Video Article

Ultrasonography in Experimental Reproductive Investigations on Rats

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Abstract

With the development of assisted reproductive technology and the ethical limitations of research on humans, rat animal models have been widely used in reproductive medicine. In the past, the study of reproductive system development in rodents has been based on one-time histological examination of excised tissues. Recently, with the development of high-resolution transabdominal ultrasound, high-quality sonography can now be performed to evaluate the reproductive organs of rats, allowing a new method for studying the reproductive system. Images were obtained using a high-resolution ultrasonographic system. Gynecological ultrasonography was performed on 28 eight-week-old non-pregnant rats and 5 pregnant Sprague-Dawley rats. We describe how to recognize organs of the reproductive system and associated structures in typical views during different phases of the estrus cycle. Color flow Doppler was used to measure uterine artery blood flow and evaluate uterine blood flow pattern changes during different stages of pregnancy. We have demonstrated that ultrasound exploration is a useful method for evaluating changes in internal reproductive organs. Its use raises the possibility of conducting additional experiments, including medical or surgical procedures, and provides the ability to monitor sonographic changes to internal organs without sacrificing animals.

Video Link

The video component of this article can be found at https://www.jove.com/video/56038/

Introduction

Rat animal models have been widely used in reproductive medicine, including in embryo and ovarian transplantation[1,2]. However, in the past, the study of reproductive system development in rodents has been based on one-time histological examination of excised tissues, and the longitudinal study of day-to-day reproductive organ changes has not been possible in rats[3]. Ultrasound has been widely used in assisted reproductive technology in humans for over 30 years, but this valuable technology has only been applied to rats recently.

Our objective was to establish an ultrasonographic approach for evaluating the reproductive organs of Sprague-Dawley rats to design experimental models for reproduction and gynecology investigation and to demonstrate the procedure because to our knowledge, there are no current visualized publications regarding this procedure. We describe the procedure of ultrasonographic examination of the female rat's reproductive system and present ultrasonographic findings on anatomy and uterine artery blood flow using high-definition ultrasound. We monitored the characteristics of the endometrium, ovaries and uterine artery blood flow in non-pregnant animals at different stages of estrus cycle to evaluate the significant differences in endometrial thickness, ovarian morphology and uterine blood flow in different stages of the estrus cycle, similar to women. We used high-quality ultrasound equipment with a 70 MHz frequency and a resolution level of 30 μm. Our other objective was to evaluate changes in the resistance of uterine blood flow in pregnant rats. This technique allows for the study of daily changes in the reproductive organs without sacrificing animals.

There are several technical difficulties in utilizing ultrasound on rats. These difficulties include: the rat endometrium is much thinner than a human female[4]. Difficulty in imaging the ovaries of rats has been attributed to thicker skin and abdominal wall musculature in rats, which resulted in near-complete attenuation of the ultrasound[5], and the uterine artery is much more difficult to find in non-pregnant rats. We have solved many technical problems with the procedure, and for those problems that remain, we show how to minimize them.

Successful monitoring of sonographic changes in the reproductive organs of rats without the need to sacrifice animals will open the possibility of building future animal models of reproductive medicine and other surgical procedures.

Protocol

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. The protocol received a license for animal experimentation complying with Directive 2010/63/EU with the authorization number A13170404 (Anexo 1). All experiments were performed in a European Union-certified laboratory following national guidelines for the ethical care of animals (RD 53/2013, EU Directive 63/2010). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Murcia.

1. Animal Preparation

Note: All experiments were supported by the Animal Experimentation Section of Murcia University and the Obstetrics and Gynecology Department of Murcia University.

1. Use 8 week-old Sprague-Dawley rats (28 female rats) weighing 200-250 g in all experiments.
   Note: Here, we also used 5 pregnant rats.
2. To obtain pregnant rats, cage eight-week-old female and fertile male rats and mate from 17:00 -23:00 h. Identification of a vaginal plug on the following morning was interpreted as mating success**.** Consider day 1 of gestation, the following day after they were mated.
3. Carry out the experiments at 9, 15 and 18 days of gestation.
4. House rats in groups of two with free access to food and water and maintain on regular 12 h light/dark cycles.
5. After acclimating to the facility conditions for a minimum of two weeks, use daily morning vaginal cytology to assess for estrus frequency and regularity.
   Note: Twenty-eight rats with a regular 4 to 5 day estrus phase were selected for inclusion in the study.

2. Preparation of Rats for Imaging

Note: Images were obtained using high-resolution transabdominal ultrasonography. Estrous cycle phases were determined by vaginal smear cytology.

