Placental ARFI elastography and biometry evaluation in bitches

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ABSTRACT

Placental rigidity and biometry of twelve pregnant bitches were evaluated using B-mode and Acoustic Radiation Force Impulse (ARFI) ultrasonography, performed once daily, from day 15 of gestation until parturition. Specific software (Virtual Touch Tissue Quantification® VTTQ and Virtual Touch Tissue Imaging Quantification® VTTIQ) were used. Values for results for variables were correlated and regression models related to gestational day were used to make evaluations. Maternal-fetal placental thickness increased to day 63 ($P < 0.0001$; $R^2 = 0.91$); maternal placental thickness increased until day 40 ($P = 0.0340$; $R^2 = 0.54$); and fetal placental thickness increased to day 50 ($P < 0.0001$; $R^2 = 0.83$) of gestation. Shear wave velocity (SWV) of the dorsal ($P < 0.0010$) was greater than lateral, which in turn was greater ($P = 0.020$) than the ventral area. The SWV of the dorsal area as determined using VTTQ, decreased from day 21 to 35 and increased to day 56 of gestation ($P = 0.0291$; $R^2 = 0.4021$); lateral SWV decreased from day 24 to 45 and increased until the time of parturition ($P < 0.001$; $R^2 = 0.6055$). The SWV of the dorsal area, as determined using VTTIQ, decreased from day 21 to 43 and then increased to day 60 of gestation ($P = 0.0016$; $R^2 = 0.5075$); and ventral area SWV increased from day 21 to 23 and decreased until the time of parturition ($P < 0.001$; $R^2 = 0.8055$). Placental alterations reflect structural and biochemical gestational adaptations and can become useful techniques for obstetrics.

Keywords: Biometry; Canine; Elastography; Placental; Ultrasonography

1. Introduction

The placenta is the first fetal organ to develop and has both primordial and critical functions because it establishes an interface for the exchange of nutrients and gases in maternal-fetal circulation, as well as modifies local immunological mediators, the cardiovascular system, and metabolic functions (Cross, 2006). In carnivores, the placenta is classified as zonary due to the characteristics of the chorionic villous (Júnior, 2015) and endotheliochorial tissues at the connection between the villi of the fetal chorion and uterine endothelium (Johnston et al., 2001).

The first stage of placental development is marked by embryonic implantation, which begins in bitches around the 18th day after ovulation of the oocyte that resulted in the subsequent pregnancy (Chastant-Maillard et al., 2010). Preceding the hatching of the blastocyst are changes that begin in the uterus and embryo, which include physical contact after dissolution of the zona pellucida between the trophoblast and the endometrium (Landim-Alvarenga, 2006).

Biometrics is an important technique for morphometrically analyzing and monitoring the development and growth of maternal, embryonic, and fetal gestational structures. It is important in predicting the gestational age of the fetus and the probably date of parturition (Lopate, 2008; Miranda and Domingues, 2010). There can be a reduction in prenatal mortality by diagnosing disorders that can negatively affect pregnancy (Johnsen et al., 2008). In medicine, elastographic evaluation of the placenta has been performed to characterize structural rigidity and identify early anomalies such as preeclampsia, placental abruption, spontaneous premature delivery, intrauterine growth retardation, and perinatal death, which are the main causes (about 5% of the neonatal population) of maternal and fetal morbidity and mortality (Gupta et al., 2008; Yu et al., 2008; Lindheimer et al., 2010).

In veterinary medicine, results of only one study have been reported regarding the applicability of elastography in animal placenta evaluation, using a murine experimental model of intrauterine growth restriction (Quibel et al., 2015). This highlights a lack of information on ultrasonic characteristics of utero-placental structure when there is a gestation period involving no abnormal occurrences or when there is a gestation with complications (i.e., pathological).

