http://dx.doi.org/10.1590/1678-4162-12626

Arq. Bras. Med. Vet. Zootec., v.74, n.3, p.419-427, 2022

High-definition ultrasonography for evaluation of embryonic and fetal development in bitch

[*Ultrassonografia de alta-definição para avaliação do desenvolvimento embrionário e fetal em cadelas*]

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ABSTRACT

In women, high-definition ultrasonography provides high-quality images of normal and diseased tissues throughout pregnancy, however there are no veterinary studies describing the use of this technology in pregnant dogs. The objective was to evaluate pregnant female dogs daily, using high-definition ultrasonography (HD) in brachycephalic, documenting daily findings of maternal-fetal structures. Twelve healthy brachycephalic females were evaluated daily by HD ultrasound until delivery. It was possible to identify and categorize all maternal, embryonic, and fetal structures and their development, including some fetal tissues not yet described in the literature. The HD exam is an excellent method to monitor the pregnancy of female dogs and identify structures early, providing safe and complete information regarding fetal development.

Keywords: canine, insemination, organogenesis, pregnancy, ultrasonography

RESUMO

Em mulheres, a ultrassonografia de alta definição fornece imagens de alta qualidade de tecidos normais e alterados durante a gravidez, entretanto não existem estudos veterinários que descrevam o uso dessa tecnologia em cadelas prenhas. O objetivo deste estudo foi avaliar diariamente cadelas braquicefálicas gestantes, por meio de ultrassonografia de alta definição (HD), documentando achados diários das estruturas materno-fetais. Doze cadelas braquicefálicas saudáveis foram avaliadas diariamente por ultrassonografia HD, desde a inseminação até o parto. Foi possível identificar e categorizar todas as estruturas maternas, embrionárias e fetais e seu desenvolvimento, incluindo alguns tecidos fetais ainda não descritos na literatura. O exame de HD é um excelente método para monitorar a gestação de cadelas e identificar precocemente as estruturas, fornecendo informações seguras e completas sobre o desenvolvimento fetal.

Palavras-chave: canino, inseminação, organogênese, prenhez, ultrassonografia

INTRODUCTION

Ultrasonographic examination has demonstrated its utility in small animal obstetrics, specifically in the evaluation of pregnancy, performing diagnosis, monitor female dogs, determine fetal viability, monitoring the development of organs and identify gestational abnormalities (Lamm and Makloski, 2012). Technological development positively impacting the quality of the ultrasound image, it is possible to observe advances in the study of pregnant bitches by obtaining a detailed assessment of fetal morphology, monitoring characteristics of the maternal-fetal tissues development (Feliciano *et al.*, 2015) and even accurately predict gestational age using fetal biometrics, for example measuring kidney length between 48 and 52 days of gestation (Gil *et al.*, 2018).

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Submitted: December 29, 2021. Accepted: March 17, 2022.

In women, HD ultrasound (HDUS) allows a more detailed description of the embryonic and fetal components in all trimesters of pregnancy, providing high quality images and more reliable characteristics of normality and abnormality during the gestational process (Araujo *et al.*, 2015). However, no studies were found in the veterinary literature describing the use of HDUS exams to assess the gestational period of domestic animals.

The HD technique is based on the use of transducers with a greater amount of small piezoelectric elements (reaching up to 4096) separated by a reduced space (pitch), different from conventional transducers (which usually have around 128 elements), resulting in sound waves smaller in width and, consequently, greater axial spatial resolution (ability to distinguish two points in the direction of the beam), lateral (ability to distinguish two points in the perpendicular plane to the beam) and elevation (ability to distinguish two points in the third spatial axis, perpendicular to the beam), in addition to better contrast and greater detail of the structures, provided by the increase in the number of pixels by area generated in the monitor, resulting in image quality far superior to conventional ones (Lieu, 2010).

