Microvascularization of Corpus Luteum of Bovine Treated With Equine Chorionic Gonadotropin

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KEY WORDS bovine; corpus luteum; equine chorionic gonadotropin; microvascular density

ABSTRACT This study aimed to evaluate the morphological changes in microvascular density and corpus luteum (CL) vascularization in cows treated with eCG during stimulatory and superovulatory protocols. Sixteen cows were synchronized and divided into three groups: control (n = 6), stimulated (n = 4) and superovulated (n = 6), one was submitted to estrous synchronization (ES) and received no eCG (control), and those that were submitted to ES and received eCG before or after follicular deviation (superovulation and stimulation of the dominant follicle, respectively). Ovulation was synchronized using a progesterone device-based protocol. After six days of ovulation, the cows were slaughtered and the ovaries and CL were collected. The CLs were processed and photomicrographs were taken under light microscopy to assess the vascular volume density (Vv) by stereology, and scanning electron microscopy (SEM) was used to perform ultrastructural analysis of the microvasculature. The Vv in stimulated and superovulated cows significantly increased (P < 0.0001) when compared to control, indicating that the eCG is able to induce angiogenic activity in bovine CL. However, no significant differences were observed between stimulated and superovulated cows. The SEM demonstrated ratings indicative of angiogenesis, marked by several button-shaped projections in the capillaries, and the presence of more dilated capillaries in CL treated with eCG. These morphological findings are evidence of an angiogenic effect of the eCG treatment in CL of cows. Microsc. Res. Tech. 78:747–753, 2015.

INTRODUCTION The corpus luteum (CL) is an endocrine gland that participates in the regulation of the ovarian cycle and fertility of the female (Schams and Berisha, 2004). Although lasting only 18 days during the bovine estrous cycle, the CL undergoes significant structural changes in this period, such as rapid proliferation and differentiation of steroidogenic cells and extensive angiogenesis (Skarzynski et al., 2013).

There is substantial evidence that the pituitary gonadotropins, FSH and LH, influence the expression of angiogenic agents in CL (Fraser and Wulff, 2003; Papa et al., 2007; Fátima et al., 2013a). LH stimulates the production of most of these factors by luteal cells in a paracrine mechanism that induces angiogenesis in the developing CL (Fraser and Wulff, 2003). FSH also increases the expression of VEGF in human luteal cells (Reisinger et al., 2007), and has caused a significant increase in the vascular density of buffalo CL (Papa et al., 2007). Other studies have also demonstrated that human chorionic gonadotropin (hCG) also promotes an increase in the expression of factors such as VEGF and Ang in luteal cells (Christenson and Stouffer, 1997; Laitinen et al. 1997; Wulff et al., 2001).

The equine chorionic gonadotropin (eCG) is a gonadotropic hormone produced by trophoblast cells of endometrial cups in pregnant mares and plays a major role in the maintenance of early gestation (Legardinier et al., 2005). In non-equine species, the eCG has both FSH and LH activity and stimulates follicular development, ovulation, and CL formation. This hormone has also been widely used together with progesterone therapy and pituitary stimulation by GnRH to shorten the postpartum anestrus in cows (Lunenfeld, 2004). There are reports that the eCG is capable of inducing growth

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Received 22 February 2015; accepted in revised form 24 May 2015

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and ovulation of follicles in pre-pubertal heifers (Duffy et al., 2004; Ferreira et al., 2013).

Recently, an increase in CL volume and plasma progesterone concentration during the subsequent luteal phase in cows treated with eCG has been reported (Ferreira et al., 2013; Rostami et al., 2011), and this fact has been related to the influence of this hormone on the differentiation and function of granulosa cells and theca after ovulation (Kenyon et al., 2012), as well as on the number of large and small luteal cells, and the volume and shape of mitochondria in these steroidogenic cells (Rigoglio et al., 2013). However, until now no studies have evaluated the microvasculature of the CL from cows treated with eCG, despite its blood supply being closely related to its steroidogenic function. Therefore, this study aimed to evaluate the changes caused by stimulatory and superovulatory treatments with eCG in microvascular density and vascularization of bovine CL.

