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### REFERÊNCIA

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## Evaluation of ovarian structures using computerized microtomography

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### ABSTRACT

Visualization and clear understanding of the ovarian structures are important in determining the stage of oestrus, helping to diagnose several pathologies and supporting advances in reproductive technologies. In this research, computerized microtomography (microCT) was used to explore and characterize the ovarian structure of seven mammalian species. Ovaries of rats, female dog, queens, cows, mares, sows and a female donkey were used. After microCT scanning, the same samples were prepared for histologic evaluation, used here as a validation criterion. It was possible to distinguish regions of the cortex and medulla, visualize the morphology and distribution of blood vessels, clearly observe corpus luteum and antral follicles, and visualize oocytes inside some antral follicles. This is the first report using microCT to explore and compare ovarian structures in several domestic mammals. MicroCT revealed great potential for the evaluation of ovarian structures. This research opens prospects for the use of computerized tomography (CT) as a non-invasive approach to studying ovarian structures in live animals, which may be especially attractive for scientific study of development of ovarian structures and/or ovarian pathologies in small animals' models.

**Key words:** ovarian follicle, blood vessels, histology, tomography, ovary.

### INTRODUCTION

The ovary is a complex and specialized organ, and visualization and clear understanding of ovarian structures are important in determining the stage of oestrus, helping to diagnose several pathologies and supporting advances in reproductive technologies.

Several imaging techniques have been used to perform ovary evaluation and diagnosis. Currently,

the most common tool for *in vivo* evaluation of human and animal ovaries is ultrasound, which allows the identification of ovarian tumours and cysts (Prater et al. 1988, Vlaisavljevic and Došen 2007). Doppler ultrasound boosted the precision of the differentiation of some malign pathologies that are frequently observed in the ovary and that normally present neovascularization regions (Giordano et al. 2009).

In the 1970's computerized tomography (CT) appeared as a new route for imaging diagnosis.

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Computerized microtomography (microCT) is a lower-scale CT, with even greater precision (Guldborg et al. 2003), developed for research studies. MicroCT has been frequently used to evaluate mineralized tissues, such as bones and teeth (Davis and Wong 1996), as well as brain tissue (Langheinrich et al. 2010), blood vessels (Langheinrich et al. 2004, Barreto et al. 2008, Nebuloni et al. 2014) and lungs (Postnov et al. 2005, Ashton et al. 2014). Concerning the ovaries, CT has only been used to visualize tumours, torsions and a few other pathologies in humans (Sawyer et al. 1985, Foshager and Walsh 1994, Hamm 1994, Saksouk and Johnson 2004, Giordano et al. 2009, Shaaban and Rezvani 2009, Moribata et al. 2015), register morphological aspects in monkeys (Jones et al. 2007, 2010) and tumours in rats (Hariri et al. 2010). Although the contribution of CT to ovarian investigation is still insufficient, the characteristics of microCT are interesting for ovary imaging.

MicroCT is a non-invasive technique that can detect less than 1% density differences, allowing micrometric evaluation of structures (Bontrager 2003). Although both ultrasonography and CT permit the visualization of ovarian structures, CT allows a three-dimensional (3D) reconstruction of the organ and the visualization of specific sections of interest in much greater detail. The images generated by CT are comparable in resolution to those of histological sections (Metscher 2009a). However, while high-resolution techniques (i.e. histology and confocal laser scanning microscopy) usually depend on biopsies for tissue evaluation, CT could be used in live individuals (Metscher 2009b). Other features of CT, such as speed, precision and interactivity, reinforce the need to verify the potential of this technique in ovarian evaluation. In these terms, the present study aimed to verify if microCT is suitable to explore and characterize the ovarian structures of seven mammalian species.

## MATERIALS AND METHODS

Whole ovaries from rats (*Rattus norvegicus*) (n=2), female dogs (*Canis lupus familiaris*) (n=2), cows (*Bos taurus*) (n=2) and queen (*Felis catus domesticus*) (n=1) and hemi-ovaries from sows (*Sus domesticus*) (n=3), mares (*Equus caballus*) (n=3) and female donkey (*Equus africanus asinus*) (n=1) were used in this study. All ovaries were collected from adult, healthy and non-pregnant animals. Ovaries from female dogs, queen, mares and female donkey were obtained from animals subjected to elective ovariohysterectomy at a local veterinary clinic/hospital. Ovaries from sows and cows were collected in certified slaughterhouse and the ovaries from rats were collected from an animal euthanized at the University Animal Facility.

