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Magnetic Resonance Imaging of Ovarian Activity in Microminipigs Showing Normal Estrous Cycles

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Abstract. *We investigated whether magnetic resonance imaging (MRI) might be applicable to evaluation of the ovarian activity of microminipigs. Firstly, using three mature microminipigs, we confirmed ovarian position and morphology by laparotomy or laparoscopy, and then acquired MRI images in various patterns to determine the most suitable condition for the acquisition. Next, using four microminipigs, we performed daily MRI, starting 10 days after ovulation and ending 10 days after the subsequent ovulation, as the starting day of standing estrus was taken as day 0. While the ovarian structure could not be discriminated on T1-weighted imaging, it was possible to confirm the follicles during estrus as hyperintense regions on T2-weighted imaging. With chronological MRI, 3-5 follicles were visible on T2-weighted imaging during the interval from day −2 to day 1, and their size immediately prior to ovulation was 3-5 mm. However, confirmation of the presence of small follicles and the corpus luteum was difficult.*

Pigs are regarded as useful experimental animals that act as a bridge from rodents to humans when extrapolating findings (1, 2). This is because pigs have many physiological and anatomical similarities to humans and the ethical barriers to the use of pigs in experiments are relatively low as they are food animals.

However, domestic pigs weigh in excess of 200 kg, and therefore have the drawback of being very large; accordingly, they are also expensive to rear and difficult to handle, and test agents must be administered in large doses. Therefore, various minipigs, such as the Göttingen Minipig, the Hanford Miniature Swine, the Panepinto Micropig, the Sinclair Miniature Swine, and the Yucatan Miniature Swine, have been developed to overcome the disadvantages of size (3-7). While minipigs are smaller than regular domestic pigs, adults nevertheless weigh 35 to 100 kg and so are not of a size that can be readily handled (4).

Fuji Micra, Inc. has developed microminipigs, which are the smallest minipigs in the world, weighing around 10 kg at 9 months of age and only 15 to 20 kg in adulthood (3, 8). This small size is of great advantage for use as an experimental animal as handling is relatively easy (9), there are savings in the cost of rearing and experimentation, and less space is needed. Microminipigs thus have tremendous potential as experimental animals. However, there is a lack of basic data in various fields because these pigs have been only recently developed.

Theriogenological expertise and techniques are central to the production of livestock. Extensive theriogenological research has been conducted in pigs to ensure stable production of pork, and advanced techniques have been established. Among these, the use of ultrasonography to understand the condition of the ovaries is a key technique for pig producion (10, 11).

Ultrasonography is an important technology for understanding the status of the female reproductive organs. However, ultrasonography has disadvantages in that imaging accuracy varies with the proficiency of the examiner, in that not everyone can observe the activity of the ovaries. Moreover, the observable field of ultrasonography is narrow, and it is possible that only limited information is obtained (12). In light of the fact that microminipigs are experimental animals, additional tools need to be developed for use in diagnosing and analyzing their reproductive status.

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Magnetic resonance imaging (MRI) uses the phenomenon of nuclear magnetic resonance, and information taken *in vivo* is converted into images. MRI renders images of soft tissue with excellent contrast. In the field of reproduction, MRI has been used not only for diagnosing tumors but also for observing normal ovaries (13, 14).

MRI might avoid the disadvantages of ultrasonography as a tool for use in microminipig theriogenology. This is because MRI can produce images that are not affected by the skill of the examiner and allows observation not only of the ovaries but also of the female reproductive organs overall.

Thus, in the present study, we performed daily MRI, starting 10 days after ovulation and ending 10 days after the subsequent ovulation, in order to assess whether MRI can become a new imaging tool for monitoring ovarian activity in microminipigs.

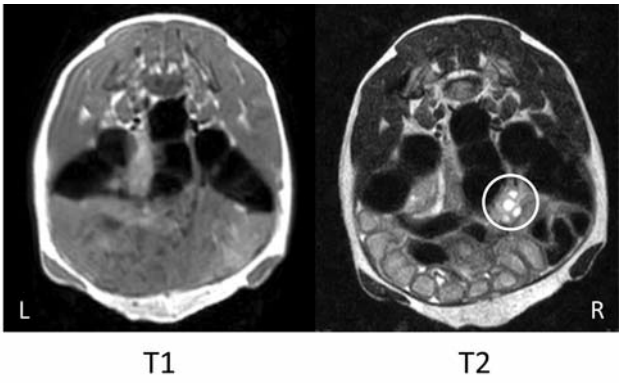

Figure 1. *Comparison of T1-weighted and T2-weighted magnetic resonance imaging acquisitions. While the structure of the ovaries cannot be discriminated in the T1-weighted image, the ovarian follicles in the T2-weighted image were hyperintense. The ovary is circled in the T2-weighted image. L: Left, R: right.*

