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Serum Levels of Cardiac Markers NT-proANP and NT-proBNP in Brachycephalic bitches at Different Gestational Stages

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Contents
The aim of this study was to determine serum levels of natriuretic peptide precursors (NT-proANP and NT-proBNP) during pregnancy in brachycephalic bitches. Fifteen healthy multiparous bitches were selected for this prospective study. Serum levels of NT-proANP and NT-proBNP were measured during anoestrous and at 14, 35, 42, 49 and 56 days (2nd, 5th, 6th, 7th and 8th weeks) of pregnancy. Fourteen animals had normal gestations, and one bitch developed single foetus syndrome. The natriuretic peptide levels of this animal were not included in this study; however, it is important to report that its NT-proANP levels were four times greater than those of normal patients. There was no significant difference ($p = 0.072$) in NT-proBNP levels between anoestrous (0.20 ± 0.10 ng/ml) and the different pregnancy weeks (0.27 ± 0.12 ng/ml). There was a positive correlation ($p < 0.0001$) between NT-proANP and gestational age, and the levels of this marker increased significantly ($p < 0.0001$) during the 6th (0.26 ± 0.06 ng/ml), 7th (0.28 ± 0.04 ng/ml) and 8th weeks (0.29 ± 0.05 ng/ml) when compared to anoestrous (0.18 ± 0.02 ng/ml). NT-proANP serum levels are correlated with gestational development and may be indicative of cardiovascular adaptation in canine brachycephalic pregnancy.

Introduction

The inactive N-terminal fragment by-product (NT) of the cleaved prohormones (pro) of the atrial natriuretic peptide (ANP) and the B-type natriuretic peptide (BNP), denominated NT-proANP and NT-proBNP, are part of the natriuretic peptide family, which aid the regulation of fluid homoeostasis (Oyama and Singletary 2010). ANP and BNP are considered the most important cardiac natriuretic peptides in dogs and cats (Kimmenade and Januzzi 2009) and are primarily produced by the cardiac muscle. These biomarkers are released in response to other neurohormonal peptides (norepinephrine and angiotensin-II) and also to a variety of stimuli, such as volume overload, cardiac hypertrophy and hypoxia. Initially, the natriuretic peptides are released into the bloodstream as prohormones and processed by specific serum and myocardial proteases into an active carboxy-terminal fragment (C-ANP and C-BNP) and a N-terminal by-product. When C-ANP and C-BNP bind to their receptors, it leads to natriuresis and vasodilation, protective responses against hypertrophy and fibrosis. The natriuretic receptors are predominantly found in the lungs, kidneys, blood vessels and adrenal glands and are responsible for counteracting the constrictive vessel and sodium retaining effects of the renin–angiotensin–aldosterone system (Potter et al. 2009; Oyama and Singletary 2010).

Assays are available for the detection of ANP, C-ANP, C-BNP, NT-proANP and NT-proBNP; and, in general, their diagnostic use for the different hormone states is similar. In Medicine, BNP and NT-proBNP are considered the best markers of heart disease. Canine ANP is the natriuretic peptide most similar to its human equivalent, with a long half-life; thus, the human ANP and its cleavage equivalent NT-proANP remain as the most important markers for canine studies (Kimmenade and Januzzi 2009). NT-proBNP is considered to be more stable *in vivo,* and a commercial canine test and recent clinical trial data are available for the validation of this assay (Kellihan et al. 2011).

ANP and BNP are considered myocardial stress biomarkers (Boswood 2009). In humans, ANP and BNP are used as markers of underlying cardiac disorders, to diagnose congestive heart failure, to differentiate respiratory signs and to provide information on morbidity and mortality risks (Rehman and Januzzi 2008). Commercial canine tests are currently available and their application holds great promise, especially in the differentiation between pulmonary and cardiac compromise in respiratory distress, cardiac failure stratification and in directing therapy (Oyama and Singletary 2010).

