Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
USE OF REAL-TIME ULTRASONOGRAPHY AS AN ALTERNATIVE METHOD FOR EARLY DETECTION, CONFIRMATION AND EVALUATION OF RAT PREGNANCY

MARIANNA STASINOPPOULOU,* GEORGE MANTZIARAS,* EUThIMIOS PARONIS,* EVANGELOS BALAFAS,* PAVLOS LELOVAS,* ATHINA SAMARA,1 and NIKOLAOS KOSTOMITSOPOULOS*

*Laboratory of Animal Facilities, Centre of Experimental Surgery, Biomedical Research Foundation Academy of Athens, Athens, Greece; yVeterinary Department, Medical Directorate of Hellenic Airforce General Staff, Athens, Greece; and zLaboratory of Endocrinology and Metabolism, Department of Clinical Research, Biomedical Research Foundation Academy of Athens, Athens, Greece

(Received 21 April 2013; revised 11 November 2013; in final form 14 November 2013)

Abstract—Researchers sometimes face difficulties in the diagnosis of pregnancy and assessment of embryonic development. Ultrasonography (US) is a non-invasive imaging method with minimal side effects on the subjects or operators. It provides real-time evaluation of the physiology of rapidly moving structures (i.e., heart) and facilitates evaluation of fetal tissue development. US discerns tissues based on composition, making it the imaging method of choice for abdominal examination. In this study we used real-time US as an alternative method for early diagnosis of pregnancy in rats. Sixty-four Wistar rats aged 16–20 wk were examined, and day 8 was the earliest point at which pregnancy could be detected. We constructed a detailed timeline of embryonic features detectable by US on days 8 to 19. We trust this index will be a valuable tool. More refined work toward a more detailed “atlas” will help to reduce animal sacrifice during embryonic development studies. (E-mail: gmantziaras@yahoo.com) © 2014 World Federation for Ultrasound in Medicine & Biology.

Key Words: Ultrasonography, Wistar, Rat, “3 Rs”, Pregnancy, Development, Fetal structures.

INTRODUCTION

Rats have been the backbone of many biomedical research studies (Aitman et al. 2008) and are one of the most common models in toxicity and developmental studies during gestation. Early and precise diagnosis of pregnancy, confirmation of the number and age of fetuses and evaluation of embryonic development are prominent in these studies (Krinke 2000). Pregnancy can be determined traditionally by the presence of a copulatory plug after mating, an enlarged lower abdomen, manual palpation (Agematsu et al. 1983; Bennet and Vickery 1970), hair loss around nipples and nipple enlargement (Bennet and Vickery 1970; Bivin 1986) and finally, monitoring of weight gain.

Ultrasonography, although widely used in human pregnancy, is not as common in laboratory animal studies. Until recently, the use of ultrasonography was limited to larger non-rodent species (Beccaglia and Luvoni 2006; Carr et al. 2012; Lenard et al. 2007; Lindahl 1966; Quintela et al. 2012; Zambelli and Prati 2006). Because of advances in ultrasound imaging technology, commercially available ultrasound systems now have the spatial and temporal resolution to obtain accurate images of rat and mouse hearts, kidneys and other organs (Coatney 2001; Golden et al. 2012; Kobayashi et al. 2001; Srinivasan et al. 1998; Stypmann 2007). For this reason, ultrasound is a practical method for the diagnosis of pregnancy in agouti mice (Sousa et al. 2012), staging of gestation and monitoring of intrauterine growth in rats (Brown et al. 2006; Mu et al. 2008; Nguyen et al. 2012; Pallares and Gonzalez-Bulnes 2009; Ypsilantis et al. 2009).

The aim of the present study was to develop a method that could be used in conjunction with the standard pregnancy detection setup in routine experiments. Additionally, this method had to be non-invasive and comply with the “3 Rs”—replacement, reduction and refinement—in animal research (Russell and Burch 1959). In this article, we describe the results of monitoring pregnancy in Wistar rats using real-time ultrasound and share the results collected relative not only to the early
determination of pregnancy, but also to the assessment of fetal development at different embryonic stages.

METHODS

Ethical statement

The study was performed in the animal facility of the Centre for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens, and was evaluated and authorized by the Veterinary Service of the Prefecture of Athens, as mandated by Greek legal requirements for animal experimentation. The facility is registered as a “breeding” and “experimental” facility according to Greek Presidential Decree 160/91, which harmonizes national legislation with European Community Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes.