Materials and Methods

Ethical statement. The protocols for the use of the animals were approved by the Animal Care and Use Committee of Gifu University (#13096). The care and use of the laboratory animals were conducted in compliance with the guidelines of Good Laboratory Practice.

Microminipigs and their husbandry. We purchased seven microminipigs for this experiment from Fuji Micra Inc. (Fujinomiya, Shizuoka, Japan) and the animals were kept in a controlled room at a temperature of 24˚C (21-27˚C) and a humidity of 70-80%. Lighting in the room was set at a 12-h light/12-h dark cycle starting at 06:00. The microminipigs were fed once daily with 200 g of MMP Pellets (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) at the recommended volume for their age. This feed was composed of the following ingredients: total digestible nutrients >74%, crude protein >13%, crude fat >2.0%, crude fiber >8.0%, crude ash <1.0%, calcium (Ca) >1.1%, and phosphorus (P) >0.9%. The animals had free access to water.

Determination of suitable condition for MRI acquisition. Prior to the MRI trial, we confirmed the ovarian position and morphology immediately before ovulation in three microminipigs aged 20, 34, and 36 months. Confirmation of ovarian position and morphology was performed by laparotomy or laparoscopy under anesthesia using isoflurane (IsoFlo, DS Pharma Animal Health Co., Ltd., Osaka, Japan). The condition suitables for MRI acquisition, obtaining accurate depictions of the ovaries, was also investigated using these microminipigs.

Observation of the estrus cycles. We used four microminipigs (pigs A to D) at the age of 4 to 6 months for the MRI trial. Firstly, we confirmed at least two estrous cycles during an interval of 2 to 3 months. During the observation period, the pigs adapted to their new environment and showed a normal growth curve according to the supplier. The pigs showed normal estrous behavior, and the estrous cycle confirmed by back-pressure testing was a median (range) of 21 days (21-23 days), consistent with previous data (15). When the microminipigs reached 8.4 (7.0-8.6) months of age, and weighed 8.7 (8.4-9.0) kg, we started the trial.

MRI trial. We performed daily MRI, starting 10 days after ovulation and ending 10 days after the subsequent ovulation. The starting day of standing estrus was taken as day 0, and the experimental period was taken as the 21-day period from day −10 to day 10.

For MRI acquisition, we treated the microminipigs with an intramuscular administration of 0.015 mg/kg medetomidine (Dorbene vet; Kyoritsu Seiyaku Corporation, Tokyo, Japan), 0.15 mg/kg midazolam (Dormicum injection 10 mg; Astellas, Tokyo, Japan), and 0.12 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma Co., Tokyo, Japan) (9). In addition, the condition of the animals throughout the trial was monitored using biomedical devices and thorough observation by veterinarians.

MRI was performed using a 0.4-Tesla (T) MR system (Hitachi Medical Corporation, Chiba, Japan) with a QD knee coil for signal reception. For ovarian morphological imaging, pelvic multi-slice T1-weighted spin-echo (SE) acquisitions and T2-weighted fast spin-echo (FSE) acquisitions were obtained in the transaxial and sagittal planes (repetition time/echo time, 12,000/104 ms; flip angle, 90˚; field of view, 180 mm; slice thickness, 3 mm).

MRI was performed over the area within a range of 180 mm from the hindmost teat, and a respiratory gating system was used to collect signals during the exhalation phase only to ensure that no motion artifacts were generated.

Estrus behavior and blood collection. Together with MRI, we observed estrous behavior, conducted back-pressure testing and performed genital observation, and determined the changes in plasma 17β-estradiol and progesterone concentrations during the trial. We collected approximately 2 ml of blood samples daily from the jugular vein and placed them into tubes containing EDTA-2Na. The plasma was immediately separated by refrigerated centrifugation (4˚C, 1,000 × *g*, 15 min) and stored at −80˚C until determination of 17β-estradiol and progesterone concentrations.