MATERIAL AND METHODS

Animals

We studied 16 crossbred cows (Angus x Nelore) aged between 2 and 5 years which were kept in pasture and supplemented with ground corn (17.05%), soybean meal (4.65%), urea (1.13%), ammonium sulfate (0.11%), minerals (1.69%), salt (0.53%) and corn silage (fodder) (74.86%). These animals were evaluated for body condition score (1-5 scale, according to Wildman, 1982 and ovarian status by rectal palpation, as described by Madureira, 2004), and those with scores between 2.0 and 3.0 and with normal ovarian activity (CL presence and/or absence of follicles and cysts) were selected according to Fátima et al., (2013b).

All experimental procedures performed in this work were approved by the Ethics Committee for Animal Use of the School of Veterinary Medicine and Animal Science, University of São Paulo (protocol n ° 1637/2009).

Hormonal Treatment

For hormone treatment, females were randomly divided into three groups: control (n = 6), stimulated (n = 4) and superovulated (n = 6). This treatment consisted of the use of a vaginal implant containing 1g of progesterone (Bovine Intravaginal Device; progesterone: 1 g; Primer, Technopec, São Paulo, SP, Brazil) and 2 mg of IM estradiol benzoate (Estrogen Farmavet, São Paulo, SP, Brazil) administered to all animals on Day 0 of the protocol. For control and stimulated cows, the implants were removed and 0.15 mg of d-cloprostenol (PGF2α; Prolise; Arsa, Buenos Aires, Argentina) was administered on Day 8. In stimulated cows, 400 IU of eCG (Novormon; Syntex, Buenos Aires, Argentina) was also administered on Day 8. The control cows did not receive any eCG. At 48 h after removal of the implants, control and stimulated animals received 0.025 mg lecirelin (GnRH; Gestran Plus, Arsa). Superovulated cows were treated with 2000 IU eCG on day 4 and 0.15 mg of d-cloprostenol on Day 6. In this group, the implants were removed on day 7, then a second dose of 0.15 mg d-cloprostenol was administered at this time and after 12 h they received 0.025 mg of GnRH (Baruselli et al. 2011). According to Baruselli et al., (2012), differences regarding hormonal protocol for superovulated cows are based on achieving more synchronous ovulations. Seven days after GnRH administration, all the females were slaughtered, their ovaries were collected and then their corporal lutea (CL) was dissected.

Histological Processing

After dissection, four CL from each group were fixed in 10% (v/v) formaldehyde in a phosphate buffer solution of pH 7.2 (PBS) for 24 h and submitted to histological processing. We made cuts of 6 μm in micrometre (Leica RM 2125 RT, Wetzlar, Germany) and stained the sections with periodic acid-Schiff (PAS) (Sigma). This substance has been used to identify blood vessels because it reacts strongly with carbohydrates of the microvascular basal membrane, thereby facilitating the measurement of microvessel density (Ferreira-Dias et al., 2006).

Vascular Density

For assessment of vascular density, we obtained four histologic sections of CL from each animal (a total of 16 sections per group). We used a Nikon DXM1200 digital camera attached to an Olympus BX41 microscope with 400× magnification to obtain eight photomicrographs of distinct randomly selected regions from each section (a total of 128 micrographs per group). For quantification, a cycloid arcs type test system consisting of 35 arches and 70 test points was superimposed onto the images as previously described by Gaytan et al. (1999) with some modifications. Each arch had a total length (l/p) of 22 cm, field analysis was delimited by a test area (A_P) of 636 cm² and constituted by inclusion (upper and right edges) and exclusion (lower left margins) lines. We only counted the blood vessels (named profiles in the stereological method) located within the test area and did not touch the exclusion lines. Volume density (Vv) is the ratio of the CL volume occupied by blood vessels: Vv = ΣP/ΣP_CL, where ΣP is the number of test points which touch the vessels and ΣP_CL is the total number of test points on the CL. In these assessments, all vessels in the luteal tissue were also assessed without distinguishing their nature (arterioles, venules, and capillaries).