Study design and experimental procedures

The animals were weighed before mating and for the duration of pregnancy. Consumption of food and water was observed during experimentation.

The first ultrasound examination was performed on the seventh day (D7) post-coitum in all animals. Thereafter, pregnant animals were examined ultrasonographically daily by the same operator (G.M.), between 1100 and 1300 h. The last ultrasound was performed on day 19. The ultrasonographer was blinded to the day the animals became pregnant.

All animals were anesthetized for ultrasound examinations. Anesthesia was induced by administration of isoflurane 5% (Wenger 2012; Flecknell 2009) via a face mask (VME Small Animal Anesthesia Machine, MDS Matrix, Orchard Park, NY, USA). Induction of anesthesia was confirmed by loss of the pedal withdraw reflex. Ultrasonography examination commenced immediately after loss of the pedal withdraw reflex of the animal. Anesthesia was maintained by administration of isoflurane 2.5% (Medical Supplies & Services International, Keighley, West Yorkshire, UK) simultaneously with oxygen 1 to 1.2 L/min.

Ultrasonography was conducted at the operating room of the facility where the vaporizer is located. All animals were examined in the supine position. The hair of the abdomen was thoroughly clipped and shaved immediately after anesthesia induction, to achieve full contact of the probe with the skin in the region concerned. Acoustic gel (Skintact, Leonard Lang, Innsbruck, Austria) was liberally applied. In this way, the phenomenon of “grimy acoustic shadow” created by the air, which holds the animal dander, was reduced to a minimum and the resolution of ultrasonography was greatly improved.

We used the Vivid I ultrasound machine (GE Medical Systems, Tirat Carmel, Israel) with the linear transducer probe 12 L-RS (GE Yokogawa Medical Systems, Tokyo, Japan), which has a variable frequency of 5–13 MHz. To achieve detailed imaging of the reproductive tract of rats and embryos, we used a frequency of 13 MHz, with one focal zone set at a depth of 0.5–2 cm. The total duration of the examination was approximately 10 min.

Immediately after the end of each study, administration of isoflurane was interrupted; all animals were receiving oxygen and recovering smoothly when returned to their cages. Use of a volatile anesthetic at the precise dosage for the vaporizer is considered a tolerable method of anesthesia (Flecknell 2009; Kohn et al. 1997). Specialized personnel (E.B.) handled the animals during vaginal smear collection and anesthesia induction and there were no adverse effects.

Experimental animals and sample size

A total of 64 female Wistar rats (HsdOla:WI) aged 18 ± 1.56 wk were studied. These animals were obtained from the breeding colony of the animal facility and were being used for the first time in reproductive studies; their estrous cycles were confirmed as normal with vaginal smears. The animals were caged in pairs in H-Temp polysulfone type III cages 425 mm long × 266 mm wide × 185 mm high (Tecniplast, Varese, Italy).

Husbandry

Two female Wistar rats were introduced into the cage of a male rat of proven fertility where they remained until the day sperm was detected on the vaginal smear. All cytologic examinations were performed by the same person (M.S.). The day on which sperm was detected was designated as embryonic day 0 (D0), and the female was returned to its cage.

Housing (food, water and bedding)

All cages were kept in the same animal room with a HEPA-filtered air supply, 15 ACH, at a room temperature of 24 ± 2°C, relative humidity of 55 ± 10%, 12 h:12 h light/dark cycle (0700/1900), light intensity of 300 lx, as measured 1 m above the floor in the middle of the room, and positive air pressure of 0.6 Pa within the room. All animals were free of a wide range of pathogens including Kilham rat virus, rat parovirus, Toolan’s H-1 virus, Sendai virus, pneumonia virus of mice, reovirus type III, murine encephalomyelitis virus, sialodacyroadenitis virus, rat min virus, Hantaan virus, lymphocytic choriomeningitis virus, CAR bacillus, MAD 1 and 2, rat rotavirus, rat coronavirus, Mycoplasma pulmonis, Clostridium piliforme, Bordetella bronchiseptica, Pasteurella spp, fur mites and pinworms.

