid include mice, rats, rabbits, and horses.

There are 2 main techniques for collecting amniotic fluid, depending on the volume in the animal studied: echographic and surgical. For large animals such as cows and mares, amniotic fluid collection is performed using echographic methods, either transabdominal or transvaginal.[15,20,25] For medium-sized animals like rabbits, collection of amniotic fluid is done either blindly or through a surgically exposed amnion.[10] In small animals, the standard procedure for amniotic sac puncture is usually performed after laparotomy through the wall of the uterus.[14,18,21] Nevertheless, Turnbull,[24] Olsson,[19] and Zhou[26] performed ultrasound- or biomicroscopy-guided injection combined with or without surgery in a mouse model. A transverse laparotomy incision is placed to the mouse abdomen to extract an embryo from the uterus. Then, the embryo is placed in a warm bath (phosphate-buffered saline), and the ultrasound transducer scans over the bath to facilitate the injection or embryonic transplantation. The same procedure could be used for amniocentesis.

Here we present a method for amniotic puncture and fluid collection under echographic guidance in rats in the last 3rd of gestation. This echographic method could be an alternative method to the surgical approach. The aim of the study is to compare 2 amniocentesis techniques: ultrasound-guided and surgical. The effects of these 2 types of procedure are compared by evaluating features of offspring. The day of parturition, number of pups, and body weight of pups are recorded and compared with those of a control group of animals that did not undergo amniotic puncture.

Materials and Methods

Animals. Animals used in this study were female Sprague–Dawley rats weighing between 200 and 270 g at the beginning of the experiment. All animals were used in accordance with the *Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* from the Council of the European Communities (86/609/EEC).

The experiments were carried out on 18 time-dated pregnant Sprague–Dawley rats (Harlan France, Gannat, France). Health monitoring of the animals by the vendor is in accordance with recommendations of the Federation of European Laboratory Animal Science Associations. The recommended minimum sampling frequency of monitoring is every 3 mo for virology (10 organisms tested), bacteriology (10 bacteria tested), parasitology (2 parasites) tests (Table 1); animals also were checked when pathologic lesions appeared. Animals were kept in a conventional animal facility. Pregnant rats were kept in individual plastic cages (800 cm^2) with open-air tops. Wood shavings were used for bedding, and animals were maintained under a 12:12-h light:dark photoperiod. They received ad libitum water and chow containing 16% protein and 3.5% fat, which supports growth and maintenance (Teklad Global, Harlan France).

Cages were changed twice per week by caretakers wearing gloves, labcoats, and overshoes inside the animal facility.

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Table 1. Organisms tested for during monitoring of rat health in accordance with the Federation of European Laboratory Animal Science Associations

Viruses	Bacteria, mycoplasma, and fungi	Parasites
Hantaviruses	*Bordetelle bronchiseptica*	Ectoparasites
Kilhian rat virus	*Clostridium piliform*	Endoparasites
Mouse adenovirus type 1 (FL)	*Corynebacterium kutscheri*	
Mouse adenovirus type 2 (K87)	*Hellicobacter* spp.	
Pneumonia virus	*Mycoplasma* spp.	
Rat parvovirus	Pasteurellaceae	
Reovirus type 3	*Salmonella* spp.	
Sendai virus	*Streptobacillus moniliformis*	
Sialodacryoadenitis (rat coronavirus)	β hemolytic (not group D) *Streptococcus* spp.	
Toolan H1 virus	*Streptococcus pneumoniae*	

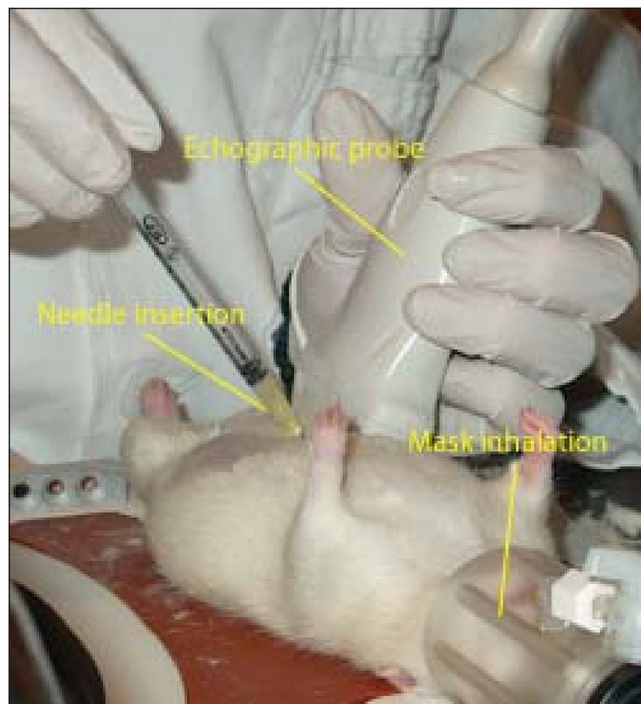

Figure 1. Setup for collecting amniotic fluid by the ultrasound-guided technique.