All samples were fixed in Carnoy solution (60% ethanol, 30% chloroform and 10% glacial acetic acid), overnight for hemi-ovaries of mares, female donkey, sows and cows, or for 8 hours for ovaries of female dogs, queen and rats, and then immersed in 70% ethanol and kept under refrigeration (4 °C).

## COMPUTERIZED MICROTOMOGRAPHY

Before scanning, all ovaries and hemi-ovaries were immersed in a solution of 70% ethanol and 1% tincture of iodine (10%) in water for 24 hours to obtain contrast. Ovaries were scanned using a Skyscan 1076 MicroCT (Skyscan, Aartselaar, Belgium) at 100 kV, 100 mA, 9µm pixel size, with Al 1mm filter, rotation step of 0.3 ° and 1µSv/h for radiation. Bi-dimensional reconstruction of the images was performed using the NRecon software (V 1.6.9, version 64 bit with GPU acceleration) and three-dimensional reconstruction using CTvox software (V 2.7, version 64 bit) and *DataViewer* (1.5.0 version 32 bit). All software used was developed by Skyscan (Kontich, Belgium). For calculation of standard x-ray attenuation units (Hounsfield units, HU) a phantom object was used. For this purpose, polypropylene tube of 50 mL

volume was filled with deionized water and scanned in the microCT device using the same parameters used for each species scanned. To calculate the mean density value of HU for the water phantom, CT software was used.

#### HISTOLOGY

Histology was used as a cross-checking technique, to validate the findings observed in microCT. After scanning, samples were individually transferred to 70% ethanol solution, dehydrated in ethanol, clarified with xylene, and embedded in paraffin wax (Paraplast, Sigma). From each ovary, 5 sections of 5µm were cut, mounted on microscopic slides and stained with hematoxylin and eosin (HE).

Microscopic slides were evaluated using a light microscope (Leica DM1000, Leica, Wetzlar, Germany); photographs were taken with a digital camera (Leica DFC280, Leica, Wetzlar, Germany) with the aid of Leica Application Suite software (version 2.7.0 R1 Copyright 2003-2007, Leica Microsystems, Switzerland). Images of all microscopic fields were captured at 4x magnification and were merged together, producing a unique histologic image (Adobe Photoshop CS6 software, Adobe Systems Incorporated-Version 13.0x64, USA). A virtual section from the scanned ovary was chosen to match the histological section and compare the structures.

#### RESULTS

In all the seven species used, the images obtained by microCT scanning were remarkably similar to the reconstructed histologic sections, validating every finding. In the ovary of queens (Fig. 1a), the female donkey (Fig. 1b) and mares (Fig. 1c) high calibre blood vessels were concentrated in the medulla, branching into thinner vessels towards the cortex. In sows (Fig. 1d), cows (Fig. 1e) and rats (Fig. 1f) the medium calibre blood vessels were spread throughout the ovarian tissue. In the ovaries

of the female donkey and (Fig. 1b) mares (Fig. 1c) the inverted position of cortical and medullary regions was clearly seen. In these two species, the medulla is the outer region of the ovary.