All Wistar rats had ad libitum access to filtered tap water in drinking bottles and pelleted chow, which
RESULTS

Presence of an embryonic sac and ability to distinguish embryo, embryonic organs and placenta were evaluated. Findings were correlated with the overall development of the fetus, as previously described by

Fig. 1. Representative ultrasound images of a rat uterus on different days of gestation: In some of the images, white crosses mark the start and end points of measurements (1, 2) of the longitudinal and transverse diameters, the values of which are given (in cm) in the inset at the top left of the image. (a) Day 8 of gestation: The embryonic sac is visible as a round or oval structure filled with anechoic to isoechoic fluid. (b) Day 9: The developing placenta can also be recognized as a hyperechoic structure attached to the inner surface of the gestational sac. (c) Day 10: The fetus is distinguishable. (d) Day 11: The heart can be imaged by B-mode. (e) Day 12: Cardiac function is detectable using both B-mode and pulse wave Doppler as imaging techniques. (f) Day 13: The extremities are apparent. (g) Day 14: The body and head can be distinguished. (h) Day 15: The body uncoils and the head is distinct from the body. (i) Day 16: The lungs appear as hyperechoic structures surrounding the heart in the thoracic cavity. (j) Day: The urinary bladder is visualized as a cystic structure filled with anechoic fluid (urine), in the abdominal cavity. Ossification of ribs is obvious. Brain ventricles are detectable as hypoechoic discontinuities in the cranial outline. (k) Day 18: The ribs are ossified and visible along with the urinary bladder. (l) Day 19: Brain ventricles are bisected from the ossified joint of the frontal cranial bones. Cranial ossification is more intense and the jawbone can be differentiated.
other authors (Theiler 1989; Witschi 1972; Ypsilantis et al. 2009). The characteristic features unique for each day were noted and are summarized in Table 1.

With D0 considered the day of detection of sperm in the vaginal smear, ultrasound examination was performed daily starting from D7. The pregnancy was first detected by ultrasound on D8 (Fig. 1a). The only visible structure was the embryonic sac, which appeared as a round to oval structure filled with anechoic to isoechoic fluid. There was no evidence of the embryo. The placenta was first evident on D9 (Fig. 1b), as a hyperechoic zonar structure attached in the inner surface of the gestational sac. On D10, the fetus became distinguishable and the heart was also obvious with color Doppler signal (Fig. 1c). The cardiac pulse appeared on the same day, but the heart rate could not be measured with pulse wave Doppler. On D11, the heart was clearly visible with B-mode imaging, but heart rate could not be estimated (Fig. 1d). The next day D12, the amniotic fluid was visible and cardiac function was evaluated with pulse wave Doppler (Fig. 1e). The tail could be distinguished and the fetal extremities were apparent on D13 (Fig. 1f). The four chambers of the heart and the umbilical cord were also visible on D13.

From D14 onward, the body and head became increasingly distinguishable as the embryo was bent dorsally, the snout lifted from the chest and the medullary tube was visible (Fig. 1g). The body uncoiled and there was clear separation between the head and body at D15, along with cranial ossification. The urinary bladder was also visible on D15 (Fig. 1h). On D16, osseous tissue
Table 1. Embryonic features detectable by ultrasonography during gestational days 8–19

| D8 | D9 | D10 | D12 | D13 | D14 | D15 | D16 | D17 | D18 | D19 |
|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Embryonic sac | + | + | + | + | + | + | + | + | + | + |
| Placenta | + | + | + | + | + | + | + | + | + | + |
| Amniotic fluid | + | + | + | + | + | + | + | + | + | + |
| Umbilical cord | + | + | + | + | + | + | + | + | + | + |
| Embryo | Visible embryo | Visible embryo | Visible embryo | Distinct embryos; recognizable body ends | Snout lifts off chest; embryos bent dorsally; visible body ends | Clear separation between head and body; body uncoils |
| Posture | | | | | | | | | | |
| Tail | | | | | | | | | | |
| Heart | Embryonic heart pulse appears; HR not yet measurable; BFI signal present | Heart visible using B-mode; no measurements when PWD is used | Clear imaging of cardiac function and estimation of HR (using both B-mode and PWD) | Appears | All four heart chambers visible | | | | | |
| Liver/ gallbladder/ kidneys/ urinary bladder | | | | | | | | | | |
| Brain/cranium/ spine | | | | | | | | | | |
| Ribs | | | | | | | | | | |
| Extremities | | | | | | | | | | |