The imaging methods developed specifically to evaluate the elasticity of tissues are collectively referred as elastography, a non-invasive imaging modality classified based on the source of the force that is exerted on the tissues, such as manual compression, acoustic radiation force impulse (ARFI) and real-time shear velocity (RSV). In addition, elastographic evaluations can be performed using the qualitative technique, strain elastography, and the quantitative technique using compression waves (Feliciano et al., 2015a). In veterinary medicine, ARFI ultrasonography technique has been used to detect various pathological changes in the dog and cat spleen and kidneys, as well as in the dog liver, prostate gland, and testes (Holdsworth et al., 2014; Feliciano et al., 2015b, 2015c; Garcia et al., 2015; Maronezi et al., 2015), dog and cat mammary gland (Feliciano et al., 2015; Feliciano et al., 2017;), dog lymph node metastasis (Silva et al., 2018a), and dog and sheep fetal lung and liver (Simões et al., 2018; Silva et al., 2018b). This technique has the advantages of being repeatable, objective, and less operator-dependent, and has promise as a valid method of assessing structural changes in dog placental tissues.

Furthermore, it was hypothesized that: i) placental biometry using B-mode ultrasonography allows for prediction of gestational age; and ii) alterations in the tissue rigidity of the placental structure during gestational development, measured by elastography. The aim of the present study was to evaluate the biometry of placental structures and determine the quantitative elastographic characteristics and reference ranges for shear wave

velocities (SWV) of dog placental structures in healthy bitches from gestational day 15 until parturition. The rationale was that the use of elastography would provide useful information on placental development during physiological gestation in dogs. Additionally, it might be a useful technique for identifying pathological conditions that may alter tissue rigidity and development during pregnancy, a period during which the restriction of uterine growth is one of the most important causes of perinatal morbidity and mortality (Bernstein et al., 2000; Aucott et al., 2004). It is expected that the results of the present study will provide a basis for future studies aiming at the early diagnosis and monitoring of female dogs with gestational complications, thereby reducing genetic and economic losses of kennels and breeders (Beccaglia, 2015).

2. Materials and methods

2.1. Ethical aspects and animals

All experimental procedures were approved by the Animal Ethics and Welfare Committee (Univ. Estadual Paulista) protocol N° 9.884/16. Twelve brachycephalic, primiparous or multiparous bitches (body weight 10.5 ± 3.3 kg and age of 2.56 ± 0.89 years), all clinically healthy (based on assessment of clinical history, general examination, hematology and biochemical ALT, creatinine and blood glucose, dosage) and of different breeds (eight French Bulldogs, two Pugs and two Shih-tzus) were randomly selected according to the inclusion criteria for commercial dog breeders were used in this study. The sample size was defined with the aim of evaluating 24 placental units, which allowed for a statistical power of at least 85% based on results from a previous study in which there was evaluation of the rigidity of the placental structure in women with an ongoing pregnancy (Sugitani et al., 2013).

126 *2.2. Experimental protocol*

127 *2.2.1. Artificial insemination*

128 Kennel owners were trained to detect early signs of proestrus, to determinate the dogs'
129 optimal mating period. The ovarian estrous cyclic phase was determined by observing the
130 signs of estrus (i.e., female's acceptance of coupling) and using vaginal cytology (detection of
131 anucleated surface cells in smears (> 80 %) (Socha et al., 2012). Following vaginal cytology
132 estrous confirmation, artificial intravaginal insemination (AIVI) with fresh semen was
133 performed, every 48 h for 3 consecutive days (Jacomini et al., 2006).

134

135 *2.2.2. B-mode ultrasonography assessment*

136 Ultrasonographic pregnancy detection (Acuson S2000™ ultrasonic device; Siemens®,
137 Munich, Germany equipped with a 9.0 MHz linear transducer) was performed 2 weeks after
138 the first AI as described for a previous study of Feliciano et al. (2007). To perform ultrasonic
139 exams the hair of the abdomen was clipped. The transducer was positioned in the caudal
140 abdominal area and all adjustable settings of the ultrasonic device (e.g., depth, gain,
141 mechanical index, and focal zones) were optimized to ensure a more precise image and left
142 unchanged for the entire study period. All ultrasonic examinations were performed by a
143 single experienced operator (5 years) to reduce the evaluation time and stress endured by
144 pregnant animals, and consistently ascertain the proper development of conceptuses and the
145 gestational age (Yeager et al., 1992; Socha et al., 2012; Simões et al., 2018).