Considering the applicability of this ultrasound modality in medicine and the lack of studies in domestic animals, the aim of this study was to evaluate the findings of HDUS of maternal and embryonic/fetal structures and functions in pregnant brachycephalic bitches after artificial insemination.

MATERIAL AND METHODS

This study was approved by the Animal Ethics Committee of the School of Agriculture and Veterinary Sciences, UNESP/Jaboticabal (3652/17). It included 12 females (1 to 4 years of age, weighing 7 to 13kg) of the following breeds: French Bulldog (n = 8); Pug (n = 2); and Shih-tzu (n = 2). All dogs were healthy based on clinical examination and ultrasonography of the reproductive tract. The dogs were from regional kennels that have a partnership with the department.

The estrous cycle was confirmed by signs of heat (i.e., the period in which females accept copulation) associated with serial vaginal cytology every day after the start of the proestrus, until most of the visualized cells (>80%) had superficial characteristics (Socha *et al.*, 2012). After confirmation, all animals underwent intrauterine artificial insemination (AI) with fresh semen every 24 h for three consecutive days. The selected males (same breed as the receiving female dogs) were suitable for reproduction after clinical and urological examination.

The ultrasonographic examinations were performed using an HDUS instrument (ACUSON S2000, Siemens, Munich, Germany) equipped with a high-resolution digital transducer (18L6HD) at a frequency of 17.0MHz. Females underwent ultrasound evaluation 15 days before being inseminated. The objective was to condition the patients previously so that the exams were carried out with a minimum of stress during pregnancy. The first day from artificial insemination was considered as 0 day of gestation and the pregnant bitches were followed by ultrasonographic examination from the 8th day (Gil *et al.*, 2015, 2018) by an experienced veterinarian, subsequently the gestational diagnosis was made at 15 days by gestational vesicle detection.

The ultrasound evaluation followed the protocols described by Gil *et al.* (2014), with the objective of evaluating the entire abdominal region and the largest number of fetuses. Maternal-fetal structures were evaluated in the longitudinal and dorsal planes. Gain, focus and depth of penetration were adjusted for each fetus during the examination.

The HDUS findings were divided into two phases (embryonic until the 35th day of pregnancy and fetal from the 35th day until delivery) (Pretzer, 2008). In both phases, attempts were made to visualize the gestational sac; detect and characterize embryonic and fetal structures; study basic functions and assess the development of the gestational tissues.

RESULTS

The 12 female dogs produced 70 puppies (5.83±1.33 puppies/litter). The average litter size in the French bulldog was 6.12±1.24, in Pug 4.50±2.12 and in Shih-tzu 6.00±0 and all puppies were born healthy without abnormalities. The mean gestational time was 61±3 days. Four bitches had normal birth and eight needed c-section.

Gestational findings of HDUS are shown in Table 1 and 2 (Fig. 1, 2 and 3). Pregnancy was confirmed between days 10 and 13 according to the detection of gestational sacs and structures. Embryos were initially visualized from the 17th to the 18th days and characterized as small pediculated echogenic structures located eccentrically to the gestational sac, initially identified as hyperechogenic structures in contact with the inner wall of the gestational sac, with no movement or heart beats. Between days 18 and 20 was possible to observe the heartbeat with a mean heart rate of 129±7 beats/min, that gradually increasing until stabilizing at 224±7 beats/min four days after initial detection.

On days 21 and 22, the yolk membrane was visualized as two parallel or circular echogenic lines separated by anechoic content. Allantoid membrane appeared as a hypoechoic membrane surrounding the embryo and yolk sac, and the umbilical cord was detected as a hyperechoic line in the central region of the embryo. At this stage, the gestational sacs had two hyperechoic layers correlated with portions of the placenta. This saccular structure changed in shape from spherical to oblong after gestational day 25.