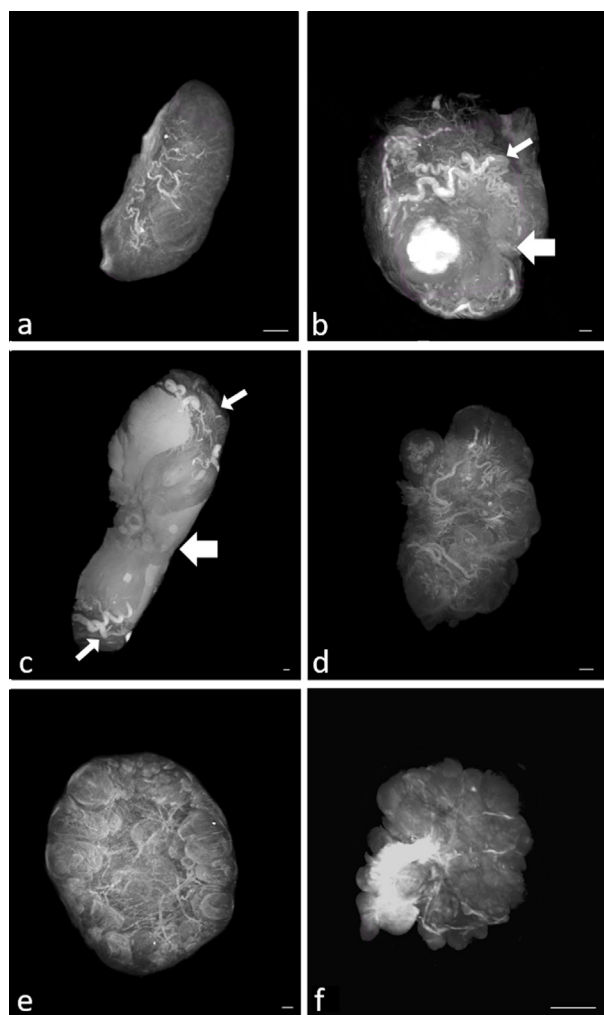
MicroCT allowed precise visualization of antral follicles and corpus luteum, confirmed by histology, in the ovaries from queens (Figs. 2a and 2b), female dogs (Figs. 2c and 2d) and sows (Figs. 2e and 2f), as well as in cows (Fig. 3), rats and mares. Additionally, microCT allowed a 3D reconstruction of the ovary permitting high-resolution visualization of specific virtual sections in any chosen axis (Fig. 3). This tool even enabled the visualization of oocytes inside some antral follicles (Fig. 4) in this study.

The contrast tool from *DataViewer* software allowed the quality of the images to be increased, especially for the visualization of the corpus luteum (Fig. 5). Corpus luteum were more abundant than antral follicles in the ovaries of mares (Fig. 5a), sows (Fig. 5b) and rats (Fig. 5c), probably due to the oestral cycle stage of each animal at the moment of sampling.

Histology showed the presence of preantral follicles in the ovaries of queen, female dogs, rats, sows and cows, even though microCT did not allow the visualization of these structures. Nevertheless, the colour device from *DataViewer* allowed the identification of the region in which histology showed high number of preantral follicles in the queen's ovary (Fig. 6). In addition, this tool improved the cortex and medulla delimitation.

#### DISCUSSION

This is the first report using microCT to explore and compare ovarian structures in several domestic mammals. In all seven species, it was possible to distinguish the regions of cortex and medulla, identify antral follicles and corpus luteum and visualize the distribution of blood vessels. Nowadays, the main method for evaluation of the

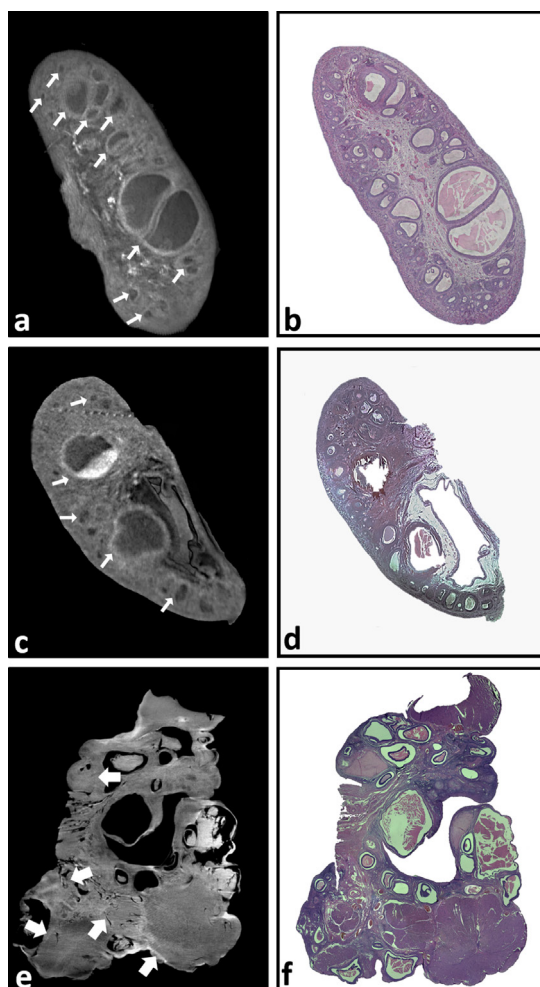


**Figure 1-** MicroCT images of the ovary with emphasis on blood vessels. Blood vessels are concentrated in the medullar region in the ovaries of queen (a), female donkey (b) and mare (c), while spread throughout the whole organ in sow (d), cow (e) and rat (f). Thin arrows: medullar region, thick arrows: cortex region. White areas reveal greater concentration of the contrast substance in b and f. Barr = 2.5 mm.

