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Effect of breed body-size on leptin amniotic fluid concentrations at term pregnancy in dogs

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ABSTRACT

Because of the need to improve the knowledge about canine perinatology, and given the major role of fetal fluids in sustaining the course of pregnancy and fetal development, an in-depth analysis to better understand the role of some hormones in these compartments is essential. Among all, leptin is recognized to play a key role not only on the energetic homeostasis, but also at multiple levels, influencing the control of reproduction, food assumption and metabolism. Even if in humans and other species it is reported the presence of leptin receptors during fetal development, very little is known about the canine species, in which the role of leptin still needs to be fully understood. The present study aimed to assess the amniotic fluid leptin (AFL) concentrations at term pregnancy in healthy dogs, and to evaluate the possible influence played by breed body-size (after assessment of correlation with maternal bodyweight and placental weight), or other maternal (age, parity, and the so-called "litter effect") and neonatal (gender, birth weight, litter size) parameters on AFL concentrations, analyzed by ELISA test. The study was performed on 90 healthy, viable and normal weighted puppies, 39 small-sized (adult body weight < 10 kg) and 51 large-sized (adult body weight > 25 kg), born by 29 purebred, healthy bitches, submitted to elective Caesarean section because of breed-related or individual high risk for dystocia. The results showed that the mean AFL concentration in the small-sized puppies was significantly ($p < 0.05$) higher in comparison to large-sized puppies (867.48 vs 698.42 pg/ml), while all the other studied parameters did not show to influence AFL concentrations. In conclusions, the present study showed significant higher at term AFL concentrations in small-sized as compared to large-sized breeds, suggesting an influence of breed body-size on fetal metabolism, as previously reported for NEFA and IGF-I.

1. Introduction

The study of canine perinatology has recently raised increased interest under a two-fold perspective: on one hand there is the need to deepen the knowledge about the canine perinatal physiology, on the other hand the availability of reference physiologic data is necessary for the further detection of possible markers for the diagnosis of diseases or abnormalities. One of the limitations to the study of canine perinatology is addressed to the difficulty to perform biological samples collection before and even after birth. This limitation represents also a concern from an animal welfare standpoint, especially when blood sampling is considered. Moreover, in dogs, although amniocentesis can be used to collect amniotic fluids in late pregnancy, it was reported that it could be difficult to achieve, because puncturing the amniotic sac is possible if the bitch stays calm and in a dorsal recumbent position [1]. However, several studies demonstrated that, in dogs, amniotic fluid could be safely collected during Caesarean section to define the fetal fluid composition at the time of birth, providing information about the normal composition [2–5] and preliminary results on differences in pathological newborns [4,5].

Among the study performed on canine fetal fluids composition [2], investigated the IGF-I and NEFA fetal fluids concentrations, and reported a breed body-size association between lower IGF-I and higher NEFA concentrations in small-as compared to large-sized breeds, raising the question about the possible influence of breed-size on some metabolic amniotic components in dogs.

Beside IGF-I and NEFA, also leptin plays a key role in the energetic homeostasis. Leptin, is a protein mainly produced by white adipose tissue [6] with lesser quantities produced also from other tissues, that acts at multiple levels with pleiotropic roles in the control of reproduction, food assumption, energy expenditure, and metabolism [7,8]. The role of leptin in the fetus did not receive an extensive investigation, although some studies described the role of leptin, or the presence for leptin receptors, in different organs in humans and some animals, but not in the dog, during fetal development [9–11]. Leptin was also reported to be involved in the process of growth and development,

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so that alterations in leptin were reported to be considered as indications of disease risk [12]. Canine leptin plasma concentrations have been investigated by Ishioka et al. [13], in relation to body condition score, age, gender and breed. Those authors found a positive correlation between plasma leptin concentrations and body condition score, but no effects of age, gender and breed, demonstrating the role of leptin as a marker for adiposity in dogs. Leptin secretion is modified in obese women and overweight pregnant bitches were reported to be more prone to dystocia [14]. Recently [15], leptin circulating concentrations have been described along dog pregnancy and lactation, highlighting a first increase from 0 to 45 days of pregnancy, with further decrease at 60 days of pregnancy and a subsequent increase in the two months of lactation. Balogh et al. [16] studied the gene expression of leptin and leptin receptors in the uterus and placenta of bitches at different stages of pregnancy, and concluded that, in the canine species, the uterus and placenta are source for leptin production but also target organs of its autocrine/paracrine actions [16].