146 Considering a variable period of gestation in dogs (between 57 to 63 days)
147 (Concannon et al., 1983), the day after the first insemination was considered the first day of
148 gestation in this study. Thickness (mm) assessments of the maternal-fetal placental structure
149 tissues were determined once daily from day 15 of gestation and the individual maternal and
150 fetal tissues from day 21 of gestation until whelping (Figure 1). To evaluate the same area of

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151 the placenta in each animal consistently, there was standardization of the evaluation of two
152 caudal placenta (one from each uterine horn).
153
154 *2.2.3. ARFI elastography assessment*
155 After completion of the B-mode ultrasonography, quantitative elastographic (ARFI)
156 determinations were made of the placental structure (dorsal, lateral and ventral) using two
157 specific types of software designed for tissue rigidity image analyses: VTTQ™ (Virtual
158 Touch™ Tissue Quantification; Siemens, Germany) and VTTIQ™ (Virtual Touch Tissue™
159 Imaging Quantification; 2D-SWE technique, Siemens, Germany). Each procedure was
160 performed once daily from 21 days of gestation until whelping. Values for real-time
161 quantitative elastographic variables of shear wave velocities (SWV; m/s) of the tissues were
162 obtained by placing an electronic calliper with fixed dimensions (VTTQ™: 5 x 5 mm, Figure
163 2; VTTIQ™: 1 x 1 mm, Figure 3) within the parenchyma of each organ at three different
164 locations (cranial, caudal, central), with the depth ranging from 5 to 20 mm.
165
166 *2.2.4. Apgar score*
167 After parturition, all neonates were clinically evaluated using the Apgar score (0-10;
168 at 0, 5, and 60 min post-partum) by quantifying the body temperature (TC), heart rate (HR),
169 respiratory effort (RF), gingival mucous color, muscle tone, irritability reflexes, and
170 vocalization (Silva et al., 2008). The morpho-physiological development of the pups was
171 verified weekly using clinical examinations until day 60 postpartum, when the pups were
172 separated from the mother.
173
174 *2.3. Statistical analysis*

Statistical analyses were performed using the R® statistical software (R Foundation for Statistical Computing; Vienna, Austria), using a block (bitches) randomized experimental design, with parcels subdivided in time (gestational days). Residual normality (Shapiro test) and homoscedasticity of variances (Barlett's test) were previously tested. The variation between measurements of the three areas of interest in each area of the placental structure was studied using the Bland-Altman concordance test. The SWV averages and placental thicknesses were compared between the evaluated areas and gestational days using the analysis of variance (ANOVA) with repeated measures. The values for variables that were different based on use of an ANOVA were further analyzed using mathematical regression models (linear, quadratic, and cubic) or orthogonal contrasts. The statistical significance was set at 95% (P value < 0.05).

3. Results

All animals had normal gestation periods and there were no anomalies in fetal or placental development at the time of pre- or postpartum examinations. Respiratory distress did not occur in any neonate and Apgar scores were > 7 (median ± IQR: 9 ± 1.5 at 0 min; 10 ± 1.5 at 5 min, and 10 ± 0.5 at 60 min after birth) during the first hour after birth. The B-mode and ARFI ultrasonography could be performed without difficulties and did not cause any morpho-physiological alterations in the puppies during the first 60 days after parturition.

Maternal-fetal placental thickness (5.59 ± 0.83 mm) determined from day 15 of gestation until the day of parturition varied with the progression of gestation (P < 0.0001); it increased in thickness gradually until the day 63, as indicated by use of a linear regression model for this variable (P < 0.0001; R^2 = 0.91); and the placental thickness was observed to vary after this period.

Maternal placental thickness (2.20 ± 0.55 mm), determined from day 21 of gestation until parturition (Figure 1) increased with the progression of gestation ($P < 0.0001$) from day 21 to 40 and subsequently plateaued. This is indicated by a cubic regression model for this variable ($P = 0.0340$; $R^2 = 0.54$). The fetal placental thickness (3.40 ± 0.63 mm) determined after day 21 of gestation until parturition also varied with the progress of gestation ($P < 0.0001$), gradually increasing until the day 50 and then stabilizing as indicated by a linear regression model for this variable ($P < 0.0001$; $R^2 = 0.83$; Figure 4).