Neural plate and primitive line were identified on day 21. Notochord appeared as a tubular structure with hyperechoic wall and anechoic content, corresponding to the primitive neural tube. Head and neck were identified on day 27, when the head was determined to be a hypoechoic focus followed by the choroid plexus, bilobed and hyperechoic, still in exordial stage, surrounded by anechoic brain ventricles. Choroid plexus gradually increased and, on day 40 it had the shape of a butterfly.

Pelvic and thoracic limbs were identified on the 25th to 26th day. Hyperechoic areas without acoustic shadow, compatible with the ossification center of some facial bones, and diaphysis of long bones were visualized two days after the identification of the limb buttons. Axial skeleton was visualized as hyperechoic structures with acoustic shadows, and the spine was subdivided into regions and structures that were visible on day 32. Nasal septum and the palate were apparent on day 35 and the limb joints were identified on day 36, along with the ribs and the cervical spine. All fetuses showed movement on day 29, suction reflexes were observed on day 31.

Stomach was identified as an anechoic focal area with different degrees of distension on day 27 and, on day 43, the stomach lumen was filled with anechoic content with hyperechoic sediment, when it was possible to identify parietal and mucous stratification. However, on the 47th day, the segments were better defined, and the luminal content was totally echogenic. Esophagus was identified on the 35th day as an anechoic tubular structure, following the entire cervicothoracic extension until its insertion in the stomach.

Intestinal structures were identified on day 38. On day 43, some intestinal segments exhibited better definition and stratification of layers. On day 47, the intestinal segments were well defined and exhibited hyperechoic intraluminal content, mucosal surface, interface, and focal peristalsis. On the 53rd day, they had peristalsis and a well-defined and stratified intestinal wall.

A hyperechoic liver structure was detected on day 27. On day 30, it was isoechoic in relation to the lung. On day 34, the liver was hypoechoic in relation to the lung parenchyma; this difference increased progressively during development. Gallbladder exhibited anechoic content and a thin hyperechoic wall different from anechoic liver vessels on day 36 and hepatic lobes appeared on day 43. Lung was identified on day 29 and became progressively hyperechoic. On the 35th day, the trachea was identified as an anechoic tubular structure with a hyperechoic wall extending to the bifurcation between the bronchi. Spleen was observed to be isoechoic to the liver on day 35 and, on day 43, it was possible to differentiate the echogenicity between them, which become hyperechogenic in relation to the liver.

Table 1. Embryonic findings observed on high-definition examination of brachycephalic female dogs, gestational day (GD) on which they were identified

Structure	GD	CI-95%
Gestational sac	10	10–13
Embryo attached to the gestational sac wall	17	17–18
Heart beats	18	18–20
Umbilical cord	21	21–22
Neural plate	21	21–23
Spinal canal (primitive line)	21	21–23
Yolk sac	22	21–22
Allantois	22	21–22
Kidney structure	24	24–27
Heart	24	24–26
Initial limb development	25	25–26
Placentation	25	25–26
Liver	27	27–29
Definition of head and raphe nuclei	27	27–29
Choroid plexus	27	27–29
Ossification center	27	27–28
Eye orbits	27	27–29
Stomach full of anechoic content	27	27–28
Lung	29	29–31
Differentiation between thorax and abdomen	30	30–32
Embryonic movement	29	27–29
Crystalline lens	30	30–32
Femur	30	30–32
Heart (septa and chambers)	30	30–34
Suction reflex	31	30–32
Bladder	31	31–32
Fetal skeleton	32	32–33
Spine	32	31–33
Tail	33	33–34
Digits	33	33–34
Echogenic difference between lung and liver	34	34–36
Aorta and caudal vena cava	34	34–36

Table 2. Fetal findings observed on high-definition examination of brachycephalic female dogs on the gestational day (GD) they were identified