1. Prior to the imaging study, anesthetize the dam in the induction chamber with 2-3% isoflurane gas.
2. Remove the animal and immediately place the snout within a nose cone connected to the anesthesia system and maintain the animal on 1.5-2% isoflurane during the ultrasonographic examination.
3. Remove the fur from the costal margin to the caudal abdomen with clippers and depilatory cream.
4. Place the anesthetized rat in a supine position on a heated table to secure the rat and ensure optimal comfort and maintenance of physiological parameters for the duration of the imaging session. All physiological parameters should be integrated with the images and data captured in real-time via the ultrasound application.
5. Gently insert a rectal probe (after lubricating) to monitor body temperature (37.5 °C ± 0.5 °C).
6. Place the transducer (30 Hz) in a stationary holder and move it along the vertical axis and horizontal axis (forward-to-back and side-to-side) using a manually operated joystick or by hand.
7. Apply petroleum-based ophthalmic balm to the eyes of the rat to avoid drying during the procedure.

3. Examination Procedure

Note: Anatomy of rats´ reproductive organs: The vagina lies dorsal to the urinary bladder and divides into two uterine horns that extend toward the kidneys. The ovaries are connected to the uterine horns via the oviducts (**Figure 1**). The uterus is located in the region posterior to the kidneys.

1. **Identification of the Uterus**
   1. By using the bladder as a landmark to find the cervix, follow the cervix to find the branch of the left and right uterine horns.
   2. Switch to two-dimensional viewing/video by selecting "B-mode". Measure the antero-posterior diameter of each uterine horn in the mid-isthmic region on a sagittal midline image. Detect measurements using the ultrasonographic system software.
   3. Measure the endometrial thickness from echogenic border to echogenic border across the endometrial cavity on a sagittal midline image in "B-mode".
   4. Change to color Doppler mode by selecting "Color Doppler". Use color Doppler to identify the blood supply of the endometrium and measure the blood flow of endometrium. Select the following parameters in color Doppler mode: high-pass filter set at 4 Hz, pulsed repetition frequency set between 4 and 48 kHz, and Pulsed Doppler gate set between 0.2 to 0.5 mm.

2. **Identification of Uterine Artery Blood Flow**
   1. Obtain Doppler waveforms in the uterine artery near the lateral-inferior margin of the utero-cervical junction close to the iliac artery on each side.
   2. Use the following parameters in Doppler mode: high-pass filter set at 6 Hz, pulsed repetition frequency set between 4 and 48 kHz, and Pulsed Doppler gate set between 0.2 to 0.5 mm.
   3. Take care to align blood flow and the Doppler beam to minimize the Doppler angle. Record the angle between the Doppler beam and the vessel. Values taken beyond an angle of 60° are inaccurate and should be avoided[6].
   4. Measure the peak systolic velocity (PSV) and end-diastolic velocity (EDV) from three consecutive cycles. Then calculate the systolic to diastolic (S/D) ratio (PSV/EDV) and resistance index (RI) ([PSV-EDV]/PSV) values for each uterine horn.
   5. Measure the uterine artery blood flow of 5 pregnant rats during the 9^{th}, 15^{th}, 18^{th} day of gestation.

3. **Identification of Ovary and Ovarian Artery Blood Flow**
   Note: Female rat ovaries are located lateral to the kidneys on both sides of the animal and reside in fat pads found at the end of the uterine horn (**Figure 1**).
   1. To image the ovary, start with the probe in a transverse plane and place it on the lateral aspect of the animal slightly below the ribs. The kidney and the fat pad have a hyperechoic appearance compared to the ovary.
   2. Measure the external boundary of the ovary and follicles. Numbers on the scale to each image are in millimeters, with 0.1 mm increments.
      Note: Color Doppler Mode and Power Doppler Mode imaging helps with identification of ovarian intensity and directional flow.

4. Design of the Study

1. Check the estrus cycle by vaginal smear cytology.
2. Divide all rats in two groups. For Group 1 or pre-fertile (or periovulatory), include the rats that were in the proestrus and estrus cycle phases. For Group 2 or post-fertile, include the rats that were in early diestrus and late diestrus cycle phases.
3. Monitor and compare the antero-posterior diameter of each uterine horn in the mid-isthmic region in Groups 1 and 2.
4. Monitor and compare the endometrial thickness and characteristics of the endometrium in Groups 1 and 2.
5. Monitor and compare the size (maximum diameters) and characteristics of the ovaries, and localize any periovulatory follicles in both ovaries in Groups 1 and 2.
6. Monitor and compare the uterine artery blood flow in Groups 1 and 2.
7. Monitor and compare the uterine artery blood flow of pregnant rats in different stages of pregnancy (days 9, 15 and 18 of gestation).
8. Perform statistical analyses using SPSS. Present data as the mean ± standard deviation (SD) or median with interquartile ranges. Analyze the results using the Student's t-test between the different groups. A P-value of less than 0.05 was considered a statistically significant difference.