ovaries is transvaginal ultrasonography. However, transvaginal and/or transrectal ultrasonography is not viable in some smaller animals, such as rodents, small primates and even queens. The alternative use of transabdominal ultrasonography is limited to identifying and measuring antral follicles (Morgan et al. 1987). The precise visualization of the ovary by ultrasound is also difficult due to the overlap of pelvic bones, or the presence

of faeces and gas (Jones et al. 2010). The results showed that microCT could be suitable for ovarian investigation in domestic mammals and may be a promising technique to explore ovarian structures, specially in research works that need monitoring the development of the ovarian structures in live animals during a certain period of time.

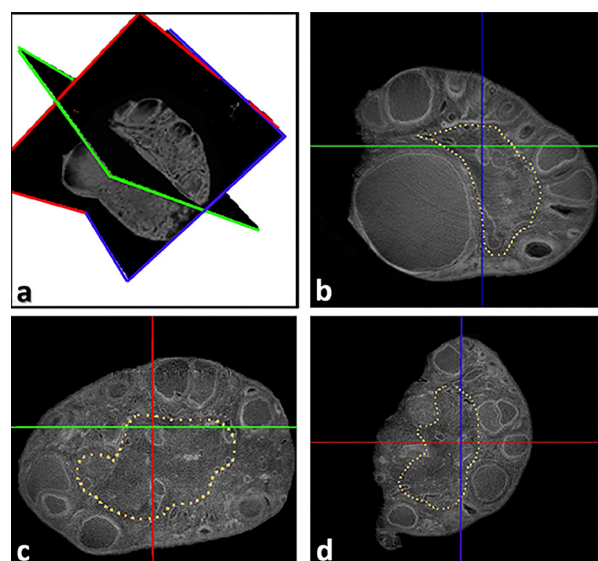
In this study, the distribution of blood vessels was different among species. Medium calibre blood vessels were distributed throughout the organ in the ovaries of cows, sows and rats, while, in queens, mares and female donkeys, the higher calibre blood vessels of ovaries were concentrated in the medulla with thinner vessels in the cortex. Moreover, in the ovaries of the female donkey and mares the localization of blood vessels proved the inverted position of cortical and medullary regions, which has been previously reported (Ginther 1992). MicroCT was shown to be applicable for morphologic evaluation of blood vessels of medium calibre, but the resolution provided by the imaging system is limited to the visualization of capillaries (Nebuloni et al. 2014). In this research, the use of a device from *DataViewer* software enabled better definition and improved images of blood vessels to be achieved. The contrast and colour tools of this software are dynamic and easy to use. Precise observation of blood vessels is important to identify the periovulatory period, since it is marked by intense neovascularization (Ginther et al. 2014). Doppler ultrasound enables the visualization of neovascularization, and it can be used to determine the fertile period in women (Vlaisavlevic and Došen 2007). Likewise, CT has been used for the evaluation of blood vessels in periovulatory stages in humans as well (Rozenblit et al. 2001, Saksouk and Johnson 2004). Meticulous *in vivo* investigation of the microvascular network is also necessary for other situations, such as in the differential diagnosis of malignant tumours, and CT seems appropriate for this type of analysis (Nebuloni et al. 2014). In fact, some researchers have used CT to identify



**Figure 2** - MicroCT and histology images of the ovaries. **a; b:** queen (Barr = 2 mm). **c; d:** female dog (Barr = 2 mm). **e; f:** sow (Barr = 1.4 mm). MicroCT images were chosen to match the histological section. Thin arrows: antral follicles, Thick arrows: corpus luteum. The structures observed in microCT were all confirmed by histology.