In the evaluation of tissue rigidity using the quantitative elastographic technique VTTQ ™ (Figure 2), there was not an intra-observational variation for SWV ($P = 0.6291$) and the data were considered to be similar because of the very small amount of variation (0.3 ± 0.2 m/s). Between the two placentas analyzed in each of the pregnant bitches, no variations were observed in the SWV ($P = 0.2023$).

Comparing the placental areas, the mean SWV of the dorsal area analyzed using VTTQ™ (2.62 ± 0.63 m/s) was greater ($P < 0.0010$) than that of the lateral area (1.68 ± 0.53 m/s), which was greater ($P = 0.020$) than that of the ventral area (1.50 ± 1.37 m/s). The SWV of the dorsal area (Figure 5) varied during the gestational period ($P = 0.0052$), as indicated by a cubic regression model ($P = 0.0291$; $R^2 = 0.40219$), such that the values for this variable decreased from day 21 to 35 of gestation, subsequently increased until day 56 and then stabilized. The SWV of the lateral area also varied ($P < 0.001$) and this variation is indicated by a quadratic regression model ($P < 0.001$; $R^2 = 0.6055$), where the values for this variable decreased gradually from day 24 to 45 and then gradually increased until parturition. The placental SWV of the ventral area did not change during the gestational period ($P = 0.9611$).

In the evaluation of tissue rigidity by the quantitative elastographic technique VTTIQ™ (Figure 3), there was no intra-observational variation for SWV ($P = 0.4774$) and the data were considered similar because of the small amount of variation (0.02 ± 0.52 m/s).

Between the two placentas analyzed in each of the pregnant bitches, there were no variations in the SWV ($P = 0.6570$).

Comparing the placental areas, the mean SWV of the dorsal area (2.60 ± 0.44 m/s) measured using the VTTIQ™ technique was greater ($P < 0.001$) than that of the lateral (2.33 ± 0.56 m/s), which was greater ($P < 0.001$) than that of the ventral (1.66 ± 0.66 m/s) area. The SWV of the dorsal area (Figure 6) varied during the gestational period ($P < 0.0010$), as indicated by a cubic regression model ($P = 0.0016$; $R^2 = 0.5075$), in which the values for this variable decreased from day 21 to 43, increased to day 60 of gestation and then stabilized. The placental SWV of the ventral area also varied ($P < 0.001$) which is indicated by a quadratic regression model ($P < 0.001$; $R^2 = 0.8055$), where the values for this variable gradually increased from day 21 to 23 of gestation and then progressively decreased until the time of parturition. Furthermore, the placental SWV of the lateral area did not change during the gestational period ($P = 0.1092$).

The values resulting from SWV evaluations using the VTTQ™ and VTTIQ™ techniques were positively correlated ($P < 0.0001$) for each of the dorsal, ventral, and lateral area ($R = 0.261$, 0.626 and 0.460; respectively) and only small variations were observed when there were comparisons of these values (- 0.08 ± 0.62, - 0.07 ± 0.026, 0.60 ± 0.79 m/s, respectively).

4. Discussion

Based on the results of the present study, implementation of B-mode and ARFI ultrasonography in brachycephalic bitches performed once a day from day 15 of gestation to parturition was applicable and did not cause any clinical change in viability and maternal, fetal, or neonatal health. These findings are consistent with results in elastographic studies

performed in humans (Sugitani et al., 2013; Karaman et al., 2016), baboons (Quarello et al., 2016) and with determination of biometrics in bitches (Maldonado et al., 2012).

The increase in maternal, fetal, and maternal-fetal placental thickness as assessed using B-mode ultrasonography in dogs apparently is indicative of the changes that occur in the histology of the placental areas of the uterus that is associated with more rapid growth during the prenatal period, after implantation (Dantzer and Leiser, 2012). The maternal-fetal placental thickness (5.59 ± 0.83 mm) determinations also allowed for the prediction of gestational age, corroborating the results of a previous study (Maldonado et al., 2012) in which there was evaluation of different breeds and sizes of bitches (small, medium, and large), and defining of an average placental thickness of 0.58 cm corresponding to the days (days 30.5, 35.2, 46, and 56.3) of gestation. Each dog was evaluated once from the third gestational week, while in the present study each animal was evaluated daily, from day 15 after the first artificial insemination until parturition, and only brachycephalic breeds of dogs being used as specimens for the study. These results also corroborate the findings of Silva et al. (2007) in boxer breeds in a study where there were determinations that there was a positive correlation between values for placental thickness and days of gestation with R^2 = 0.91, similar to results from the present study.