BFI = blood flow imaging; HR = heart rate; PWV = pulse wave Doppler.
started to develop, especially at the body ends, with clear discrimination of the phalanges. The ribs started to develop, and eye sockets were visible. D16 was also the first day the liver became visible (Fig. 1i). Brain ventricles became detectable on D17, along with the thoracic and abdominal aorta. Ossification of the spine and ribs was in progress, whereas the phalanges were clearly ossified (Fig. 1j). On D18, the heart ventricles became evident, while ossification of the spine and ribs continued. The liver and other abdominal organs were also distinguishable (Fig. 1k). Finally, on D19, the jaw bone could be differentiated from the rest of the skull and the kidneys were also discernible (Fig. 1l).

**DISCUSSION**

Because of advances in ultrasound imaging technology, commercially available ultrasound systems now have the spatial and temporal resolution to obtain accurate images of rat and mouse hearts, kidneys and other target tissues (Coatney 2001; Golden et al. 2012; Stypmann 2007). The primary aim of this study was to evaluate the use of real-time ultrasound examination in the accurate detection of rat pregnancy. According to the data obtained, D8 is the earliest day on which pregnancy could be detected, and it was based on the observation of the embryonic sacs. Detection of pregnancy is just as important as detection of non-pregnancy; it is crucial for researchers in terms of time management and planning, reducing the number of mother animals sacrificed and waiting time between experiments. Such information might prove vital to decision making in each case. In addition, ultrasound imaging of the reproductive tract during pregnancy provides valuable information relative to the developmental stage of embryos, embryo viability and progression of fetal development.

Figure 1 is a sequence of representative images of embryonic development. Information on fetal age and development may be gathered from both somatometric measurements and indexing of other detectable features, such as the heart rate on each day of pregnancy (Zambelli and Prati 2006). Ultrasonographic detection of the heart rate at day 12 was also considered a clear indicator of embryonic age. Days 14 and 15 were milestones with respect to anatomic changes in the fetus, as fetal skeletal morphology could easily be distinguished and evaluated. Furthermore, another useful finding was the detection of fetal abdominal organs from day 15 onward.

We created an index as a reference for researchers in their everyday laboratory animal science praxis. The results obtained by ultrasonography (Table 1) were in accordance with anatomic descriptions reported in textbooks (Theiler 1989; Witschi 1972), can be non-invasively obtained by ultrasound examination and may prove to be a valuable tool for future studies.

The limitations of the present study must, however, be recognized. Only one person performed the ultrasound examinations using a single ultrasound scanner. The intrinsic spatial resolution of the imaging system and the absence of reproducibility analysis may limit the generalizability of our results. Further work is necessary to expand the dataset obtained from this study.

Ultrasonography is an examination method with minimal biological or other side effects for the patient, the operator and assisting personnel (Shaw et al. 1999). Furthermore the interventions used in the present study were in compliance with the principle of refinement of the 3 Rs for humane use of animals in research. Furthermore, this effort may be of benefit in future experimentation in reducing the numbers of animals used or sacrificed, specifically in protocols concerning pregnancy or embryo transfer. Finally, it is worth mentioning that use of ultrasonography in embryonic development studies provides the unique opportunity to monitor the same pregnant animal throughout the whole gestational period, in parallel with real-time monitoring of the viability and somatometric characteristics of the fetuses.