Structure	GD	CI-95%
Esophagus	35	35–37
Trachea	35	35–37
Carina	35	35–37
Spleen	35	35–37
Nasal septum	35	35–36
Palate	35	35–37
Renal pelvis	36	36–38
Gallbladder	36	35–37
Liver vessels	36	35–37
Joints	36	35–36
Scapula	36	36–37
Ribs	36	36–38
Heart (atrial/ventricular chambers)	37	37–40
Corticomedullary differentiation and kidney diverticulum	37	37–39
Humerus	38	38–39
Tibia	38	38–39
Radius	38	38–40
Amorphous intestinal structure	38	38–41
Adrenals	39	39–43
Fetal sexing	39	39–48
Tongue	39	39–41
Alveolar processes	40	40–42
Thymus	40	40–42
Pancreas	41	41–44
Intestinal stratification	43	43–46
Tympanic structure	43	43–46
Stomach stratification	43	43–45
Differentiation of hepatic lobes	43	40–43
Differentiation between liver and spleen	43	43–46
Focal intestinal peristalsis	47	47–50
Stomach with echogenic content	47	47–50
Generalized bowel motility	53	51-53

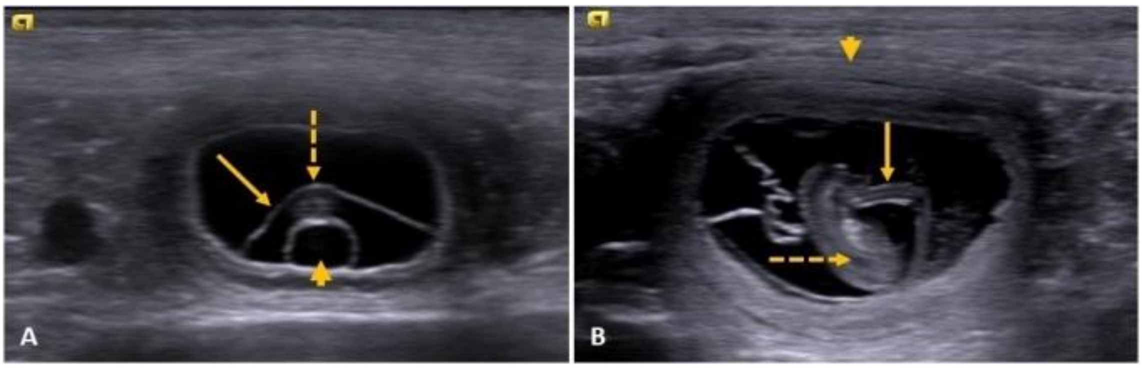

Figure 1**.** High-definition ultrasonography of canine embryos. (A) Embryo in cross section (interrupted arrow), presence of allantoic membrane (continuous arrow) and yolk sac (arrowhead); (B) Embryo in longitudinal section, presence of neural tube (interrupted arrow), umbilical cord (continuous arrow) and placentation (arrowhead).