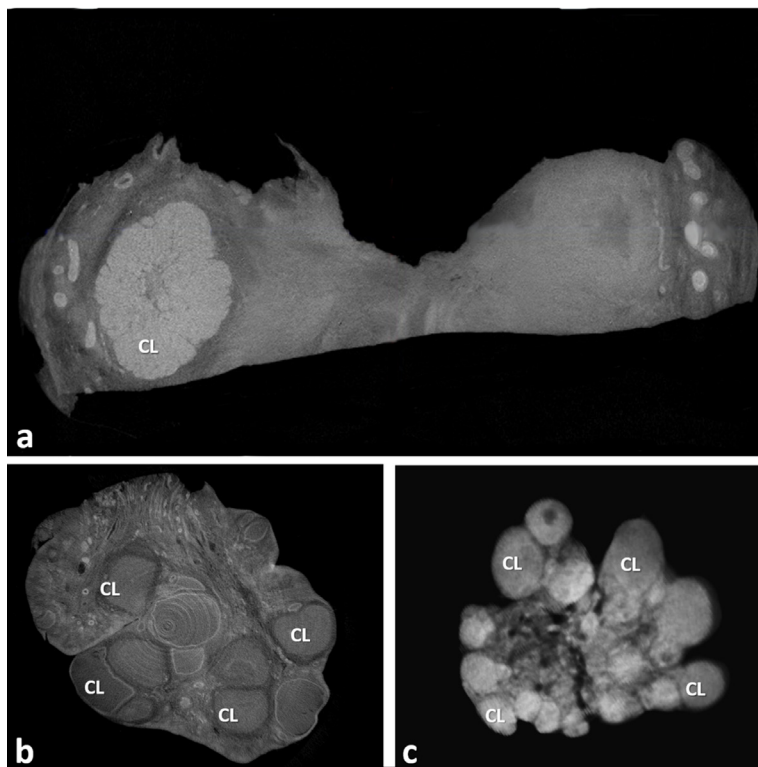
**Figure 4** - MicroCT image showing oocytes inside antral follicles (white arrows) in the ovary of a queen. Barr = 2 mm.



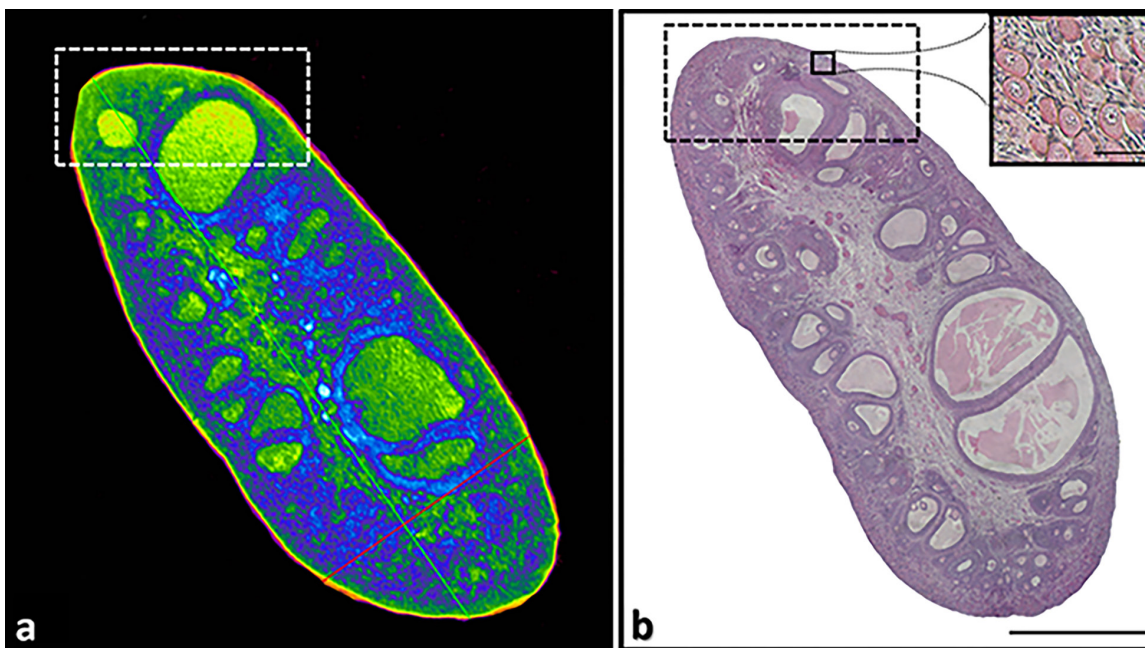
**Figure 3** - Reconstructed microCT images of the cow ovary in 3D showing good definition of cortex, medulla and antral follicles. **a:** 3D image showing virtual sections of the three axis: sagittal (red), axial (blue) and coronal (green). **b:** Virtual section of the sagittal axis. **c:** Virtual section of the axial axis. **d:** Virtual section of the coronal axis. The medullar region is highlighted by a dotted line. Several antral follicles are present in the cortex.

ovarian tumours (Hamm et al. 1999, Tempany et al. 2000, Togashi 2003, Tsili et al. 2008, Perera and Prabhakar 2015) and even differentiate malignancy (Kurtz et al. 1999). MicroCT may be a valuable tool in researches concerning ovarian tumor using small animals models.