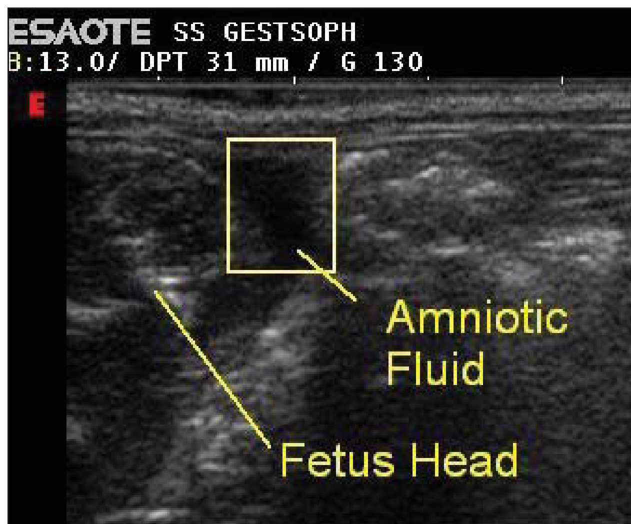

Figure 2. Echographic image of a gestational sac. Amniotic fluid and the head of a fetus are shown.

Animals were removed from the dirty cage and were placed in a clean one; the water bottle and the chow were also changed twice each week. Females were allowed to deliver normally, and the day of delivery was recorded as postnatal day 1. At birth, litters remained with the birth mother and were adjusted (when necessary) to a maximum of 12 pups, equivalent to the number of teats (equal numbers of male and female mice were represented in the litter). The number of pups per dam was counted on the day of parturition. The pups were weighed on postnatal day 2 instead of day 1, to avoid stress to the mother.

Ultrasound system. An ultrasound Esaote Technos MPX system (ESAOTE France, Fontenay-sous-bois, France) connected to a 14-MHz linear transducer was used during amniotic fluid collection. The field of view was set to an imaging depth of 21 mm and width of 32 mm.

Experimental procedures. On day 18 of gestation, anesthesia was induced using mask inhalation that delivered 3% isoflurane (AErrane, Baxter, Lessines, Belgium) in oxygen (2 l/min); anesthesia was maintained with 1% isoflurane. The use of a rapid-acting and safe inhalation agent, such as isoflurane, provides stability during the procedure and allows rapid recovery from anesthesia after the surgery. The duration of anesthesia never exceeded 60 min. Animal temperature was monitored and maintained at 37.5 ± 0.2 °C by use of a feedback temperature controller connected to 2 incandescent lights for regulation.

Sac puncture. Two techniques of needle insertion were evaluated: insertion after surgical exposure of the uterine horn (group I, n = 7) and echographically guided amniotic puncture (Figures 1 through 4; group II, n = 6). For the 3rd group, no amniocentesis was done (group III, n = 5).

For group I, each animal was placed in supine position; the abdomen and lower back region were shaved, cleaned with iodine solution, and aseptically draped. A 3-cm ventral midline incision was made through the skin and peritoneum. Then the bicornuate uterus was exteriorized while any traction on the ovarian arteries was avoided, in order to prevent ischemia. During the entire surgical procedure, the uterus and exposed fetus were moistened continually with prewarmed sterile saline solution (37 °C). A maximum of 5 sacs were sampled, with exclusion of the gestational sacs just above the cervix and above the ovary.[21] Transuterine insertion of a sterile 24-gauge needle was performed; 0.1 ml of amniotic fluid was withdrawn from each sac, and 0.1 ml of sterile saline solution (prewarmed, 37 °C) was reinjected to keep the volume unchanged. Care was taken to avoid puncturing the fetuses themselves, and no escape of fluid was noted. Then uterine horns were gently pushed back into the abdominal cavity. The maternal laparotomy was sutured by use of 2-0 Vicryl (Ethicon, Issy les Moulineaux, France).

