Evaluation of serum concentrations of cortisol and thyroxine in bitches with early-stage mammary carcinoma

Ricardo L. Salomão, Leandro Z. Crivellenti, Ana Paula R. Simões, Marina B.S. Brito, Paloma E.S. Silva, Paula B. Costa, Aureo E. Santana e Sofia Borin-Crivellenti


In view of the fact that cancer is considered a chronic disease that can interfere with hormonal homeostasis by means of cytokines, we hypothesized that, even at early stages, mammary carcinoma is able to alter the balance of the hypothalamic-pituitary-thyroid and hypothalamic-pituitary-adrenal axes. To test this hypothesis, the serum concentrations of basal cortisol, thyroxine (T4), free thyroxine (fT4), and thyroid-stimulating hormone (TSH) were evaluated in 20 unspayed bitches that had a histopathological diagnosis of grade 1 mammary carcinoma at clinical stage 1 according to the World Health Organization (WHO) classification (T1N0M0). The control animals comprised 10 unspayed bitches in perfect health conditions that were matched with those with mammary carcinoma by age. No significant differences regarding the concentrations of basal cortisol, TSH, T4, and fT4 were found between the bitches carrying early stage mammary carcinoma when compared to the control group. This suggests that, even if malignant, early-stage mammary carcinomas do not exhibit the ability to alter the concentrations of hormones produced by the hypothalamic-pituitary-adrenal or hypothalamic-pituitary-thyroid axes.

INDEX TERMS: Serum concentration, cortisol, thyroxine, bitches, mammary carcinoma, dogs, hormone quantification, mammary cancer, endocrine tests, clinics.
This prospective study included 20 unspayed bitches with a histological diagnosis of mammary carcinoma at clinical stage I according to the TNM classification of the World Health Organization. These bitches were treated at the “Governador Laudo Natel” Veterinary Hospital of FCAV/Unesp, Jaboticabal campus or at the Veterinary Hospital of University of Franca (Unifran), and were subjected to mastectomy and ovariohysterectomy (OH). The control group (CG) was composed of 10 healthy unspayed bitches that were selected and matched by age with their counterparts in the mammary carcinoma group (MCG). All the patients were evaluated by means of clinical and laboratory exams before mastectomy and/or OH. Animals were excluded from the CG whenever they exhibited any type of tumor, signs of metastasis in nearby or distant lymph nodes, and/or endocrinopathy within six months after the initial hormonal evaluation. Additional exclusion criteria in study were previous history of treatment with exogenous corticosteroid (or any other type or hormone replacement therapy), concomitant disease that involved thyroid or adrenal glands, pregnancy, presence of any tumors other than mammary carcinoma, and evidence of pulmonary, abdominal, and/or adjacent lymph node (axillary and/or inguinal) metastasis as assessed by radiographic, ultrasonographic or histopathological exams.

Sample collection. Before mastectomy, peripheral blood samples were collected by venipuncture of the jugular vein. A fraction of the blood was transferred to vacuum tubes containing K2-EDTA for hematological analyses; another fraction of the blood was transferred to vacuum tubes containing no anticoagulant, which were then centrifuged for 15 minutes at 800xg in a refrigerated centrifuge to provide serum. Serum samples were frozen at -80°C for subsequent biochemical analysis, including quantification of hormones and cytokines.

Laboratory analyses. All the hematological and biochemical laboratory analyses on samples of all the patients were performed at the “Professor Dr. Joaquim Martins Ferreira Neto” Veterinary Clinical Pathology Laboratory at “Governador Laudo Natel” Veterinary Hospital at UNESP, Jaboticabal campus, SP, Brazil, and at the Clinical Pathology Laboratory of Franca (UNIFRAN), Franca/SP, Brazil. Complete blood count was performed using automated cell counters (ABC Vet - HORIBA ABX - e poch-100 Iv-Diff - MS Diagnóstica). Serum samples were processed using commercially available kits (Reagentes - Labtest Diagnóstica) following the manufacturer’s instructions in order to determine the concentrations of creatinine, urea, total protein, albumin, alanine aminotransferase, and alkaline phosphatase.