Hormone assay. We determined plasma 17β-estradiol and progesterone concentrations using enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). All assays were performed according to the manufacturer's protocol. In order

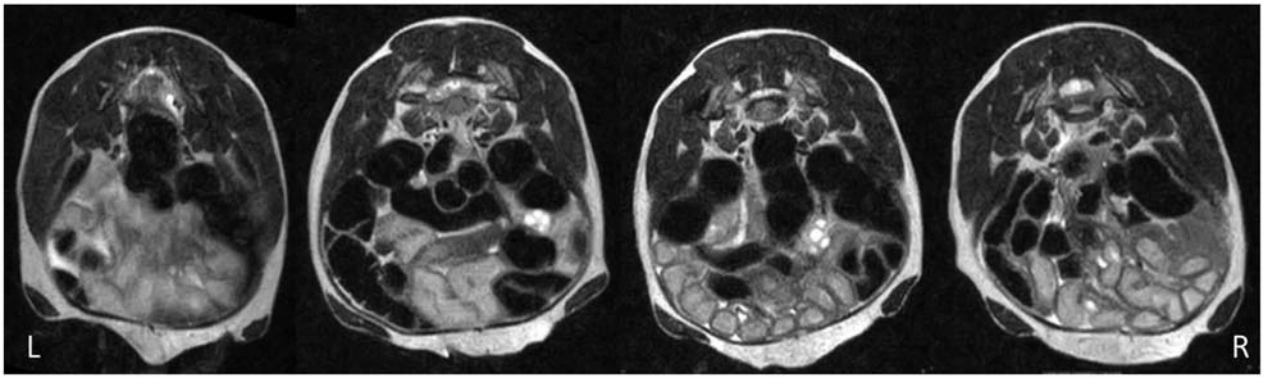

Figure 2. *Magnetic resonance images from appearance until disappearance of the ovarian follicles. The ovarian follicles, shown as hyperintense circular structures, began to appear on day −1 and disappeared on day 1. L: Left, R: right.*

to perform the plasma EIA assays, appropriate aliquots were extracted with 1.0 ml diethyl ether.

Prior to the determinations, the EIAs were validated for pig samples in our laboratory. The minimal detection limit of 17β-estradiol EIA was 7.9 pg/ml, and the intra- and inter-coefficients of variation (CVs) were less than 5.4% and 9.7%, respectively. Moreover, the minimal detection limit of the progesterone EIA was 25.9 pg/ml, and the intra- and inter-CVs were less than 8.9% and 13.9%, respectively.

Analyses. The acquired images were analyzed using OsiriX software v 4.1.2 (Pixmeo Sàrl, Bernex, Switzerland) to measure the diameter and number of ovarian follicles. We also confirmed changes in plasma hormone concentrations during the trial. In this present study, all data are shown as the median (minimum-maximum).

Results

Suitable condition for MRI acquisition. The microminipig ovaries are located within a range of about 5 cm from the hindmost teat, and the sizes of the ovarian follicles immediately prior to ovulation ranged from approximately 3-5 mm. While the structure of the ovaries could not be discriminated on T1-weighted imaging, the ovarian follicles, including the follicular fluid, were confirmed during estrus as hyperintense regions on T2-weighted imaging (Figure 1). In MRI acquisition, less than 5 min was required for acquisition when a single cross-section was imaged, and less than 15 min for two cross-sections.

MRI trial. With chronological MRI, it was not possible to see ovarian follicles from day −10 to day −3, but 3-5 ovarian follicles were visible on T2-weighted imaging during the period from day −2 to day 1 (Figure 2). The sizes of the ovarian follicles visible during this period ranged from 1.1 to 4.7 mm, and the maximum size of each ovarian follicle was 3.3 mm (2.3-4.7 mm). Out of the four pigs that underwent MRI, the ovarian follicles disappeared by day 1 in two pigs (pigs A and D) and by day 2 in the other two pigs (pigs B and C) (Figure 3). However, the *corpus luteum* was not distinguishable throughout the entire experimental period.