For group II, each animal was placed in supine position, and the abdomen was shaved, cleaned with iodine solution, and draped aseptically. Freehand echographic amniocentesis was

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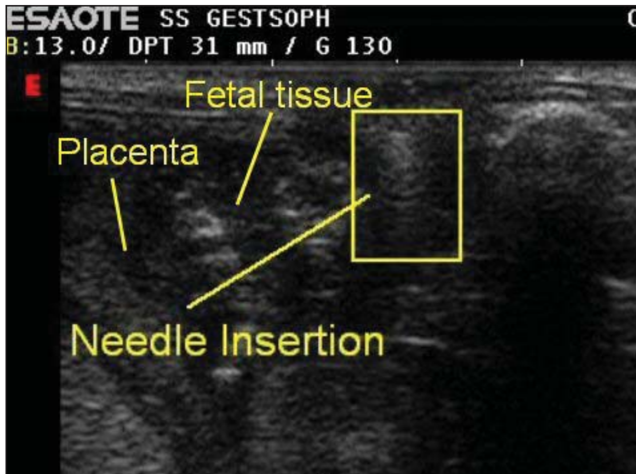

Figure 3. Progression of the needle through the skin of a rat on day 18 of gestation.

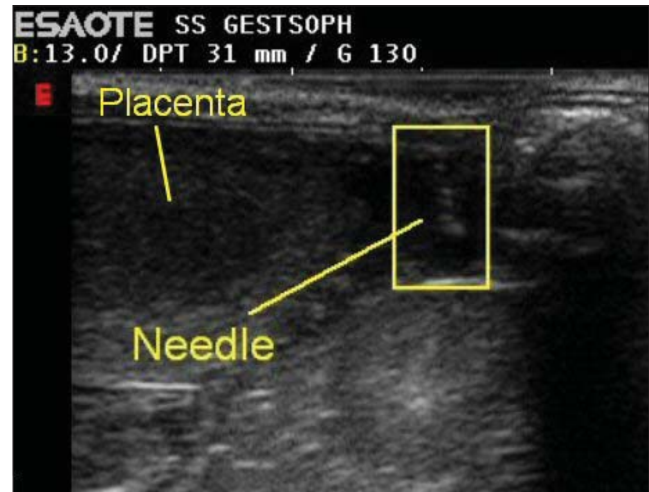

Figure 4. Needle positioned in the gestational sac to collect amniotic fluid.

performed by transabdominal insertion of a sterile 24-gauge needle. Echographic guidance was done by positioning the probe transversely and manually driving the needle progression into the middle of the field of view (Figures 1 through 3). As in the laparotomy procedure, 0.1 ml of amniotic fluid was removed from each sac, and 0.1 ml of sterile saline solution (prewarmed, 37 °C) was reinjected to maintain the initial volume.

In group III, female rats were anesthetized during the same time as were sampled animals but no further manipulation was done. After the experimental procedure, rats were allowed to recover in individual cages, fed ad libitum, and monitored for signs of infection or labor. Maternal body temperature and weight were unchanged postsurgery, and no sign of pain (abnormal temperature, coat, activity) or vaginal loss was noted. Therefore, postoperative analgesia was not provided.

Statistical analysis. Analysis of variance (SigmaStat 3.1, Systat Software, Richmond, CA) was performed to compare the 3 groups. *P* values were considered significant when less than 0.05. The results were expressed as mean ± standard error.

Results

Parturition day. Table 2 describes the percentages of dams delivering on days 21, 22, and 23 postmating. As expected, all of the control rats (group 3) delivered on day 22 of gestation (GD22). The majority of rats in group II (echographic collection of amniotic fluid) delivered on day 22 of gestation (GD22, 83%; GD21, 17%). In group I (collection through surgically exposed uterine horn), 14% delivered on GD21, 14% on GD23, and 72% on GD22. None of the dams failed to deliver. There was no statistically significant difference among the groups ($P = 0.75$).

Number of pups at delivery. The 7 females in group I delivered a total of 76 pups, whereas 66 were born in group II (6 dams) and 65 in group III (5 dams). The mean number of pups per dam (Table 2) tended to be lower in group I (10.85 ± 0.93) and II (11 ± 1.09) than in control group (13 ± 0.63). The mean number of pups is decreased by 16.48% in the surgical group and by 15.38% in the echographic amniocentesis group. Despite this trend, there is no significant difference among the 3 groups.

Weight of newborns. The mean body weight of pups is shown in Table 2. Weight of newborns at day 2 postpartum is 7.10 g ± 0.14 in control group (n = 65), 7.11 g ± 0.13 in group I (n = 76) and 7.47 g ± 0.18 in group II (n = 66). Analysis of variance showed that the mean weight of pups did not differ significantly among the 3 groups.