Hormonal analyses. The samples were submitted to a Brazilian veterinary endocrinology reference laboratory (Provet, Medicina Veterinária Diagnóstica, São Paulo/SP), where they were processed routinely, and the hormonal analyses were performed in duplicates. All the quality tests for these analyses were done according to the directions of the international scientific community. Cortisol, total T4, free T4 by equilibrium dialysis, and TSH were quantitated in all of the samples included in the experiment. TSH was quantified using chemiluminescence, while the other hormones were quantified using radioimmunoassay (RIA). These hormonal quantifications were carried out using commercially available solid-phase immunoassay kits. Cortisol was quantified using a kit from MP (MP Biomedicals, Orangeburg/NY, USA). Total T4 and free T4 by equilibrium dialysis were quantified using kits from IVD (IVD, Santa Ana/CA, USA). TSH was quantified using a kit from Siemens (IVD, Santa Ana/CA, USA). All of these commercial kits were validated for use in dogs;

INTRODUCTION
Breast cancer is considered one of the worst health issues affecting women in the western world (Lacombe et al. 2013). Likewise, it is a neoplastic disease that frequently affects adult female dogs (De Nardi et al. 2009), which makes this species an excellent model for understanding the physiopathology, diagnostics, treatment, and prognostics of mammary carcinoma in humans; the most frequent forms of this disease in both humans and dogs are epithelial (carcinoma), and mixtures of epithelial and connective tissue (carcinosarcomas or mixed malignant tumors) (Ferreira et al. 2003, Lana et al. 2007).

Studies indicate that tumor size, involvement of lymph nodes, and histological grade are among the factors associated with a negative prognosis in cases of mammary neoplasia (Novosad 2003). Several authors agree that early detection and early therapeutic intervention are essential for preventing spreading of this cancer to nearby or distant sites in the body (Novosad 2003, Lana et al. 2007, De Nardi et al. 2009).

Even though bitches exhibit high mortality rates (Schneider et al. 1969) and reduced average survival time after diagnosis, mammary cancers at early stages are frequently overlooked by clinicians and tutors owing to the scarcity of research data on the early stages of this disease (Sorenmo et al. 2003).

There is evidence that other factors are involved in the progression of mammary cancer; even in early stages. The influence of hormones on the onset, maintenance, and progression of mammary tumors has been intensely studied, especially in the last two or three decades (Ruttenman & Misdorp 1993, Peña et al. 2003, Queiroga et al. 2005, Hardee et al. 2006, Illera et al. 2006, Obr & Edwards 2012, Brisken 2013, Perry et al. 2013). Mammary cancer is a chronic disease that elicits an inflammatory response; hormones and cytokines potentially related to the occurrence of this disease include cortisol, thyroxine, and proinflammatory cytokines (Baumann & Gauldie 1994).

From the endocrinological standpoint, and similar to what happens in other chronic diseases, changes in the balance of these hormones are speculated to mislead interpretation of hormonal tests, which could result in false positives for important endocrine diseases such as hypothyroidism and hypercortisolism (Buckingham et al. 1992, Carvalho & Jericó 1994), and, ultimately, lead to inadequate procedures and treatments.

Therefore, since mammary carcinoma is a malignant neoplasm, we hypothesized that, even at its early stages, this disease is able to change the hormone production balance of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid (HPT) axes.

MATERIALS AND METHODS
This experiment has been approved by the Ethics Committee on Animal Use (CEUA) of São Paulo State University (UNESP), Jaboticabal campus, SP, Brazil (protocol no. 010289/14).
reference values were provided by the manufacturer or by the reference laboratory.

**Statistical analysis.** The data were submitted to Kolmogorov-Smirnov normality test. Comparisons between the two experimental groups were made using the Student's t-test for variables with parametric distribution, and the Mann-Whitney test for non-parametric variables (band neutrophils, eosinophils, monocytes, creatinine, ALT, UPC, and TSH). P values less than 0.05 were considered significant. The variables are presented as mean ± standard error of the means (parametric distribution), or median with minimum and maximum interval (non-parametric variables).

**RESULTS**

Age and weight were used as criteria for selection of the animals for the control group (CG), which were matched with the animals of the mammary carcinoma group (MCG). The average ages were 9.18±1.8 years in the MCG, and 7.80±2.97 years in the CG (P=0.1290). The average weights in the MCG and in the CG were 10.90±6.97 kg and 10.54±4.56 kg, respectively (P=0.8834).