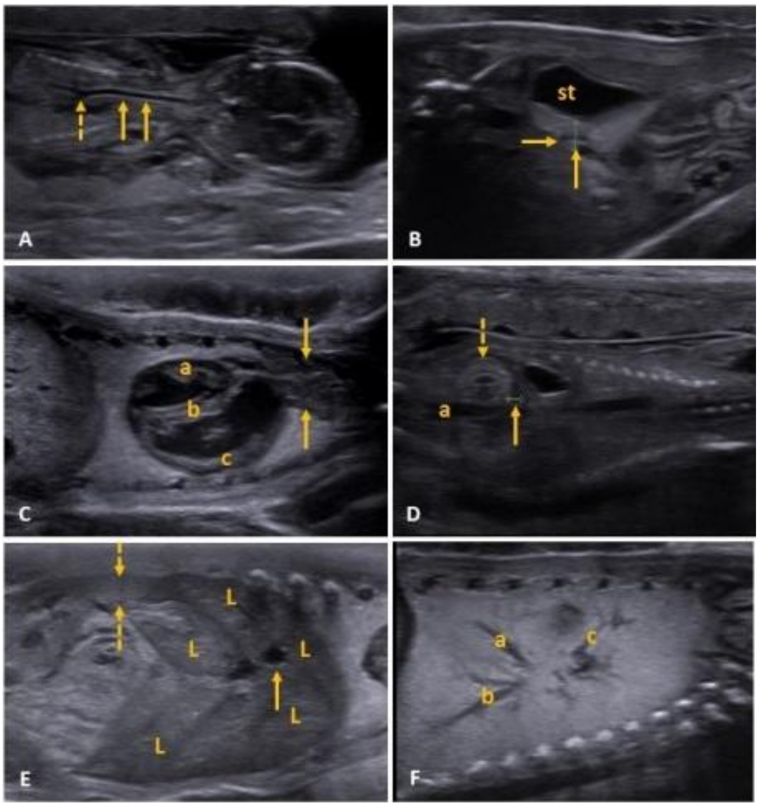

Figure 2. High-definition ultrasonography of canine fetuses: A) trachea (continuous arrow) bifurcating into the carina (interrupted arrow) and hyperechoic lung on day 36; B) pancreas (arrows) and stomachal (st) on gestational day 41; C) cardiac chambers with (a) right ventricle (a), interventricular septum (b), left ventricle (c), hyperechoic lung, and hypoechoic thymus (arrows) on gestational day 47; D) adrenal (continuous arrow), kidney (interrupted arrow) and fetal aorta (a) on gestational day 41; E) liver with formed hepatic lobes (L), gallbladder (continuous arrow) and spleen (interrupted arrow) on day 47; F) hyperechoic lung and bronchial branching to the right caudal (a), left caudal (b) and right cranial (c) pulmonary lobe on day 47.

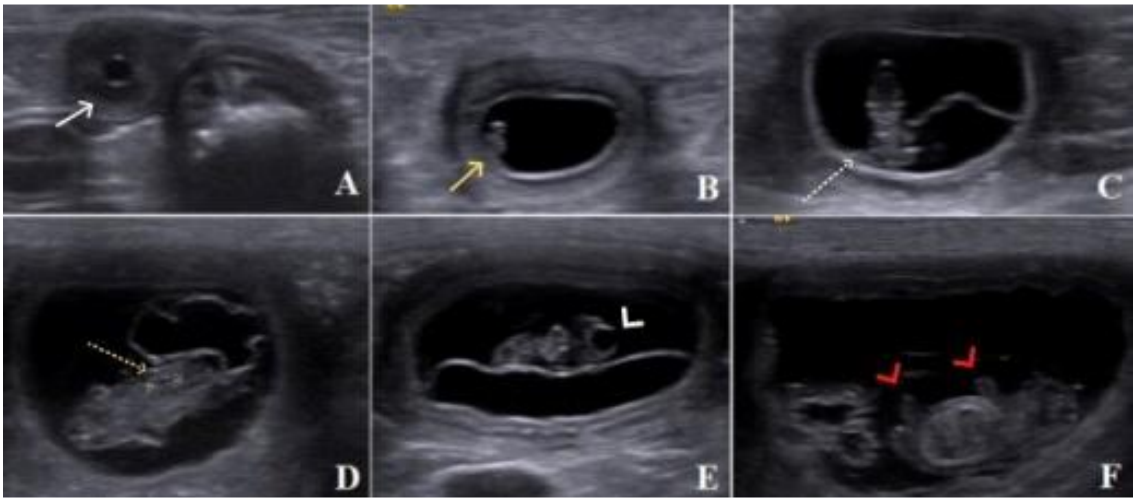

Figure 3. High-definition ultrasonography of canine fetuses: A) image of the gestational vesicle (white arrow); B) small echogenic and pediculated area adhered to the wall – embryo (yellow arrow); C) presence of an embryo (white dotted arrow); D) detection of primitive renal structure – mesonephro (yellow dotted arrow); E) detection of anechoic structure in the cranial portion - neural tube (white arrowhead); F) visualization of the members' buttons (red arrow heads).