In all mammalian species studied, microCT was suitable for the observation of ovarian structures (i.e. corpus luteum and antral follicles). The images registered were confirmed by histology. Only one research team validated the CT findings with histology (Jones et al. 2007, 2010), using primate ovaries. Antral follicles were observed in those studies (Jones et al. 2007, 2010), as well as in ours, suggesting that CT could be applied in monitoring the oestral cycle and the diagnosis of some ovarian pathologies. In humans, the visualization of antral follicles with CT was used to diagnose cysts and polycystic ovaries (Foshager and Walsh 1994, Saksouk and Johnson 2004, Shaaban and Rezvani



**Figure 5** - MicroCT image obtained using the contrast tool (*DataViewer* software) that permits a better view of corpus luteum in the ovary. **a**: mare. **b**: sow. **c**: rat. CL: corpus luteum. Barr = 2.5 mm.



**Figure 6** - MicroCT image using the color tool of the software *DataViewer* (**a**) and reconstructed histological section (**b**) of queen ovary (Barr = 2 mm). The region with preantral follicle is highlighted in green in the microCT image (dotted box), as well as the medullar area and the antrum of antral follicles. Insert: Amplified region with many preantral follicles (Barr = 50  $\mu$ m).

2009). Corpus luteum was accurately seen as well. The identification of corpus luteum and albicans have been reported by other authors using CT (Rieber et al. 2001, Hariri et al. 2010). In contrast, follicles and corpus luteum were poorly identified using magnetic resonance imaging (Nöthling et al. 2006). Beyond the most commonly evaluated structures, in our study, oocytes could be observed within antral follicles. Thus, CT may complement or sometimes replace the ultrasonography diagnosis.

Although preantral follicles could not be adequately seen in the present study, probably because of their small size and the absence of liquid in these microstructures, the cortex area presenting a significant concentration of preantral follicles was observed in the queen's ovary with the use of *DataViewer* software. The use of the software highlighted preantral follicle regions, possibly due to its hypodense characteristic, as in the medullary region. Further studies are necessary to better investigate these findings, which might open interesting prospects for future applications of microCT.

Although we used surgically removed ovaries in this work, tomography can be used *in vivo* as well, as already carried out in humans (Foshager and Walsh 1994, Rozenblit et al. 2001). In this study, the ovaries were immersed in iodine solution, which provides good contrast to visualize different ovarian structures using microtomography. Contrasting substances are also used *in vivo* by intravenous injection, resulting in a good visualization of blood vessels and other structures. Contrast agents other than iodine may be used as well, such as iohexol and barium sulphate (Gregor et al. 2012). Alternatively, other contrast methods based in nanoparticles are being developed (Samei et al. 2009, Ashton et al. 2014).

The results of this research suggest the further use of CT as a non-invasive approach to study ovarian structures in small animals. This may be especially attractive for researches that

need to follow the development of the ovarian structures and/or tumors in live animals. In those cases, invasive techniques usually require animals' euthanasia, not allowing the monitoring of the same animal throughout time, and the non-invasive techniques available, such as ultrasound, do not provide high spatial resolution. Of course, *in vivo* scanning brings up many additional challenges compared to *ex vivo* scanning, including exposure to radiation, which needs some consideration.

### CONCLUSIONS

MicroCT presented great resolution to analyse ovarian structures, allowing perfect visualization of corpus luteum and antral follicles, even with the observation of oocytes. All tomography findings were confirmed by histology. Thus, this study draws attention to microCT as a promising technique for scientific study of development of ovarian structures and/or ovarian pathologies in small animals' models. We emphasize that further *in vivo* experiments will be necessary to check the feasibility of visualization of ovarian structures in live animals using microCT.

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