In pregnant females, cardiovascular adaptation is essential for foetal development (Blanco et al. 2011). In women, volume overload during pregnancy induces cardiac hypertrophy, with corresponding augmentation of the left ventricular mass and increased cardiac output (Kametas et al. 2001; Desai et al. 2004). Due to cardiac adaptation, these patients show a progressive increase in atrial natriuretic concentrations (Castro et al. 1994); however, extreme increase in blood volume during gestation has been correlated to pre-eclampsia and restricted intrauterine growth (Ville et al. 1994; Resnik et al. 2005).

The cardiovascular adaptation of pregnant bitches is similar to that of humans. The cardiac hypertrophy developed in canine pregnancy supposedly occurs in response to volume overload and is classified as eccentric hypertrophy (Blanco et al. 2011), similarly to other mammals (Eghbali et al. 2005). Alterations in plasma renin activity and vasopressin, angiotensin-II, ACTH

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and cortisol levels in pregnant bitches are indicators of the vascular system response during pregnancy (Brooks and Keil 1994).

There are currently no studies on the behaviour of natriuretic peptides during pregnancy in canines, and therefore, the hypotheses of this study were as follows: (i) the levels of NPs are different from non-pregnant bitches and are associated with cardiovascular remodelling associated with pregnancy; (ii) the levels of NP will be constant throughout pregnancy.

Considering the importance of these markers on the study of the haemodynamic changes associated with pregnancy and gestational disorders in humans patients and the greater occurrence of gestational abnormalities in brachycephalic females, this study aimed to determine serum levels of natriuretic peptide precursors (NT-proANP and NT-proBNP) in pregnant brachycephalic bitches to assess the cardiovascular adaptations of these animals.

Material and Methods

This study was approved by the Ethics Committee on the Use of Animals of the College of Agriculture and Veterinary Sciences of the Universidade Estadual Paulista (FCAV/UNESP), Jaboticabal – SP, Brazil (protocol 017314/10).

Fifteen brachycephalic bitches from the same kennel were selected based on the following criteria: non-pregnant; multiparous; Shih Tzu; English Bulldog, French Bulldog or Pug; weighing between 5 and 25 kg; aged from 4 to 6 years and considered healthy following physical and reproductive examination, which included inspection of the reproductive tract by of vaginoscopy, vaginal cytology, abdominal palpation and ultrasonography.

The kennel owner was trained to detect the first signs of pro-oestrus (vaginal bleeding) and brought the bitches to the Department of Obstetrics and Gynaecology of the Univ Estadual Paulista (UNESP) so that the optimal fertility period could be determined by vaginal cytology (akaryotic cell levels and the presence of metoestrum cells and leucocytes; Socha et al. 2012) and by observing signs of oestrus (i.e. female's acceptance to mounting). The females in oestrus were either allowed to mate with the male for 3 days or were artificially inseminated. Males were considered fit for breeding following andrological (inspection and testicular ultrasonography) and semen (spermiogram) evaluation. Artificial insemination was carried out according to the technique described by Jacomini et al. (2006), with intrauterine deposit of the ejaculate (fresh semen).

Pregnancy diagnosis was performed 14 days (2 weeks) after mating, according to the method described by Feliciano et al. (2007). Foetal development was evaluated at 14, 35, 42, 49 and 56 days of pregnancy (2nd, 5th, 6th, 7th and 8th weeks) by ultrasound, using MyLab VET 30 ultrasound equipment (Esaote, Italy) with a 12.0-MHz linear transducer. These dates were determined according to the foetal ultrasound measures (biometry of biparietal diameter) as suggested by Socha et al. (2012), who reported high accuracy in determining gestational age by this method.

Blood samples were collected into dry tubes by cephalic vein puncture at anoestrous (control time) and on the days of ultrasonographic scanning (14, 35, 42, 49 and 56 days after mating). Serum was separated and stored at −80°C. Anoestrous was determined as the period subsequent to pregnancy and weaning (approximately 3 months after the oestrous period), considering that the animals have a history of regular oestrous cycles (every 6 months). NT-proANP and NT-proBNP levels were determined by a canine-specific immunoenzymatic assay kit (ELISA Kit; CSB-EQ027254DO and CSB-E17577c; CUSABIO Biotech Co., Wuhan, China) according to the manufacturer's instructions. The assays were performed at Genesis laboratory, São Paulo – SP, Brazil.