Comparing individual maternal and fetal thicknesses, the fetal placental thickness (3.40 ± 0.63 mm) was greater than that of the maternal thickness (2.20 ± 0.55 mm), indicating there was a gradual and constant increase until the second third of gestation and subsequently stabilizing of placental thickness; however, there have been limited evaluations in previous studies. As observed in humans (Schwartz et al., 2012), the measurement of placental thickness may be an important clinical evaluation in the prediction of uteroplacental blood flow restriction in dogs.

A thick heterogeneous placenta is a disorder strongly associated with maternal, fetal, and neonatal complications, including the end-stage of a compensatory process caused by an underdevelopment of the uteroplacental circulation (Raio et al., 2004), placental calcification (Maldonado et al., 2012), early signs of fetal hydrops, uncontrolled maternal diabetes and congenital infection (Kuhlmann and Warsof, 1996).

The placenta is one of the most important parenchymal organs in obstetrics and studies evaluating placental elasticity are limited. Sugitani et al. (2013) were the first to evaluate *ex vivo* placental tissue in pregnant women with fetal growth restriction and in which there was identification of an increase in placental rigidity compared to the healthy control group. Karaman et al. (2016) also evaluated placental elasticity *in vivo* using the ARFI technique in pre-eclamptic and normal hypertensive pregnant women, concluding that this diagnostic approach allows for accurate assessments in detection of these complications.

The present study is the first conducted to evaluate the chronology of placental physiological elasticity in bitches, defining normal placental patterns for brachycephalic bitches, with the precept that these abnormalities may be an important factor in fetal morbidity and mortality due to its importance in fetal development and growth (Bowman and Zennedy, 2014). Pathophysiological studies, therefore, are extremely important in validation of this diagnostic parameter in different species. Results from some studies indicate there are possible long-term fetal adverse effects of ultrasonography, such as thermally induced teratogenesis due to tissue temperature increases, associated with the absence of fetal vascular perfusion during early pregnancy (Abramowicz et al., 2008). In the present study, however, there were no malformations observed even with the daily evaluation using this technique.

The ARFI elastography was performed without discomfort to the pregnant bitches and there was no evidence of alterations in the factors being evaluated as result of the use of this

technique, considering it a safe and a non-invasive approach. There were similar observations by Tabaru et al. (2012) in a study on the biological effects of ultrasonography in fetal evaluation. These findings were verified by Sugitani et al. (2013) in women when there was reporting that, microscopically, there were no structural, thermal, or mechanical changes in the tissues studied. The ARFI elastography was also effective because of the ease of applicability and reproducibility, that is, any operator should be able to obtain the same image quality without difficulty. There was no difference between repeated measures, even with in-depth interference in the measured SWV, consistent with the results in a report of Ohmaru et al. (2015) in which there was evaluation of the second and third gestational period in women.

Li et al. (2012) reported there was no difference between the borders and central areas of placenta in terms of placental elasticity values in the third trimester of physiological gestation in humans. The SWV of the dorsal area that corresponds to the free edge area of the maternal-fetal placental unit was evaluated using both software to distinguish a well-defined tissue function during the gestational process, similar to that reported in women by Ohmaru et al. (2015). The findings indicated that although the SWV values for placenta assessments did not correlate with the week of gestation, however, it gradually increased during pregnancy. Furthermore, in the third trimester of gestation, results from histological evaluations indicated there were alterations such as a greater than typical abundance of collagen fibers and the presence of fibrosis. These conditions could increase tissue rigidity; and additional factors such as inflammatory changes that could be the result of vascular dysfunction associated with gestational hypertension and/or infarction could not be excluded; however, complications were not detected during the observation period.