In the MCG, 40% of the bitches exhibited nodules only in the left mammary chain (n=8), 25% (n=5) of the bitches had nodules only in the right mammary chain, and 35% (n=7) had nodules in both chains. Most of the bitches exhibited a single mammary nodule (40%, n=8), while 30% (n=6) had 6 nodules, 15% (n=3) had 2 nodules, and 5% had 4, 6, and 10 nodules each (n=1). The average diameter of the largest mammary nodule of each bitch was 1.62±1.09 cm. Only one bitch had a nodule in the thoracic cranial mammary gland, and two bitches had nodules in the thoracic and caudal breasts. The remaining animals (n=17) had nodules in the central, abdominal and/or inguinal breasts. Histological grade evaluation categorized 19 tumors as grade 1, and 1 tumor as central, abdominal and/or inguinal breasts.

**DISCUSSION**

The ability of chemical mediators such as cytokines to effect alterations on the productions and secretion of adrenal and thyroid hormones has been known for decades (Drucker & Shandling 1985, Baumann & Gauldie 1994). Since mammary carcinoma (10%, n=2), solid tubular carcinoma (5%, n=1), papillary carcinoma (5%, n=1), and complex tubulopapillary carcinoma (5%, n=1).

The results of the hematological, biochemical and urinary analyses of the animals of the MCG and the CG are shown in Tables 1 and 2.

Complete blood count (CBC) results revealed that, albeit within normal values for dogs, the variables hemocrit, red blood cells, and hemoglobin were significantly reduced in the MCG when compared to the CG (P=0.046, P=0.0042, and P=0.046, respectively). The mean corpuscular volume (MCV) was slightly higher in the MCG when compared to the CG (P=0.0008). With the exception of lymphocytes, the remaining variables of the leukogram did not differ significantly between the experimental groups. Even though absolute lymphocyte counts were within normal values for dogs, they were significantly reduced in the MCG when compared to the CG (P=0.0088).

No qualitative or quantitative alterations were detected in the urine samples from both experimental groups. With the exception of urea, the serum biochemical variables exhibited no difference between the experimental groups (Table 2). Albeit within the normal range for dogs, urea concentration in the serum was significantly higher in the MCG when compared to the CG (P=0.0294).

The hormonal evaluations revealed no alterations regarding concentrations of cortisol (basal) or hormones that represent the HPT axis, namely TSH, total T4, and free T4 by equilibrium dialysis, in any of the experimental groups; all these values were within the normal range for dogs according to the reference values provided by the laboratory where these analyses were performed (Table 3).

**Table 1. Means and standard error of the means (minimum-maximum) obtained for the erythrogram and leukogram of bitches in the control and mammary carcinoma groups**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group (n=10)</th>
<th>Mammary carcinoma group (n=20)</th>
<th>P value</th>
<th>Reference values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>48.73±1.81</td>
<td>42.98±1.78</td>
<td>0.00465</td>
<td>37-55</td>
</tr>
<tr>
<td>Red blood cells (x10⁶/µL)</td>
<td>7.27±0.33</td>
<td>5.97±0.24</td>
<td>0.0042</td>
<td>5.5-8.0</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>16.26±0.77</td>
<td>14.30±0.55</td>
<td>0.0459</td>
<td>12.0-18.0</td>
</tr>
<tr>
<td>MCV(fL)</td>
<td>67.33±2.94</td>
<td>71.47±2.68</td>
<td>0.0008</td>
<td>60-77</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33.29±2.07</td>
<td>34.04±2.18</td>
<td>0.3833</td>
<td>31-34</td>
</tr>
<tr>
<td>Total leukocytes (cells/µL)</td>
<td>8.840±700.3</td>
<td>8.437±777.1</td>
<td>0.2419</td>
<td>6000-18000</td>
</tr>
<tr>
<td>Band neutrophils (µL)</td>
<td>0 (0-183)</td>
<td>0 (0-378)</td>
<td>0.1172</td>
<td>0-500</td>
</tr>
<tr>
<td>Segmented neutrophils (µL)</td>
<td>5.701±458.2</td>
<td>6.460±699.8</td>
<td>0.4661</td>
<td>3600-13800</td>
</tr>
<tr>
<td>Eosinophils (µL)</td>
<td>375 (0-1500)</td>
<td>273 (0-1001)</td>
<td>0.4478</td>
<td>120-1800</td>
</tr>
<tr>
<td>Lymphocytes (µL)</td>
<td>2.459±362</td>
<td>1.439±181</td>
<td>0.0088</td>
<td>720-5400</td>
</tr>
<tr>
<td>Monocytes (µL)</td>
<td>191.5± (0-510)</td>
<td>91 (0-684)</td>
<td>0.7387</td>
<td>180-1800</td>
</tr>
<tr>
<td>Platelets (x10⁵/µL)</td>
<td>406.5±44.5</td>
<td>322.95±265.1</td>
<td>0.0913</td>
<td>180-400</td>
</tr>
</tbody>
</table>