R Software® (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis. Data were not normally distributed, and thus, comparison between the weeks of gestation and anoestrous was performed by Friedman and Dunn's *post hoc* tests. Spearman correlation between natriuretic peptides and gestational weeks was also performed. Significance was considered at 5% ($p < 0.05$).

Results

Mean pregnancy length (±SD) was 60 ± 2.5 days, and the mean number of puppies per pregnancy was four. Fourteen animals had normal gestation, with no complications during pregnancy or parturition. One bitch developed single foetus syndrome, diagnosed by ultrasound at 35 days of gestation (the presence of a foetus was observed by ultrasound examination). The natriuretic peptide levels of this animal were not included in this study; however, it is interesting to note that the NT-proANP levels of this patient (1.15 ng/ml) were 4 times greater than those of normal patients (0.26 ng/ml) (Table 1); nevertheless, concomitant cardiorespiratory diseases in this patient were not discarded and more specific studies would be needed to correlate these findings with a gestational disorder.

Serum NT-proANP and NT-proBNP levels of each animal, at the different collection times, are detailed in Table 1. No significant difference ($p = 0.072$) was observed in NT-proBNP levels between anoestrous (0.20 ± 0.10 ng/ml) and pregnancy (0.27 ± 0.12 ng/ml) (Fig. 1). There was a significant positive correlation, moderately representative by the linear regression model ($p < 0.0001$, $R^2 = 0.43$) between NT-proANP and pregnancy age, and the levels of this marker increased significantly ($p < 0.0001$) on the 42nd (0.26 ± 0.06 ng/ml), 49th (0.28 ± 0.04 ng/ml) and 56th days (0.29 ± 0.05 ng/ml) in relation to anoestrous (0.18 ± 0.02 ng/ml). However, no significant difference was observed between the 14th (0.23 ± 0.05 ng/ml) and

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Table 1. NT-proANP and NT-proBNP serum levels (ng/ml) in 15 brachycephalic bitches during anoestrous and on the 2nd, 5th, 6th, 7th and 8th weeks of gestation

	NT-proANP (ng/ml)						NT-proBNP (ng/ml)					
		Gestation weeks						Gestation weeks				
Animal	Anoestrus	2nd	5th	6th	7th	8th	Anoestrus	2nd	5th	6th	7th	8th
1	0.191	0.203	0.270	0.288	0.326	0.298	0.198	0.271	0.551	0.304	0.298	0.290
2	0.182	0.214	0.286	0.294	0.324	0.314	0.266	0.135	0.163	0.211	0.196	0.295
3	0.182	0.224	0.294	0.304	0.298	0.299	0.193	0.062	0.099	0.080	0.359	0.139
4	0.178	0.203	0.270	0.298	0.313	0.304	0.138	0.129	0.293	0.459	0.285	0.315
5	0.143	0.292	0.260	0.297	0.300	0.298	0.379	0.216	0.095	0.471	0.171	0.277
6	0.201	N/A	0.189	0.316	0.280	0.194	0.274	N/A	0.326	0.231	0.331	0.304
7	0.208	0.185	0.260	0.224	0.298	0.288	0.111	0.234	0.256	0.124	0.296	0.277
8	0.190	0.244	0.258	0.278	0.296	0.306	0.241	0.067	0.226	0.242	0.326	0.625
9	0.161	0.280	0.272	0.189	0.281	0.296	0.178	0.132	0.178	0.270	0.297	0.710
10[a]	0.168	0.232	0.283	0.336	0.283	0.284	0.274	0.296	0.295	0.304	0.289	0.205
11	0.293	0.553	1.154	N/A	N/A	N/A	0.242	0.226	0.302	N/A	N/A	N/A
12	0.153	0.186	0.280	0.185	0.228	0.174	0.225	0.186	0.280	0.185	0.170	0.174
13	0.161	0.290	0.186	0.185	0.191	0.280	0.284	0.302	0.293	0.298	0.312	0.381
14	0.158	0.172	0.176	0.182	0.270	0.326	0.264	0.331	0.321	0.285	0.304	0.322
15	0.178	0.298	0.289	0.316	N/A	0.347	0.200	0.273	0.304	0.301	N/A	0.339