Representative Results

There were no significant differences in antero-posterior uterine horn diameters or in the thickness of the endometrium between the two sides of the uterine horn (**Table 1**). Compared with the group 2, the mean endometrial thickness in the group 1 was thicker, but no significant differences (P>0.05) were found between the two groups (**Figure 3**). Nevertheless, we found fluid inside the uterus (in 8 out of 28 rats) near the estrus cycle associated with changes in endometrium morphology (**Figure 2**).

Doppler studies also showed no significant changes in flow velocity waveform patterns in each side of the uterine horn or in different phases of estrus cycle in non-pregnant rats (**Tables 1** and **2**, **Figure 4A**). However, in pregnant rats, as gestation advanced, peak systolic and end-diastolic velocities increased significantly, and the calculated resistance index decreased significantly (**Table 3**, **Figure 4B**).

The mean diameter of the ovary was not significantly different (**Table 1**). When the morphology of the ovary was compared between the two groups, periovulatories follicles and fluid around the ovary were seen after ovulation (**Table 2**, **Figure 2**).

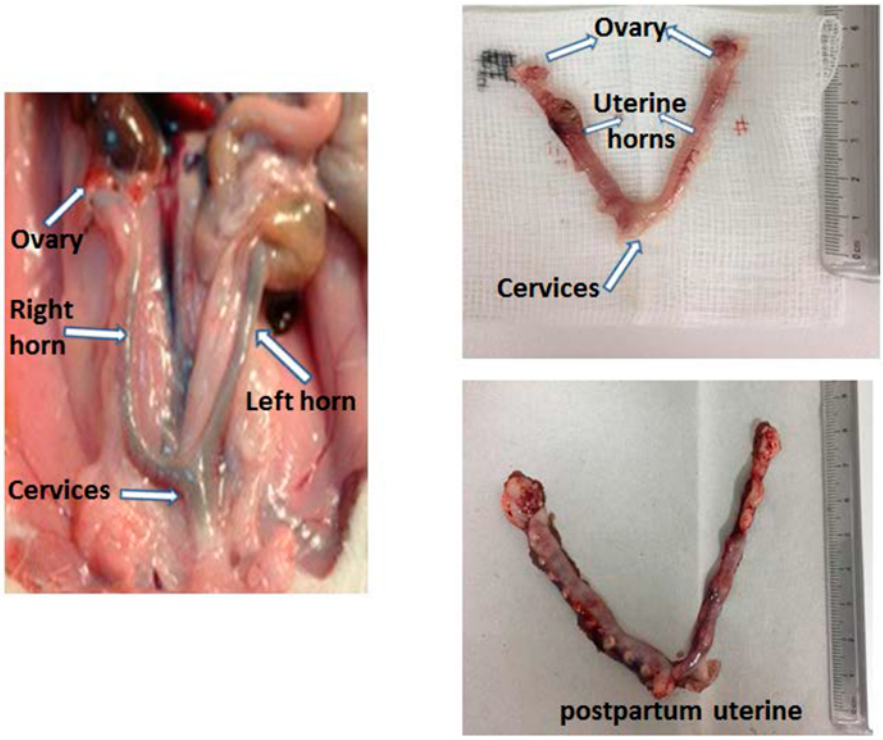

Figure 1: Anatomy Please click here to view a larger version of this figure.