The SWV values for the ventral area using the VTTQ™ software that refers to the umbilical cord insertion area, and the lateral area that corresponds to the free edge area of the placental unit with use of the VTTIQ™ software, did not change. To the best of our

knowledge, there are no previous reports that allow for comparison of the current with previous results. It, therefore, is considered advisable to evaluate the SWV of the dorsal area using any of the software types used and described for conducting the present study.

In veterinary medicine, the results from the study by Quarello et al. (2015a), in which there was assessment of placental elasticity in baboons in the first, second, and third gestational trimesters and assessment of SWV, could be explained using a quadratic regression model with $R^2 = 0.196$ during gestation. There were similar similar results in the previous study to those in the present study for the free edge area of the placenta. Nevertheless, the results of the present study indicated there was a greater coefficient of determination $R^2 = 0.60$ due to the larger sample size and repeated evaluation at regular intervals in the same animals. The results of the present study indicate the approaches used are promising for pathophysiological evaluations in future studies.

It is important to emphasize and recognize some limitations of the present study. All fetuses were born healthy; indications for caesarian-section were based on ultrasonic detection of changes in fetal heart rate (Gil et al., 2014a). The small sample size may be considered a limiting factor of the present study, however, is consistent with the methodology used by Gil et al. (2014, 2015a, 2015b) in previous studies in which there was determination of values for several clinical variables that are commonly used as reference values for dog pregnancies.

In addition, the results of the present study could have been affected by peristaltic movements occurring in pregnant bitches, variability in fetal organ depth, fetal movements, acoustic shadowing generated by fetal ribs, and the proportions of areas of interest evaluated. These limitations of the imaging technique have been previously described by Quarello et al. (2015b) and Simões et al. (2018) and may affect the relationships between elastographic features of placental tissue.

5. Conclusion

The biometric and elastographic evaluation of the dog placenta from day 15 of gestation until parturition was possible and did not cause any apparent alterations in maternal, fetal, or neonatal viability. The patterns found in placental thickening and placental SWV assessments reflect the structural and biochemical adaptations that occur during the gestational stages and can become a useful technique in clinical obstetrics. The technique and values elucidated in this study provide a reference for biometric and elastographic analyses of normal gestational tissues and a promising basis for future pathophysiological studies in different species of veterinary interest.

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Conflict of Interest

None of the authors has any conflict of interest to declare.

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Quibel, T., Deloison, B., Chammings, F.,Chalouhi,G.E., Siauve, N., Alison, M., Bessières, B., Gennisson, J.L., Clément, O., Salomon, L.J., 2015. Placental elastography in a murine intrauterine growth restriction model. Prenat. Diagn. 35, 1106-1111. https://doi.org/10.1002/pd.4654.

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534

535 **Figures Legends**

536 **Fig. 1.** High resolution ultrasonographic image in a longitudinal section of the placental
537 structure (maternal unit: x, fetal: ◊, and maternal-fetal: +) in a bitch on day 31 of gestation
538

539 **Fig. 2.** Image of quantitative ARFI VTTQ™ elastography in a longitudinal section of the
540 dorsal area of the dog placenta (green calliper) on day 40 of gestation (SWV = 2.40 m/s,
541 depth = 1.3 cm)
542

Fig. 3. Image of quantitative ARFI VTTIQ™ elastography in a longitudinal section of the dorsal, lateral, and ventral dog placenta on day 25 of gestation

Fig. 4. Graphic representation of placental maternal-fetal (A) and fetal (B) thicknesses (Y) during the physiological gestational period (X) in brachycephalic bitches; Continuous line corresponds to the regression model (maternal-fetal thickness (mm) = 0.12 X gestational day + 0.52; fetal thickness (mm) = 0.083 X gestational day – 0.13) adapted to each variable and the dots indicate the 95% confidence interval

Fig. 5. Graphical representation of the shear wave velocity (SWV; m/s) of the placental dorsal and lateral areas using the quantitative elastographic technique VTTQ™ during the physiological gestational period in 12 brachycephalic bitches

Fig. 6. Graphical representation of the shear wave velocity (SWV; m/s) of the placental dorsal and ventral areas of the placenta determined using the quantitative elastographic technique VTTIQ™ during the gestational period in 12 brachycephalic bitches