[a]Pathological pregnancy, diagnosed by ultrasound as single foetus syndrome on the fifth week of pregnancy (35th day), and the natriuretic peptide levels of this animal were not included in the study.
N/A: not available.

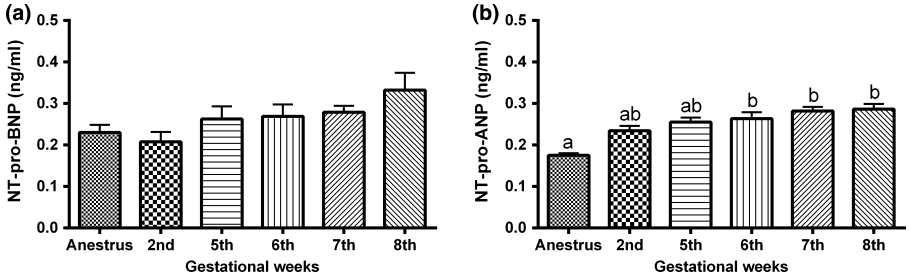

Fig. 1. Mean ± SE NT-proBNP (a) and NT-proANP (b) serum levels (ng/ml) in brachycephalic bitches during anoestrous and on the 2nd, 5th, 6th, 7th and 8th weeks of gestation. Different letters indicate significant difference between times ($p < 0.05$)

35th (0.26 ± 0.04 ng/ml) days and the other collection times (Fig. 1).

Discussion

Although serum natriuretic peptides can be measured in their active form, the evaluation of their precursors has higher sensitivity in identifying and differentiating heart diseases in humans and dogs (Sisson 2004; Kellihan et al. 2011); thus, NT-proANP and NT-proBNP analysis was selected for this study.

The extremely different natriuretic peptide levels reported in healthy and sick dogs may be a result of the type of assay used for analysis, as different laboratory techniques are available (Schellenberg et al. 2008). Comparison between normal serum levels of NT-proANP and NT-proBNP and those observed in non-pregnant bitches was challenging as the literature reports highly variable results, and different measurement units (pmol, fmol, pg, ng) and conversion factors are not always available (Asano et al. 1999; Häggström et al. 2000; Tarnow et al. 2009; Oyama and Singletary 2010; Kellihan et al. 2011).

In women, cumulative sodium and water retention are observed from the first gestational trimester to delivery, resulting in up to 45% increase in plasma volume and, consequently, cardiovascular adaptation to this haemodynamic change (Longo 1983). In pregnant bitches, haemodynamic changes (increase in cardiac output and decrease afterload) and cardiac adaptation (cardiac hypertrophy) seem to occur similarly to that in women (Abbott 2010; Blanco et al. 2011). Castro et al. (1994) reported a gradual increase in plasma ANP levels during human pregnancy (86% of analysed studies), with maximum levels in the third trimester and the first week post-partum. These findings are in agreement with those obtained in this study, in which the highest levels of NT-proANP were observed on the last 3 weeks of gestation. Furthermore, Tihtonen et al. (2007) reported similar results for prohormone in pregnant women.

The high serum ANP and NT-proANP levels during pregnancy are attributed to the greater atrial pressure resulting from the increase in blood volume (Castro et al. 1994; Tihtonen et al. 2007). In dogs, increases in ANP have been described as a response to increased atrial volume (Paganelli et al. 1986). Therefore, it is

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believed that the NT-proANP profile observed in this study may be indicative of the cardiovascular changes taking place in pregnant brachycephalic dogs.