To the authors knowledge no studies on canine amniotic leptin concentrations have been, so far, reported. However, amniotic fluid leptin was investigated in other species. In humans, amniotic fluid leptin concentrations were reported to originate by amniotic cells, but also from fetal adipose tissue and subsequent transfer to the fetal circulation [17,18]. The maternal concurrence to amniotic fluid leptin concentrations is considered scarce in humans, because of its high molecular weight, and the consequent process of leptin compartmentalization [17,19]. When the association between amniotic fluid leptin concentrations and some fetal and maternal parameters have been investigated in humans, mid trimester amniotic fluid leptin levels resulted positively correlated to maternal body mass index, and negatively correlated to parity [19], while no associations were found between amniotic fluid leptin concentrations and placental or birth weights, maternal age, ethnicity, fetal gender and pregnancy outcome [19].

Previous studies showed associations of some amniotic fluid biochemical parameters [5] and amniotic fluid IGF-I and NEFA [2] with canine breed body-size, suggesting that breed body-size could have an influence on homeostasis. Aware that many parameters can specifically influence the energetic homeostasis, the present study focused on the measurement of amniotic leptin concentrations in non-obese dogs belonging to different body-size, in order to provide an additional, although limited, information about the complex contribution to energetic homeostasis in canine fetuses/neonates. Because of the reported possible impact played by other parameters on amniotic leptin concentrations, beside the effect of breed body-size, the possible effect of some other maternal (age, parity, and the so-called "litter effect") and neonatal (gender, birth weight, litter size) on amniotic leptin concentrations was also assessed. The "litter effect" is considered as a possible parameter referring variation among litters: (i.e. differences between puppies belonging to different bitches).

2. Material and methods

2.1. Animals

The study was performed on 29 purebred, healthy bitches, aged 2–7 years, 8 primiparous and 21 pluriparous (2–5 parturitions). According to breed body-size, 15 bitches were small-sized (body weight <10 kg) (7 Chihuahua, 4 Spitz, 2 Miniature Bull Terrier, 1 Maltese, and 1 Bouledogue Francaise), and 14 were large-sized (body weight >25 kg) (4 Maremma, 2 Bernese Mountain, 2 Labrador Retriever, 2 German Shepherd, 2 Newfoundland, 1 Hovawart, and 1 Saint Bernard).

All the bitches were healthy, regularly vaccinated and dewormed. They showed a pre-pregnancy body condition score of 3/5, and were fed twice-a-day during pregnancy with a commercial diet for dogs, meeting the metabolic energy needs on the base of the nutritional guide-lines recommendations for dogs and cats. The amount of food was calculated for each bitch, on the base of breed body-size and body weight, and modified along pregnancy according to the number of fetuses. Food assumption was regularly checked twice-a-day along the entire pregnancy. At the end of pregnancy, none of the bitches showed body weight increases exceeding 30% of the pre-pregnancy body weight, so that all of them were considered as non-obese dogs.

They were clinically monitored from the time of mating/artificial insemination, along pregnancy, until whelping. Considering the breed-related or individual high risk for dystocia, all the bitches were submitted to elective Caesarean section at the estimated date of parturition, for the health of mothers and puppies. The date of parturition was calculated according to previous reports [2–5], taking in consideration the blood progesterone concentrations at the sole mating/AI, the embryonic/fetal biometry, the clinical and ultrasonographic maternal and fetal monitoring at term, and the blood progesterone concentrations at impending parturition. Caesarean section was performed using an anesthesia protocol, aimed to minimize the possible side-effects on newborn puppies' viability, as previously reported by Ref. [2].

Two expert neonatologists took care of the puppies immediately after uterine extraction, providing first assistance and evaluating neonatal viability by measuring the Apgar score within 5 min after birth [20], assessing the absence of gross malformations or physical defects, recording newborn gender and measuring birth weight before nursing. Birth weights within the breed reference range reported by the Italian Kennel Club (Ente Nazionale della Cinofilia Italiana) were considered as normal. According to Veronesi et al. [20], only normal viable (Apgar score $\geq$7), and normal weighed newborns were considered. Individual placentae were weighted according to Ref. [21].

Before surgery the owners signed an informed consent to allow anesthesia and surgery, but also to specifically allow the collection of amniotic fluid and its use for research purposes.

2.2. Amniotic fluids collection

At fetal extraction, one person was dedicated to amniotic fluid collection from each amniotic sac, as previously reported [2]. Individual amniotic fluid samples were immediately centrifuged at room temperature at $1000\times g$ for 10 min, and the supernatant placed in plastic vials and frozen at −20 °C until leptin analysis.

2.3. Leptin amniotic fluid analysis

At the time of analysis, amniotic fluids were centrifuged for 15 min at $1000\times g$ to remove debris.