Kidneys were visualized on day 24 without differentiating the cortex and medulla. On day 36, exhibited a thickened hyperechoic cortex and an enlarged pelvis with anechoic content; on day 37, the cortex and medulla became differentiated, the diverticulum was visualized, and the pelvis became less dilated. On day 50, the fetuses had kidneys like those of an adult. Early heart development was detected on the 24th day, on the 30th it exhibits hypoechoic echogenicity with linear septations representing the walls of the chambers and valves. Complete cardiovascular system was apparent on day 42.

Fetal sexing was possible after day 39, when a sagittal section detected a hyperechoic medial line in the perineal region of male (n= 36) and a genital protuberance characterized by piriform hyperechoic lines and a central line in the ventral region of a female tail, which represented the labia majora (n= 32).

DISCUSSION

It documents important information on canine organogenesis and gestational development, as well as detailed detection of maternal and fetal structures. The present investigation highlights the need to reevaluate gestational development not only in dogs, but also in many animal species, and to update the parameters of embryonic and fetal development previously published in the veterinary literature.

In the fetal phase, HDUS identified new structures that, as far as we know, are possibly due to the characteristics of ultrasound images, which have improved in line with technological advances. The image is presented in digital form, in which the number of pixels per square inch is high (1080) compared to traditional methods (approximately 255), correlating positively in contrast and observed quality (Richardson *et al.*, 2001).

Confirmation of pregnancy occurred between the tenth and thirteenth day after insemination, when it was possible to view the gestational sac properly. Other studies used the day of insemination as day zero (Feliciano *et al.*, 2015; Simões *et al.*, 2018) and, even without the hormonal dosage, obtained early gestational diagnoses. The authors agree with the importance of monitoring LH and progesterone to perform insemination and correlate with the results obtained, but hormonal evaluation is not always available in the routine, therefore, rapid confirmation of pregnancy in this study brings beneficial responses to breeders generating the possibility of gestational planning in the absence of hormones measurement.

HDUS anticipated the identification of the large cardiac vessels in the four cardiac chambers when compared to the study by Mattoon and Nyland (2015). The thymus was detected in the cranial mediastinal space in the middle third of gestation and changes in echogenicity were monitored. The evaluation of the fetal thymus is poorly described in all species, but studies in humans suggest the introduction of evaluation of this organ in prenatal care, as there is a direct correlation with growth and body weight (Diemert and Tutschek, 2018).

HDUS provided information about the neural plaque, primitive neural tube, development of the yolk sac and allantois, which until now have not been reported. These coincided with the period of fetal organogenesis, when the embryo showed such structures on the 21st and 22nd days of gestation (Pieri *et al.*, 2015). These are considered early detection data when compared to results obtained using the B method (Feliciano *et al.*, 2007).

At the 5th week of gestation, a small amount of excess brain is identified in canine fetuses using mode B, gradually increasing over the following weeks (Feliciano *et al.*, 2013). With HDUS, visualization was anticipated to the moment when the cerebral vesicles (forebrain, midbrain, and hindbrain) are already formed (Pieri *et al.*, 2015). In addition, the technique resulted in better delineation of tissues and formation, with an ultrasonographic description of normality obtained through an image of brain mass in the shape of a “butterfly”, similar to that described in normal human fetuses (Campo and Hwang, 2018). These findings are especially useful in diagnosing brain abnormalities in humans (Harigovind *et al.*, 2019) and may be useful in evaluating canine fetuses.

The femur and humerus of dogs can be identified on conventional mode B ultrasound in the final third of pregnancy (Teixeira *et al.*, 2009). However, it was possible to identify and differentiate these bones earlier in this study, probably because of the image quality provided by HDUS. The authors identified other bones of the appendicular skeleton that had not been described in the ultrasound assessment so far, such as the scapula, radius, and tibia. The early identification of these structures presents an important finding, because just as in humans, they can be correlated with fetal growth (Arduini and Giacomello, 2009).