In humans, the cardiovascular markers, specifically the NT-proBNP, have been associated with early diagnosis of pulmonary hypertension and its increase is a major risk factor for death of patients (Warwick et al. 2008). Specifically in the brachycephalic female dogs, it is common to observe the presence of respiratory discomfort at the end of gestational process due to the characteristics of these breeds, and this in the long term or constant mode can cause pulmonary hypertension (Simonneau et al. 2004), corroborating the importance of evaluating this cardiovascular marker in these pregnant females. However, NT-proBNP levels remained fairly constant throughout the different pregnancy stages, corroborating the reports by Resnik et al. (2005) and Hameed et al. (2009). Although Franz et al. (2009) reported an increase in serum NT-proBNP levels in mid-pregnancy, the reference values were not specified. Based on the results from this study and considering that the NT-proBNP is an important indicator of ventricular overload in patients with cardiomyopathies (Yoshimura et al. 1993; Warwick et al. 2008; Wolf et al. 2013), it may be suggested that animals do not have severe cardiovascular or haemodynamic abnormalities that compromised the gestational process and its cardiovascular status.

In summary, the different behaviours, observed for the plasma levels of cardiovascular markers in brachycephalic bitches, demonstrate the importance of evaluating these elements together and that these may indicate different cardiovascular or haemodynamic abnormalities in pregnant brachycephalic bitches. Specifically, the abnormalities associated with atrial change and haemodynamic adaptation (physiological or pathological compensation) correlate with the levels of NT-proANP (Castro et al. 1994; Tihtonen et al. 2007; Abbott 2010; Blanco et al. 2011); and abnormalities related to ventricular change and pulmonary hypertension correlate with the levels of NT-proBNP (Yoshimura et al. 1993; Warwick et al. 2008; Wolf et al. 2013).

In pregnant women, a severe increase in ANP precursors is indicative of a risk pregnancy (Ringholm et al. 2011). In pre-eclampsia and hypertension, the levels of ANP and BNP are higher than in healthy pregnant women due to an increase in the left ventricular mass and volume (Borghi et al. 2000; Resnik et al. 2005). In this study, it was observed that the levels of these natriuretic peptides were elevated in one case of single foetus syndrome, and although concomitant diseases that can lead to an increase in these markers have not been discarded, these findings may suggest that this gestational abnormality can promote cardiovascular and haemodynamic changes in pregnant animals and open the door to new research in canine pregnancy pathophysiology. However, the lack of scientific evidence and accurate diagnosis of single foetus syndrome and stillbirths do not allow any correlation to be drawn and further studies on these peptides in pathological pregnancy are needed.

It is important to comment on some limitations observed in this study: 1) brachycephalic animals were not assessed animals with other characteristics or other breeds. However, this study can provide important information about evaluation and reference values to compare with other breeds pregnant bitches; 2) further studies are needed to correlate the cardiovascular markers in pregnant bitches with echocardiographic findings and other pregnancy abnormalities.

Conclusion

This study provides unpublished information on the applicability of the evaluation of cardiovascular markers in pregnant brachycephalic bitches that can be used to assess cardiovascular status of these animals. NT-proANP serum levels are different from non-pregnant animals and at different stages of gestational process in brachycephalic bitches, it can be correlated with gestational age and may be indicative of cardiovascular adaptation in these animals. Additionally, NT-proANP could potentially be used as a marker for pregnancy disorders in brachycephalic bitches. In contrast, NT-proBNP showed no correlation with pregnancy.

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Conflict of interest

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

Author contributions

Marcus Antonio Rossi Feliciano, Ricardo Ramirez Uscategui and Giovana Serpa Maciel designed the study, analysed the data and drafted the manuscript. Vívian Tavares de Almeida, Matheus Folgearini Silveira, Guilherme Albuquerque Cavalcanti de Oliveira and Wilter Ricardo Russiano Vicente analysed the data and drafted the manuscript.

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