MCV = Mean corpuscular volume, MCHC = mean corpuscular hemoglobin concentration; * Veterinary Clinical Pathology Laboratory, Veterinary Hospital, Unesp-Jaboticabal, SP, Brazil, and Clinical Pathology Laboratory of Franca, Unifran-Franca, SP, Brazil. Variables followed by different letters in the same row are significantly different between the experimental groups (P<0.05).
cancer is a chronic disease, this work aimed at assessing the possible hormonal alterations in bitches with mammary carcinoma at an early stage. None of the hormonal variables used to evaluate the afore-mentioned hormone production axes exhibited alterations in this study. These results suggest, therefore, that mammary carcinomas at an early stage are not yet able to influence the hormonal homeostasis of cortisol and thyronine.

Integrity of the HPA axis is the major determinant of the body’s response to stress (Reichlin 1993), and its response is essential for the regulation of homeostasis and survival of patients affected by chronic, critical, and inflammatory diseases (Drucker & Shandling 1985, Sam et al. 2004). Although several studies in humans (Annane et al. 2000, Bollaert et al. 2003, Sam et al. 2004) and animals (Martin & Groman 2004, Schoeman et al. 2007) have found a positive correlation between high basal cortisol concentrations and mortality of critical patients (Peterson et al. 1997), no data regarding hormonal homeostasis in patients with mammary cancer at an early stage has been found in the literature, which highlights the importance of the data obtained in this study.

In view of the fact that chronic inflammatory diseases such as mammary cancer are frequent in the small animal medical routine, it is important to consider that these conditions may mislead interpretation of hormonal tests. False-positive results for endocrine diseases such as hypothyroidism, hypoadrenocorticism, and hypercortisolism could lead to implementation of inadequate proceedings and treatments (Buckingham et al. 1992, Carvalho & Jericó 2014), which could minimize or reduce the chances of determining the underlying problem, and even put the patient’s life at risk.

Part of the diagnosis of endocrinopathies is done through observation of compatible clinical signs, and also by detection of hematological, biochemical, and urinary alterations related to the suspected hormonal alteration (Meuten 2012). In this regard, the fact that the blood and urinary variables evaluated were not out of the normal range for the canine species is not suggestive of any specific endocrine disease. However, it should be noted that some of these variables were significantly different between the experimental groups; therefore, they might be related to mammary cancer. The difference regarding absolute lymphocyte counts between the experimental groups could have been influenced by the stress caused by the cancer, a factor that could be intimately connected to circulating cortisol concentrations (Gilor & Graves 2011). On the other hand, significantly lower erythrocyte-related variables in the CG could be indicative of increased production of cytokines and inflammatory interleukins able to interfere with erythropoiesis (Bordin-Crivellenti & Santana 2015), as a recent work has shown that the levels of TNF-α, IFN-γ, IL-1, and IL-3 are significantly higher in bitches affected by mammary carcinoma, whether the magnitude of the malignancy is low or high (Machado et al. 2015).

Confirmatory diagnosis of hypercortisolism depends on the demonstration of excessive production of cortisol and/or decreased sensitivity of the HPA axis to the negative feedback provided by glucocorticoids, and the main tests used for this purpose are the ACTH stimulation test (ACTHST) and the low dose dexamethasone suppression test (LDDST), respectively.
Evaluation of serum concentrations of cortisol and thyroxine in bitches with early-stage mammary carcinoma


REFERENCES