The concentration of leptin in the amniotic fluid samples was analyzed by competitive canine leptin ELISA kit (MyBioSource, San Diego, USA) utilizing a polyclonal *anti*-leptin antibody and a leptin-HRP conjugate. The ELISA was performed according to manufacturer's instructions. The sensitivity of the leptin ELISA was 0.1 ng/ml. The intra-assay and inter-assay coefficients of variation were $<10\%$ and $<10\%$, respectively. The relationships among amniotic fluid sample and the standard curve, determined through linear regressions, was linear with correlation coefficients of $r = 0.99$. The empirical regression line is given by the equation $y = 0.79 + 1.01x$.

2.4. Statistical analysis

Firstly, we calculate two regression functions, between maternal body weight and breed body-size and between placental weight and breed body-size, respectively, in order to corroborate the choice of breed body-size as bitches grouping factor. Since the breed body-size is a qualitative covariate, it was incorporated into the regression using a dummy variable X (i.e. $X = 0$ for the large breed body-size and $X = 1$ for the small breed body-size). Then, we interpreted the coefficient of determination R squared, which ranges from 0 to 1 with values closer to 1 indicating a better fit to the regression model. Moreover, consider that in case of a simple linear regression R squared corresponds to the squared Pearson correlation coefficient. Afterwards, in order to verify if there was a difference in the amniotic fluid leptin concentrations in dependence of a breed body-size, a welch-test was conducted for small-sized against large-sized dogs, under assumption of normality and of heteroscedasticity (i.e., different variances in the two groups) of the leptin concentrations. We can easily assume data normality due to the central limit theorem and the fact that our samples, containing more than 30 puppies, can therefore be considered large (rule of thumb). Variance heterogeneity may occur naturally; tests allowing heterogeneity are more robust and should be used in routine. Statistical significance was set as usual at $p < 0.05$.

To assess if other parameters could also be responsible for the amniotic fluid leptin concentrations, a linear mixed model was fit for it, in dependence not only of the breed body-size but also of other maternal (age, parity, "litter effect") and neonatal (gender, birth weight, litter-size) parameters.

All analyses were made by R (version 3.6.0) as reproducible report by the R package Knitr and could be made available upon request.

3. Results

3.1. Clinical findings and bitches grouping

A total of 90 healthy, viable and normal weighted puppies, 39 small-sized and 51 large-sized, were born; litter size ranged between 1 and 5 in small-sized bitches, and between 1 and 8 in the large-sized ones. Based on gender, 40 puppies (44%) were females, and 50 (56%) were males. Mean ± SD birth weight was 191 ± 77.57 g in the small-sized, and 583 ± 132.83 g in the large-sized newborns.

In small-sized bitches the mean ± SD maternal body weight was 5.02 ± 2.93 kg (range: 2–9.3 kg), while in large-sized bitches it was 44.0 ± 10.46 kg (range: 29–62 kg). Mean ± SD placental weight in small-sized bitches was 25.3 ± 7.26 g (range: 19.1–45.8 g), while in large-sized bitches it was 64.3 ± 10.34 g (range: 47.7–80 g). The statistical analysis showed a strong correlation between maternal body weight and breed body-size (Rsquared = 0.85, $p < 0.001$), as well as of placental weight and breed body-size (Rsquared = 0.76, $p < 0.001$), corroborating the usefulness of breed body-size for bitches grouping, thanks to its easier and more practical recording in comparison to body weight and/or placental weight measurement.

3.2. Amniotic fluid leptin concentrations

The distribution of leptin amniotic fluid concentrations in the 39 small- and in the 51 large-sized puppies is reported in Fig. 1.

The mean amniotic fluid leptin concentrations was 867.48 pg/ml in the small-sized puppies and 698.42 pg/ml in the large-sized newborns, with standard deviations of 209.32 pg/ml and 161.71 pg/ml, respectively. Correspondingly, the 95% confidence interval was 867.48 ± 65.69 pg/ml in the small-sized puppies and 698.42 ± 44.38 pg/ml in the large-sized newborns. According to the welch-test, this is a significantly different concentrations ($p < 0.05$).

On the other side, the fitted mixed model for amniotic fluid leptin concentrations in dependence of other parameters did not show significant influence played by any of the other maternal (age, parity, "litter effect") and neonatal parameters (newborn gender, birth weight, and litter size).

4. Discussion

This study showed that leptin is detectable in canine amniotic fluid collected at term of pregnancy, with concentrations very similar to those reported in humans by > 33 weeks of gestation (median value: 519 pg/ml, range: 380–761 pg/ml) [22]. Based on a previous study in which differences between small- and large-sized canine breeds in IGF-I and NEFA amniotic fluid concentrations were reported [2], the present study investigated the possible influence of breed body-size on leptin amniotic fluid concentrations, as a result of the role of canine breed body size on metabolic hormone concentrations in amniotic fluid collected at term of pregnancy. Thanks to the strong correlation between breed body-size and bitches body weight and placental weight, breed body size was demonstrated to be a useful parameter to assess the effect on leptin amniotic concentrations. Breed body size represent a more practical and easier to be recorded parameter instead of measuring maternal body weight and placental body weight, under normal conditions.