**REFERENCES**

Agematsu Y, Ikadai H, Amao H. Early detection of pregnancy of the rat. Jpn J Rats 1983;32:209–212.
Aitman TJ, Critser JK, Cuppen E, Dominiczak A, Fernandez-Suarez XM, Flint J, Gauguer D, Geurts AM, Gould M, Harris PC. Progress and prospects in rat genetics: A community view. Nat Genet 2008;40:516–522.
Beccaglia M, Luvoni GC. Comparison of the accuracy of two ultrasonographic measurements in predicting the parturition date in the bitch. J Small Anim Pract 2006;47:670–673.
Bennet JP, Vickery BH. Rats and mice. In: Hafez ESE, (ed). Reproduction and breeding techniques for laboratory animals. Philadelphia: Lea and Febiger; 1970. p. 299–315.
Bivin WS. The rat. In: Morrow DA, (ed). Current therapy in theriogenology. 2. Diagnosis, treatment, and prevention of reproductive diseases in small and large animals. London: Saunders; 1986. p. 1015–1021.
Brown SD, Zurakowski D, Rodriguez DP, Dunning PS, Hurley RJ, Taylor GA. Ultrasound diagnosis of mouse pregnancy and gestational staging. Comp Med 2006;56:262–271.
Carr DJ, Atikin RP, Milhe JS, David AL. Fetoplacental biometry and umbilical artery Doppler velocimetry in the overnourished
adolescent model of fetal growth restriction. Am J Obstet Gynecol 2012;207:141.e6–141.e15.
Coatney RW. Ultrasound imaging: Principles and applications in rodent research. ILAR J 2001;42:233–247.
Flecknell P. Laboratory animal anaesthesia. London: Academic Press; 2009.
Golden HB, Sunder S, Liu Y, Peng X, Dostal DE. In utero assessment of cardiovascular function in the embryonic mouse heart using high-resolution ultrasound biomicroscopy. Methods Mol Biol 2012;843:245–263.
Kobayashi K, Lenke RP, Greer JJ. Ultrasound measurements of fetal breathing movements in the rat. J Appl Physiol 2001;91:316–320.
Kohn DF, Wixon SK, White WJ, Benson GJ. Anesthesia and analgesia in laboratory animals. London/New York: Academic Press; 1997.
Krinke GJ, (ed). The handbook of experimental animals: The laboratory rat. London: Academic Press; 2000. p. 199–225.
Lenard ZM, Hopper BJ, Lester NV, Richardson JL, Robertson ID. Accuracy of prediction of canine litter size and gestational age with ultrasound. Aust Vet J 2007;85:222–225.
Lindahl IL. Detection of pregnancy in sheep by means of ultrasound. Nature 1966;212:642–643.
Mu J, Slevin JC, Qu D, McCormick S, Lee Adamson S. In vivo quantification of embryonic and placental growth during gestation in mice using micro-ultrasound. 2008. Reprod Biol Endocrinol 2008;6:34.
Nguyen TM, Nakamura H, Wakabayashi A, Kanagawa T, Koyama S, Tsutsui T, Hamasaki T, Kimura T. Estimation of mouse fetal weight by ultrasonography: application from clinic to laboratory. Lab Anim 2012;46:225–230.
Pallares P, Gonzalez-Bulnes A. Use of ultrasound imaging for early diagnosis of pregnancy and determination of litter size in the mouse. Lab Anim 2009;43:91–95.
Quintela LA, Barrio M, Pena AI, Becerra JJ, Cainzos J, Herradon PG, Diaz C. Use of ultrasound in the reproductive management of dairy cattle. Reprod Domest Anim 2012;47(Suppl 3):34–44.
Russell WMS, Burch RL. The principles of humane experimental technique. London: Methuen; 1959.
Shaw A, Pay NM, Preston RC, Bond AD. Proposed standard thermal test object for medical ultrasound. Ultrasound Med Biol 1999;25:121–132.
Sousa FCA, Alves FR, Fortes EAM, Ferraz MS, Machado Júnior AAN, de Menezes DJA, de Carvalho MAM. Pregnancy in Hysticomorpha: Gestational age and embryonic fetal development of agouti (Dasyprocta prymnolopha, Wagler 1831) estimated by ultrasonography. Theriogenology 2012;78:1278–1285.
Srinivasan S, Baldwin HS, Aristizabal O, Kwee L, Labow M, Artman M, Turnbull DH. Noninvasive, in utero imaging of mouse embryonic heart development with 40-MHz. Echocardiogr Circ 1998;98:912–918.
Stypmann J. Doppler ultrasound in mice. Echocardiography 2007;24:97–112.
Theiler K. The house mouse: Atlas of embryonic development. Berlin/New York: Springer-Verlag; 1989.
Wenger S. Anesthesia and analgesia in rabbits and rodents. J Exotic Pet Med 2012;21:7–16.
Witschi E. Characterization of developmental stages: Part II. Rat. In: Biology data book. 2nd ed. Washington, DC: Federation of American Societies of Experimental Biologies; 1972.
Ypsilantis P, Defteros S, Prassopoulos P, Simopoulos C. Ultrasonographic diagnosis of pregnancy in rats. J Am Assoc Lab Anim Sci 2009;48:734–739.
Zambelli D, Prati F. Ultrasonography for pregnancy diagnosis and evaluation in queens. Theriogenology 2006;66:135–144.