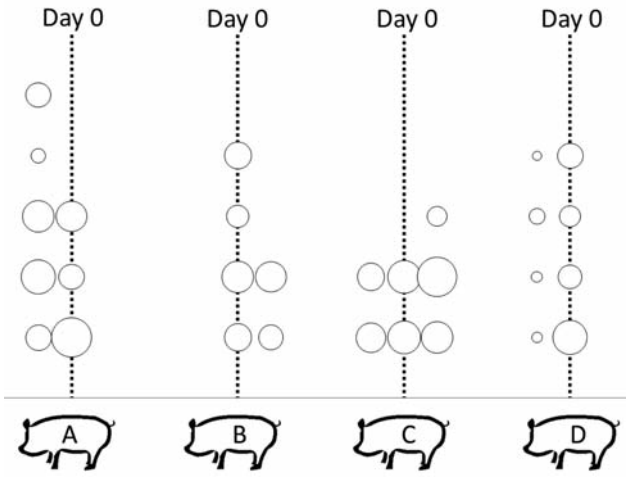

Figure 3. *Changes in follicle sizes and numbers in individual microminipigs A-D. Each circle represents an ovarian follicle. Pigs A, C, and D were found to have 5, 2, and 4 follicles, respectively, from day −1, and pig B was found to have 4 follicles from day 0. Ovarian follicles that had been observed until the previous day disappeared on day 1 in pigs A and D, and on day 0 in pigs B and C.*

Hormone dynamics. The plasma 17β-estradiol concentration remained at ≤15 pg/ml from day −10 to day −2; rose from day −1 to its maximum value of 30.9 pg/ml (24.0-35.4 pg/ml)

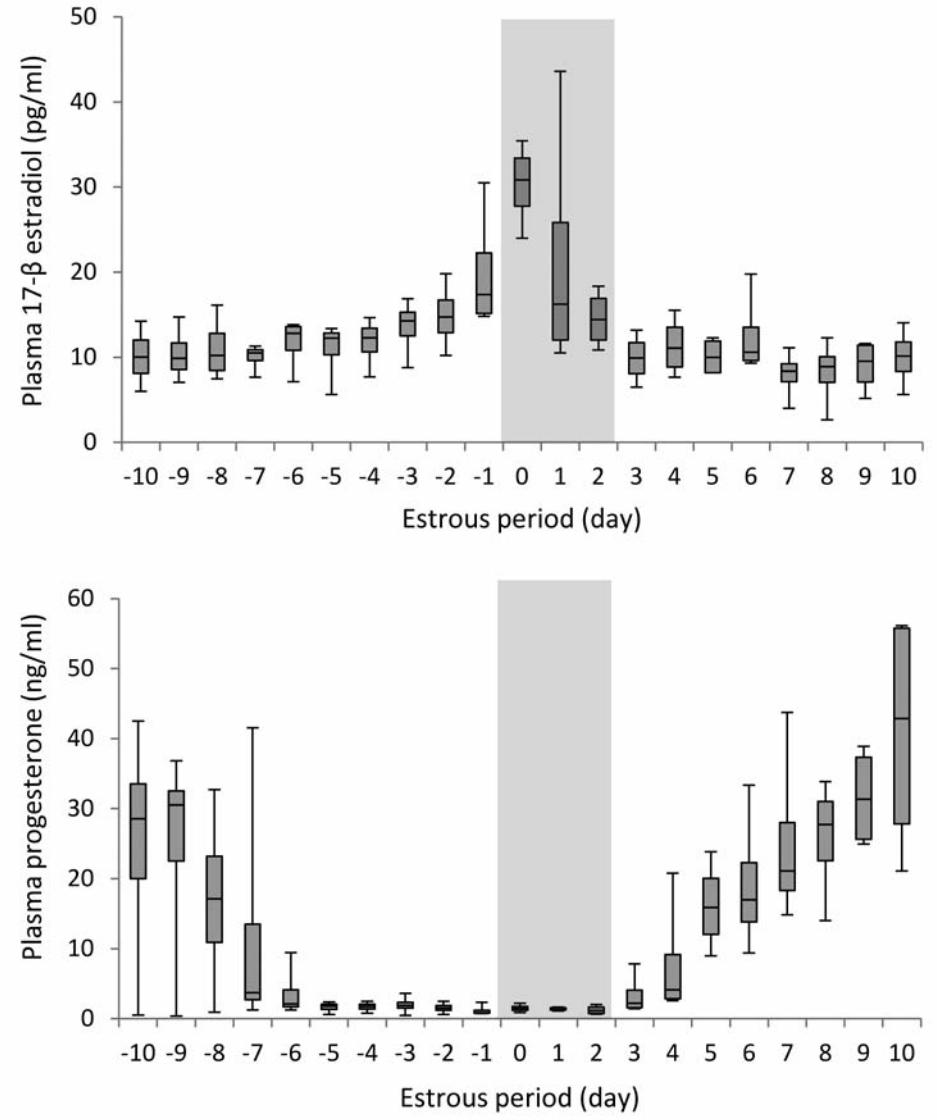

Figure 4. *Change in concentrations of plasma 17-β estradiol (upper) and progesterone (lower) in microminipigs (n=4). The data are shown as box plot with whiskers from min to max. ■: Standing estrus.*