Scanning Electron Microscopy and Morphometric

The effect of eCG on the microvasculature was assessed by comparing the capillary plexus of two superovulated CL and another of two animals in the control group. To do this, the ovaries of four animals (n = 2 control and n = 2 superovulated group) had the ovarian artery cannulated along with its pedicle for the Merox resin injection (CL-2R, Okenshoji, Tokyo, Japan). To complete polymerization of the resin, the samples were maintained at 60°C for 3–4 h. Then, the corrosion of extravascular elements were performed by means of immersion in a 30% sodium hydroxide aqueous solution for 20 h in a warm oven at 37°C. We then soaked the dry molds in distilled water for 24 h to remove residual tissue, s and we glued them onto aluminum supports (stubs) for embedding in gold and
used a LEO 435 VP Scanning Electron Microscope for visualization. For differentiating arteries and veins, the criteria proposed by Macchiarelli (1995) were adopted regarding the negative impression orientation of the nuclei of endothelial cells: narrow/thin nuclei oriented lengthwise corresponding to arteries, and randomly-oriented rounded nuclei representing the veins. Button-shaped projections in the capillaries were considered indicative of angiogenesis and partially-filled capillaries representative of degeneration (Macchiarelli, 1995; Jiang et al., 2004). The numbers of angiogenic and degenerative structures were defined per area as previously described by Jiang et al. (2003) with some modifications. In all, eight different microscopic fields of the capillary plexus from each CL were evaluated. The diameter of ten capillaries was measured in the same microscopic fields. To perform these counts and measure capillary diameter, we used Image-Pro Plus® software, version 4.5.0.29 for Windows.

**Statistical Analysis**

We analyzed the vascular density for the normality of residuals using the Anderson–Darling test, transformed into base 10 logarithm when necessary and submitted to one-way ANOVA followed by Tukey or Kruskal–Wallis test. Student’s T test was used to compare differences in capillary diameter and the number of angiogenic and degenerative structures of the capillary plexus in control and superovulated group. P values $P \leq 0.05$ were considered to determine differences between means. The statistical analyses were performed using the GraphPad Prism software (version 4.00 for Windows; GraphPad Prism Software, San Diego, CA).

**RESULTS**

**Microscopy and Vascular Density**

The reaction of PAS with the endothelium allowed for the visualization of blood vessels with a circular profile, as well as those which have irregular morphology in the luteal tissue sections. Figure 1 shows the vessels with irregular morphology, in the intercellular spaces in animals stimulated with eCG. In superovulated CL, vessels with large diameter were observed in these intercellular spaces.

The vascular volume density (Vv) increased significantly in the stimulated and superovulated cows when compared to control ($P \leq 0.0001$). Nevertheless, vascular density was not different in the stimulated compared to the superovulated group (Fig. 2).
Microvasculature

The CL presented irrigation derived from ovarian cortical arteries and drainage directed to ovarian cortical veins. In the control group, a primary luteal artery was observed showing a spiral arrangement derived from the ovarian arterial component, which is evident on the surface of the CL (Fig. 3a). The angioarchitecture of the CLs accompanied the lobular structure of the gland, particularly in animals treated with eCG whose CL showed pronounced interlobular septa and a dense capillary network (Figs. 3a and 3b).

The luteal vascular plexus showed to be highly developed, comprised of numerous typical sinusoidal...
TABLE 1. Capillary diameter and number of angiogenic or degenerative structures per area of capillary plexus of CL in cows

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Supernovulated</th>
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<tbody>
<tr>
<td>Capillary diameter (μm)</td>
<td>11.10 ± 1.91*</td>
<td>25.52 ± 5.05*</td>
</tr>
<tr>
<td>Number of angiogenic structures (number per mm²)</td>
<td>2.6 ± 1.2*</td>
<td>22.2 ± 6.7*</td>
</tr>
<tr>
<td>Number of degenerative structures (number per mm²)</td>
<td>6.3 ± 2.9</td>
<td>5.4 ± 2.2</td>
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*Values with different superscripts are significantly different (P < 0.0001).

capillaries in the superovulated CL and control group (Figs. 3c–3d), although the diameter of these capillaries in the control group ranged between 8 and 15μm, whereas in the superovulated CL it was 18–35μm. We also observed numerous figures indicative of sprouting angiogenesis, especially in CL treated with eCG (Fig. 3f), whereas many degenerating capillaries in the CL control group (Fig. 3e) were found (Table 1).