The dog is a unique species in which hundreds of different breeds are included, with body size ranging between the smallest Chihuahua (body weight max 3 kg), to the largest Saint Bernard (body weight up to 120 kg). This implies a diverse metabolism among breed sizes, that can be displayed at different age or at different physiologic conditions. A recent study by Cardinali et al. [15], showed a low correlation between pregnant dogs' plasma leptin and body weight, but the evaluation of a breed influence was not possible, because all the bitches belonged to the same (Bloodhound), large-sized breed. In humans, amniotic fluid leptin mid-trimester concentrations were positively correlated to maternal body mass index, and negatively correlated to maternal parity, but not related to fetal gender, birth weight or pregnancy outcome [19]. In the present study, leptin amniotic fluid concentrations measured at term pregnancy were not significantly influenced by maternal age, parity, "litter effect", fetal gender, birth weight, and litter size. The different result concerning the influence of maternal parity in humans, as compared to dogs, may be related to the different characteristics of the placenta in the two species. Unfortunately, a comparison with similar studies on dogs is not possible, also considering leptin maternal plasma concentrations. In fact, in the study from Cardinali et al. [15], the possible effect of parity on leptin maternal plasma concentrations was not assessed. It is clear that a weakness of the present study is the lacking of maternal blood leptin concentrations measurement at parturition. In fact, although the bitches were vein cannulated for anesthesia, most of the owners, especially those of small-sized bitches, did not allow for blood sampling for research purposes. Measuring mater-

Fig. 1. Distribution of leptin amniotic fluid concentrations in the 39 small- and in the 51 large-sized puppies rendered by boxplots. Each point represents one puppy.

nal blood leptin concentrations at the time of parturition would be interesting to evaluate the possible correlation between maternal and offspring leptin concentrations.

Similar to what reported in humans, also in dogs it could be interesting to investigate the gestational age-related changes in amniotic fluid leptin concentrations, even if, as stated before, amniocentesis remains a procedure that could be difficult to achieve. However, Balogh et al. [16] reported the different expression of leptin and leptin receptors in the canine uterus and placenta during different stages of pregnancy, and suggested the possible regulatory role of leptin around the time of parturition.

It is interesting to note the absence of significant associations between amniotic fluid leptin concentrations and puppy's birth weight. In fact, although the mean birth weight was different between small- and large-sized breed's puppies, amniotic fluid leptin concentrations were not significantly influenced by birth weight. It must be highlighted that, in all the puppies, the birth weight was in the normal ranges reported for the breed, and no under- or over-weighted newborns were born. In humans, the relation between higher leptin concentrations in maternal serum, fetal serum, amniotic fluid or placenta, and growth perturbation, have been reported, even if it was suggested that the leptin levels in different biological specimens could represent diverse markers for interconnected, but different, process of development along pregnancy [22]. Some authors found a positive correlation between cord blood leptin concentrations and body weight in all the human neonates studied [23] or only in the prematures [24], but others [22] did not find an association between amniotic fluid leptin concentrations and birth weight, with amniotic fluid leptin concentrations only marginally higher in small for gestational age newborns.

Some authors [25] reported higher human amniotic fluid leptin concentrations in female than in male fetuses, suggesting a possible sexual dimorphism as an additional regulatory growth mechanism. However, similar to the present study results, other authors did not find significant differences in human amniotic fluid [22] and in cord blood [26,27] leptin concentrations between females and males, arising the doubt about a different, gender-related, body fat amount in newborns.

It was interesting to note that the “litter-effect”, did not show to influence amniotic leptin concentrations, suggesting that the differences among litters in amniotic fluid leptin concentrations unlikely could concur to explain the wide standard deviations found in the present study.

5. Conclusions

In conclusions the present study showed that leptin is detectable in canine amniotic fluids collected at term, from healthy purebred puppies, born by non-obese bitches. Significant higher concentrations were detected in small-sized as compared to large-sized breeds, suggesting an influence of breed body-size on fetal metabolism.

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CRediT authorship contribution statement

M.C. Veronesi: Conceptualization, Funding acquisition, Project administration, Writing - original draft. **J. Fusi:** Data curation, Visualization. **A. Comin:** Methodology. **P.G. Ferrario:** Software, Formal analysis. **B. Bolis:** Investigation. **A. Prandi:** Supervision, Resources, Validation, Writing - review & editing.

Declaration of competing interest

None.

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