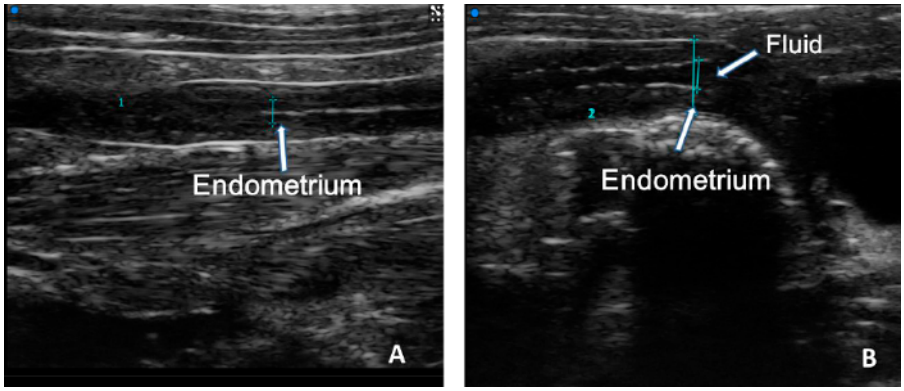

Figure 2: Measurement of the endometrial thickness (B-mode). The thickness of the endometrium (blue line) (**A**). The antero-posterior uterine horn diameters (large blue line) and thickness of the endometrium (short blue line) during estrus cycle (B). Please click here to view a larger version of this figure.

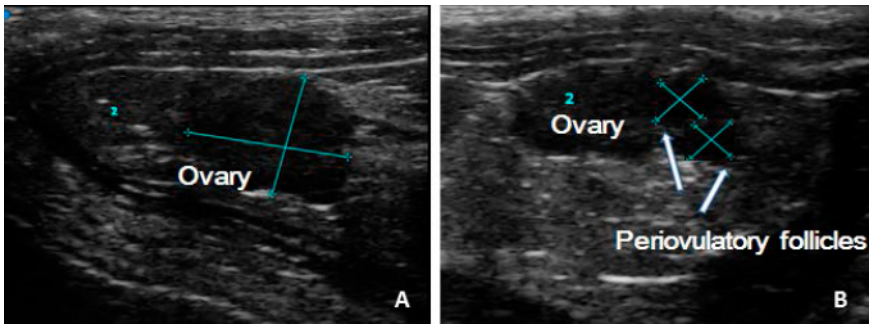

Figure 3: (**A**) Measurement of the diameter of the left ovary; (**B**) Ovary and follicles during estrus phase.

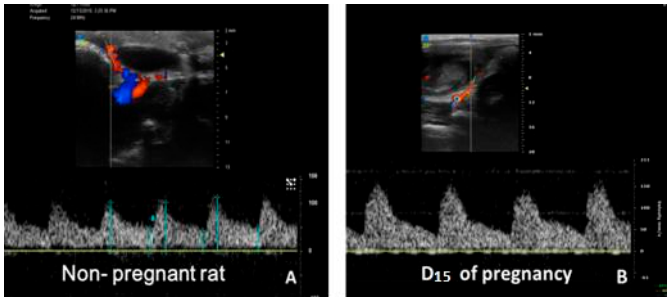

Figure 4: Measurement of uterine artery blood flow. (**A**) Uterine artery blood flow in non-pregnant rats. (**B**) Uterine artery blood flow in 15 th day of pregnant rats.

(P >0.05, no significant difference in each group).SD: Standard DesviationPSV: peak systolic velocityEDV: end-diastolic velocityS/D: Systolic to diastolic ratioRI: resistance index. ((RI)=[PSV–EDV]/PSV)

Variable (mm±SD)	**Left side**	**Right side**	**P. value**
Horn diameter (mm)	1.78±0.24	1.73±0.28	0.626
Endometrium thickness (mm)	0.75±0.06	0.76±0.05	0.752
Ovary diameter (mm)	3.69±0.52	3.62±0.32	0.107
Follicle size (mm)	1.68±0.31	1.74±0.29	0.859
PSV (mm/s)	91.52±17.91	93.07±22.87	0.055
EDV (mm/s)	34.18±9.36	36.67±11.14	0.178
S/D	2.78±0.59	2.62±0.50	0.294
RI	0.62±0.08	0.60±0.08	0.876
(P >0.05, no significant difference in each group).			
SD: Standard Desviation			
PSV: peak systolic velocity			
EDV: end-diastolic velocity			
S/D: Systolic to diastolic ratio			
RI: resistance index. ((RI)=[PSV–EDV]/PSV)			

Table 1: The differences in the left and right uterine horn and ovary.

Variable (mm±SD)	**Estrus phase (Group 1)**	**Non-estrus phase (Group 2)**	**P.value**
Horn diameter (mm)	1.71±0.18	1.83±0.23	0.433
Endometrium thickness (mm)	0.78±0.04	0.72±0.05	0.168
Ovary diameter (mm)	3.71±0.56	3.66±0.47	0.515
PSV (mm/s)	92.05±17.93	94.15±20.62	0.886
EDV (mm/s)	37.81±9.64	34.72±5.38	0.096
S/D	2.61±0.58	2.77±0.44	0.249
RI	0.60±0.08	0.63±0.06	0.232
(P >0.05, no significant difference in each group).			
SD: Standard Desviation			
PSV: peak systolic velocity			
EDV: end-diastolic velocity			
S/D: Systolic to diastolic ratio			
RI: resistance index. ((RI)=[PSV–EDV]/PSV)			

Table 2: The differences between different estrous cycle phases in non-pregnant rats.