In humans, the nasal septum of the fetus and its size are associated with Down syndrome (Mazzoni *et al.*, 2006) and visualization of the palate is essential to identify the fetal cleft palate in the uterus (Vaccari-Mazzetti *et al.*, 2009). This is the first work that evaluates and clearly describes such structures in canine fetuses. The identification of these regions can contribute, as well as in medicine, in the screening of future pathologies. As there were no changes in the fetuses evaluated, it was not possible to determine a correlation, but the findings open the way for further studies.

On the 27^{th} day, a stomach full of anechoic content was seen, as reported by Beccaglia *et al.* (2016); however, this is the first study that mentions the development of the stomach and stratification of the wall in canine fetuses. In medicine, gastric changes seen in endoscopy and associated with histological results, such as metaplasia, are predictive factors in the development of gastric neoplasms (Shichijo and Hirata, 2018).

The pancreas and esophagus were evidenced and, as far as we know, these structures have never been evaluated by ultrasound in this specie. Bricout-Neveu *et al.* (2017) performed a histological evaluation of the canine pancreas ex vivo from the 30 days after conception and suggested that the expression of insulin starts in the middle of pregnancy. Thus, even though there are no anatomical and histological experiments simultaneously with our study, it can be said that the evaluation of this structure was reliable, since at 41 days of gestation, as seen, the pancreas already has a considerable morphology to be seen on ultrasound.

The identification of intestinal loops occurred on days like those reported by Gil *et al.* (2014). This finding may not be used as a predictor of gestational age, as intestinal motility is described after day 57, unlike the present study, which identified motility before the one described, suggesting that peristalsis is not an indicator of complete fetal organogenesis (Gil *et al.*, 2014).

The lung and liver are relatively isoechoic when first seen and can be clearly identified after day 35 (Aissi and Slimani, 2008). In this study, we obtained the same result earlier, with the structures being visualized at 30 days of gestation. These findings coincide with organogenesis, because on the 23^{rd} and 25^{th} the embryo presents hepatic structure and primitive lungs, respectively (Pieri *et al.*, 2015). Thus, the authors indicate that this assessment should be instituted in the routine of pregnant bitches to diagnose possible congenital anomalies early.

No ultrasound descriptions were found in the veterinary literature regarding the splenic fetal evaluation in dogs, however, the use of HDUS allowed to detect the structure satisfactorily. In human fetuses, changes in size, echogenicity and malformations are correlated with hematological, infectious, and developmental syndromes (Diemert and Tutschek, 2018). Therefore, the description of this structure can provide valuable information for veterinarians regarding fetal anomalies as well as other organs scored here.

HDUS identified a hyperechoic renal structure on day 24, which corresponds to the development of primitive renal tissue or mesonephros (Beccaglia *et al.*, 2016). The renal development of canine fetuses using high-resolution ultrasound is described on the 39^{th} day without definition of the cortex and medulla (Araujo *et al.*, 2015). This study suggests that HDUS has a superior image compared to high-resolution exams, even using different frequencies, as it was possible to identify the cortex and spinal cord on the 37^{th} day of pregnancy. Finally, the authors were successful in fetal sexing on the 6^{th} week of gestation, two weeks earlier than reported in the literature (Gil *et al.*, 2018).

CONCLUSIONS

HDUS made it possible to identify gestational structures in brachycephalic dogs early and with quality, to study the development of embryonic and fetal organ systems and to provide information not yet described in the veterinary literature. Thus, HDUS appears to be a promising tool for embryological sciences and routine veterinary clinical services.

ACKNOWLEDGMENTS

The authors would like to thank CNPq for the scholarship and productivity scholarship and FAPESP n. 2017/14957-6 for the financial support to the research group, and Jair Matos and Siemens Healthineers for the technical assistance.

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