on day 0; then subsequently decreased and from day 2 it stayed at or below 15 pg/ml (Figure 4).

The plasma progesterone concentration gradually decreased from day −10, and from day −6 remained ≤2.5 ng/ml; from day 3, which marked the end of standing estrous behavior, it increased again, reaching its maximum value of 42.9 ng/ml (21.1-56.1 ng/ml) on day 10 (Figure 4).

Discussion

In the present study, we clarified that MRI is a useful tool in addition to ultrasonography for observing ovarian activity of microminipigs. This is because although confirmation of the presence of small ovarian follicles and the *corpus luteum* was difficult by the 0.4-T MRI system used in the present study, the presence of ovarian follicles, once above a certain size, and ovulation can be confirmed with T2-weighted imaging.

MRI is superior to ultrasonography in that it does not depend on the skill of the examiner, and the observation field is wider (12). In the present study, there were no hyperintense circular structural images of 10 mm or less within the abdominal cavity other than the ovarian follicles, and so the follicles were seen distinctly with MRI. Moreover, the position of the ovaries in the abdominal cavity were confirmed with MRI observations. In addition, it was also

possible to estimate the general size of the uterus. From these results, MRI is considered a tool that can compensate for the disadvantages of ultrasonography in the theriogenology of microminipigs.

On the other hand, MRI also has disadvantages in observing the ovaries, one being that the ovaries cannot be observed in real time (12), and another that with the open-type MRI system used in the present study, for which resolution is not particularly high, the detailed ovarian structure cannot be observed.

In fact, MRI does not permit real-time examination. However, since less than 5 min was required for acquisition when a single cross-section was imaged, and less than 15 min with two cross-sections, we did not feel any great disadvantage in using observations obtained by MRI.

It was not possible to carry-out detailed ovarian monitoring in the microminipigs because it was not possible to confirm the presence of small ovarian follicles or the *corpus luteum*, the reason being that the ovaries of microminipigs are smaller than those of domestic pigs, and the open MRI used in the present study lacked the resolution needed to observe the details of the ovaries. However, it has been reported that even mouse ovarian follicles can be observed using 11.7-T MRI (14), so a more detailed understanding of the condition of microminipig ovaries could probably be obtained using a super-conducting MRI with higher resolution.

There may be some concern regarding the need for sedative treatment when observing the ovaries with MRI. However, except for cases when the search is carried-out by an examiner who is very skilled in using an ultrasonography for well-trained microminipigs that are raised as a companion animal, sedative treatment is essential. This is because, as is apparent from the size of microminipigs, it is necessary to carefully search for the ovaries using ultrasonography, even with color Doppler imaging, as the size of the ovaries, which might be mistaken for blood vessels or other structures, is small (3-5 mm) even near the time of ovulation. Thus, it is not only with MRI, but also with ultrasonography that sedation is required when we observe microminipig ovaries. Moreover, the physical risk using the sedation to the animals is not thought to be great, as an antagonist exists (9) and the time of administration of the sedative drug until awakening is relatively short at one hour or less. In addition, the administration of anesthetics is thought to have very little effect on the estrous cycle because the estrous cycle, as well as sex hormone dynamics, is not affected even with consecutive sedative treatments.

The present study yielded a new technique for examining ovarian function using MRI, relating to the reproduction of microminipigs. Despite the limits of equipment performance, there will be huge leaps in the understanding of the reproductive physiological characteristics of microminipigs if a more detailed understanding of ovarian activity can be gained by using high-resolution MRI. Of course, MRI equipment itself is expensive and is not available at all facilities. However, considering that the microminipig is an experimental animal that provides a link between rodents and humans, any increase in the number of techniques that can be used to obtain biological information is important. Therefore, continued development of new techniques and further acquisition of knowledge of microminipigs as an experimental animal are required to promote translational research.

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