Variable	D9	D15	D18	P value
PSV(mm/s)	111.08±5.93[a,b]	122.64±7.49[c]	131.91±3.50	<0.05
EDV(mm/s)	38.80±3.37[d,e]	56.43±3.10[f]	79.29±5.47	<0.05
S/D	2.87±0.12[g,h]	2.17±0.16[i]	1.67±0.14	<0.05
RI	0.65±0.02 [j,k]	0.54±0.04[L]	0.39±0.05	<0.05
PSV= Peak systolic velocity				
EDV=End-diastolic velocity.				
S/D=Systolic to diastolic ratio (PSV/EDV).				
RI=Resistance index ((RI)= ([PSV–EDV]/PSV).				
D9= Day 9 of gestation				
D15= Day 15 of gestation				
D18=Day 18 of gestation				
SD:The errors indicate Standard Deviation (±).				
(P<0.05, no significant difference in each group)				
P value: D9 vs D15 a=0.03; d=0.001; g=0.01; j=0.01. D9 vs D18 b=0.003; e=0.001; h=0.01; k=0.001. D15 vs D18 c=0.03; f=0.001; i=0.03; L=0.04.				

Table 3: The differences in uterine artery blood flow in pregnant rats.

Discussion

Due to the procedural modifications and troubleshooting that was required in this study, despite our aim of identifying all the phases of the estrus cycle in rats using ultrasound, we were unable to find any significant differences. We hypothesize that these difficulties may be because the estrus cycle only lasts a few days in rats, unlike the cycle in women. We are sure that all measurements were done at exactly the right time to determine any differences. Therefore, we regrouped the estrus cycle phases into only two phases to evaluate for any differences, but none were observed. As expected, we found no significant difference between the measurements of each uterine horn, which will allow us to choose either horn to perform measurements in the future. Studies on the effects of isoflurane use on rodents are contradictory. It may be teratogenic, but only if administered at anesthetic concentrations for many hours or several days. In our experiment, the examination time in the pregnant rats was less than 30 min, so we did not find any adverse effects in any pregnant rats or their offspring.

We would like to have found more similarities to ultrasound evaluation of the reproductive tract in women, in which there are clear echogenic differences between the follicular phase, periovulatory and secretory phases, but such changes were not seen in the rat model. This fact can be explained because the rat estrus cycle phases only last four to five days. Short estrus cycle phases and gestation periods make the rat an ideal animal for research on reproduction[5]. Nevertheless, this fact may be the reason why there are no significant differences in the diameter of the uterine horn, endometrial thickness and ovarian diameter in the estrus and non-estrus phases. It is difficult to take the measurements at exactly the right time to ascertain differences, and despite taking measurements every day, we did not find significant changes.

Despite the limitations above, we propose the use of ultrasonography to monitor changes in the reproductive system, including thickness and morphology of reproductive organs. We can affirm this use of ultrasonography because the thickness of the endometrium in 8-week-old Sprague-Dawley rats as measured histopathologically (one layer 359.13 ± 49.70 mm) by Jing *et al.*[7] is similar to the results here. In spite of the various techniques for measuring endometrial thickness histopathologically and sonographically, we obtained similar results. Though power Doppler and color Doppler have been used on pregnant rats for several years[8,9], there have been few investigations measuring uterine artery blood flow in non-pregnant rats. Now with the development of the ultrasonography, we can take advantage of this technique for monitoring changes in the reproductive tract during each different stage, even at the very beginning of pregnancy.

Future applications of ultrasound may include exploration of the mechanism of embryo implantation and treatment of thin endometrium in rat animal models. Also, by monitoring the characteristics of developing follicles, we can obtain more comprehensive knowledge about their function in an ovarian transplantation model. Currently, there are few investigations using 3D imaging and molecular ultrasound imaging of the reproductive system of rats[10], and we will apply this technique on a thin endometrium model in the future.

We can draw the conclusion that the rat is a suitable model for the study of reproductive organ dynamics using transcutaneous ultrasound bio-microscopy, which does not require sacrifice of the animal.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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