DISCUSSION

The present study evaluated vascular changes of CL in cows for the first time, subsequent to i.m. administration of eCG at doses commonly used in stimulatory and superovulatory treatments of cattle. Based on the results of the density and vascular arrangement obtained by stereology and scanning electron microscopy, respectively, we found that the use of eCG was able to induce increased angiogenic activity in bovine CL. Other studies have demonstrated that gonadotropin also increased the formation of blood vessels in ovarian follicles of mice (Sato et al., 1982) and rats (Koos and LeMaire, 1983). In more recent studies, this eCG angiogenic effect was related to the ability to regulate VEGF expression in granulosa cells, which induced proliferation of endothelial and perivascular cells by a paracrine mechanism, allowing for the formation of new blood vessels in the follicular wall (Barboni et al., 2000; Jiang et al., 2004). Moreover, other research that assessed the effects of FSH in vitro buffalo cells showed results of increased expression of VEGF and FGF2 by granulosa cells (Fátima et al., 2013b). This increased microvasculature of CL treated with eCG in the present study may be explained by similar mechanisms, such as other gonadotropins with increased expression of VEGF in luteal cells (Christenson and Stouffer, 1997; Laitinen et al., 1997; Wulff et al., 2001; Fatima et al., 2011). The use of eCG has resulted in improved gestational success for fixed time embryo transfer, independent of the protocol employed for synchronization (Bo et al., 2011). These satisfactory results can be associated with higher progesterone plasma (P4) found in these animals (Soumano et al., 1998). P4 is essential to ensure an adequate uterine environment for growth, implantation, placentation and development of the fetus to term (Spencer et al., 2008). This increase in the synthesis of P4 has been explained by the greater volume of CL changes in the number of large luteal cells (Ferreira et al., 2013; Rostami et al., 2011), and more recently the changes in the number of spherical mitochondria, as well as mitochondria volume pro-
However, the bovine luteal tissue, treated with eCG, was not increase in mRNA expression of VEGF and FGF2 assessed by quantitative real-time PCR (Fatima et al., 2012). Therefore, this angiogenic effect of eCG in bovine CL needs to be further investigated.

In addition to the blood supply, microvascular permeability also influences the exchange of substances between cells and the ovarian vascular compartment (Mitsube et al., 2013). In vascular molds analyzed by SEM, we observed that capillaries were more dilated in CL treated with eCG, and a similar effect was also demonstrated with the use of hCG in the CL of rabbits (Macchiarelli, 1995). This action of eCG must also be associated with an increased expression of angiogenesis factors, as Ang-2 and VEGF are known for their ability to induce increased vascular permeability (Reisinger et al., 2007; Tuominen et al., 2014).

The morphometric analysis of vascular casts showed a slight reduction in the number of capillaries in the degeneration in treated CL. This result may indicate that eCG also influences the apoptosis of endothelial cells in the CL of cows. These cells are the first to undergo programmed cell death (PCD) during luteolysis (Friedman et al., 2000). There are reports showing that endothelial cells from bovine CL express tumor necrosis factor (TNFα) and its type 1 and type 2 (TNFR1 and TNFR2) receptors, and the decline of progesterone preceding the structural luteolysis is a prerequisite for apoptosis initiation in these cells induced by TNFα via TNFR1 (Friedman et al., 2000; Petroff et al., 1999; Zhao et al., 1998). According to Peluffo et al. (2009), LH decreases the expression of members of the TNFR family in CL mediated by high levels of progestins, and therefore reduces the PCD of the endothelial cells. Therefore, our morphological findings indicate that eCG may act through a similar mechanism of inhibition of capillary degeneration, but its effect on these cytoxines needs to be confirmed by molecular assays.

In summary, the treatment with eCG increased the number of figures indicative of angiogenesis and vascular density in cows’ CL. These morphological findings are evidence of an angiogenic effect of gonadotropin in the luteal parenchyma.

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