Discussion

Most previously published studies describe the use of laparotomy in puncture of the amniotic sac in a small animal. In our study, we used a transabdominal ultrasound-guided technique to withdraw amniotic fluid from fetal sacs. According to analyses of variance, this technique did not disturb the date of delivery, number of pups per dam, or weights of pups 2 d after parturition. However, even though no statistically significant difference between the groups was found, 72% of group I dams delivered on GD22 versus 83% in group II and the expected 100% of dams in the control group. Unlike in Singh's study,[21] none of our animals failed to deliver, and none of the animals died. Singh[21] observed that in the group with unpunctured sacs at the vaginal end of the uterus, 75% of deliveries occurred on GD21, whereas in the group with unpunctured sacs at the ovarian ends, 75% of deliveries occurred on day GD23. In our study, we chose not to sample sacs at either the ovarian end or vaginal end, and we noted that 17% of parturitions occurred on GD21 in the group with ultrasound-guided fluid collection compared with 14% in the surgically sampled group. We also noted delay of parturition until GD23 for 14% of dams in group I, a finding that may be related to insufficient injection of saline solution into gestational sacs. Reduction in the volume of amniotic fluid and restricted movement of the fetus may prolong the gestational period.[22,23] The situation was reversed when sacs were left unsampled at the vaginal ends. The amniocentesis induced myometrial contractions, which facilitate the process of parturition. The combined effect was either normal or premature delivery of the fetuses.[21]

When the collection was performed, the same volume of physiologic saline was reinjected to avoid oligohydramnios, which can lead to malformations, fetal growth retardation,[2] skeletal defects,[12] and pulmonary hypoplasia[18] due to neonatal morbidity from preterm prelabor rupture of membranes.[13] Moessinger[18] and Blachford[2] used 22-gauge and 20-gauge needles, respectively, to withdraw amniotic fluid. Blachford[2] showed subsequent leakage related to amniotic fluid collection and resulting in fetal growth retardation. In our study, a 24-gauge needle was used; this smaller external diameter was used in order to minimize amniotic fluid leakage and subsequent oligohydramnios. In the control group (65 pups), no morphologic abnormalities were encountered. In the 2 groups with amniotic fluid collection (142 pups), only a single pup showed hypoplasia

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Table 2. Delivery day, number of pups, and weight of newborns on day 2 postpartum

	Group I	Group II	Group III
Delivery on (% of dams)			
gestational day 21	14%	17%	0%
gestational day 22	72%	83%	100%
gestational day 23	14%	0%	0%
Number of pups per dam[a]	10.85 ± 0.93	11.00 ± 1.09	13.00 ± 0.63
Weight of newborns on day 2 (g)[a]	7.11 ± 0.13	7.47 ± 0.18	7.10 ± 0.14

[a]Data presented as mean ± standard error of the mean.

of the tail, perhaps because the needle of the syringe touched this animal; there were no other abnormalities. Prewarmed sterile saline solution (37 °C) was used to avoid thermal shock, which could lead to fetal hypothermia.

In our study, there were no noteworthy effects of amniotic puncture on sex ratios of pups, almost 50% of which were female (data not shown). There was no statistically significant difference among the 3 groups regarding the weight of newborns on day 2 postpartum. No fetal growth retardation was noted. The numbers of pups did not significantly differ from those of controls.

Surgical amniocentesis is an invasive method to withdraw amniotic fluid. It requires laparotomy and exposure of the uterine horns to ambient air. Such exposure increases the risk of maternal infection. The abdomen is closed in layers with surgical staples or suture, further increasing the postsurgery infection risk. Although the etiology of preterm premature rupture of membranes is probably multifactorial, infectious processes (intrauterine infection[17] or bacterial vaginosis[6]) may play an important role in this complication.[16] Another limitation of the surgical method is that fluid collection can be performed only once in an animal and induces decreased dam movement during the 1st hour or so after the procedure. In contrast, the ultrasound-guided method involved transabdominal needle insertion, which decreases the risk of maternal infection. In addition, animals are not exposed to potentially harmful side effects of surgery, and amniotic fluid can be collected several times from the same animal.

We here present a method for ultrasound-guided collection of amniotic fluid that is suitable for use in rats. This technique is less disruptive to animal well-being, compared with surgical techniques that require the exposure of the uterine horns. With the surgery method, the postsurgery recovery of the animals is longer and more traumatic than is the collection itself. The novelty of our method resides in the ultrasound-guided intervention, in terms of the comparison between the guided approach and a surgical approach. Despite some limitations regarding the period during which it can be used for amniotic fluid collection (only between GD13 and GD19), the echographic method is less invasive than the surgery method and allows repeated amniocenteses on the same animal.

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