



## Early development and function of the corpus luteum and relationship to pregnancy in the buffalo



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### ABSTRACT

A detailed study on the structure and function of the CL in the Day-5 to Day-10 window of development, and relationship to the likelihood of pregnancy, was undertaken in Italian Mediterranean buffaloes. In experiment 1, buffaloes underwent synchronization of estrus and fixed-time artificial insemination ( $n = 23$ ). Features of the CL were measured from Days 5 to 10 after fixed-time artificial insemination, and pregnancy was confirmed on Day 70. Buffaloes that established a pregnancy ( $n = 14$ ) had a larger CL area ( $1.31 \pm 0.1$  vs.  $1.09 \pm 0.1$  cm<sup>2</sup>;  $P < 0.01$ ) and greater progesterone ( $P_4$ ) concentrations ( $1.90 \pm 0.1$  vs.  $1.48 \pm 0.1$  ng/mL;  $P < 0.01$ ) during Days 5 to 10 compared with nonpregnant buffaloes. In the same period, blood flow measured as time average medium velocity tended to be greater ( $P = 0.059$ ) in buffaloes that were subsequently pregnant versus nonpregnant buffaloes ( $10.8 \pm 0.8$  vs.  $8.4 \pm 0.9$ ). There was a relationship ( $R^2 = 0.136$ ;  $P < 0.05$ ) between CL area,  $P_4$ , and time average medium velocity from Days 5 to 10. Logistic regression analysis showed that  $P_4$  concentration on Day 10 had a significant influence on pregnancy (odds ratio, 19.337;  $P < 0.01$ ). In experiment 2, highly vascularized CLs (HVCLs,  $n = 3$ ) and lowly vascularized CLs (LVCLs,  $n = 3$ ) on Day 5 were examined by contrast-enhanced ultrasonography and then subjected to histologic investigation. Blood flow was greater in HVCLs than in LVCLs. Highly vascularized CLs showed intense staining for factor VIII and had many small, irregular-shaped blood vessels, whereas LVCLs had low factor VIII staining and relatively few large, regular-shaped vessels. Luteal cell expression of vascular EGF was greater for HVCLs compared with LVCLs. The study has shown that greater development and function of the CL from as early as Day 5 is related to an increased likelihood of pregnancy in the buffalo. Corpus lutea that show early development at Day 5 have greater expression of vascular EGF and factor VIII, increased vascularization, and higher blood flow.

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### 1. Introduction

The CL has an important role in early embryonic development and pregnancy [1–3]. Buffaloes that established a pregnancy had a faster rate of CL growth from Days

5 to 10 (measured as the difference between Day 10 and Day 5) after artificial insemination (AI), and they showed an earlier rise in circulating concentrations of progesterone ( $P_4$ ) compared with buffaloes that did not establish a pregnancy [4]. Studies in cattle demonstrated that an early rise in blood  $P_4$  was associated with embryonic growth and elongation [5,6]. It was also associated with uterine endometrial gene expression [7,8] related to nutrient-sensing

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pathways, growth factors, and extracellular molecules [9] and immune-modulating factors [10].

Pregnancy in buffaloes was associated with greater blood flow to the CL on Days 10, 20, and 25 after AI [4,11]. There is evidence to suggest that greater activity of the CL in buffaloes that establish a pregnancy is related to increased angiogenesis [12]. In cattle, vascularization of the CL and blood flow is closely linked with P<sub>4</sub> synthesis and release [13,14]. Similar relationships between vascular density and P<sub>4</sub> synthesis were observed in buffaloes at the mid- and late-luteal phases [15,16]. In a more recent study in buffaloes, the expression of vascular endothelial growth factor (VEGF) by the CL varied during the estrous cycle and was related to circulating concentrations of P<sub>4</sub> [17]. This was similar to findings in cattle [18]. The normal role for VEGF in the developing CL is to act as a mitogen at endothelial cells to induce vascular permeability and stimulate angiogenesis [19,20].

There has not been a systematic and detailed study on the structure and function of the CL in the Day-5 to Day-10 window of development and the relationship to pregnancy outcome in buffalo. The aim of the present study was to define when, in the Day-5 to Day-10 window of CL development, differences emerge between animals with regard to VEGF and factor VIII expression, angiogenesis, blood flow, and P<sub>4</sub> secretion. Differences between animals in these CL parameters were related to the likelihood of pregnancy in buffaloes. In experiment 1, CL growth, P<sub>4</sub> secretion, and blood flow kinetics were monitored from Days 5 to 10 after AI in buffaloes that subsequently established a pregnancy and those that did not establish a pregnancy. In experiment 2, CLs with either high blood flow or low blood flow on Day 5 were compared for VEGF and factor VIII expression, vascularization, and blood flow kinetics using color Doppler and contrast-enhanced ultrasound. The study would contribute new information on the biology of early CL development in the buffalo, and it would also help to inform the development of more targeted and precise strategies to enhance CL function and increase pregnancy.

## 2. Materials and methods

### 2.1. Experiment 1

#### 2.1.1. Animals

The experiment was conducted in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes and was approved by the Animal Ethics Committee of the University of Naples, Federico II (Permit Number: 2013/010858). The experiment used 30 multiparous, nonsuckled Italian Mediterranean buffalo cows at 145 ± 7 days in milk (latitude: 40.5°N–41.5°N parallel). The animals were selected from a larger group of buffaloes by clinical examination that included (1) rectal palpation of the ovaries for follicular development (follicle ≥ 1.0 cm), (2) the presence of a CL to confirm cyclicity, and (3) the absence of gross abnormalities of the reproductive tract such as uterine fluid. The buffaloes were maintained in open yards that allowed 15 m<sup>2</sup> for each animal. A total mixed ration consisting of 50% to 55% forage and 45% to 50% concentrate, containing 0.90 milk forage units/kg of dry

matter and 15% crude protein/dry matter, was fed daily in a group pen situation.

#### 2.1.2. Estrus synchronization and AI

Stage of the estrous cycle was synchronized using the Ovsynch protocol with fixed-time AI (TAI) that was developed in cattle [21] and previously used in buffaloes [22,23]. Briefly, a GnRH agonist (buserelin acetate, 12 µg; Receptal, Intervet) was administered on Day 0, a PGF<sub>2α</sub> analog (luprostiol, 15 mg; prosolvlin, Intervet) on Day 7, and GnRH agonist (12 µg) again on Day 9. Cows were mated using TAI by the same operator at 20 hours after the second injection of GnRH. Because of the relatively low intensity of estrus in buffaloes [24], animals were palpated per rectum (immediately before TAI) to assess estrous status (follicle ≥ 1.0 cm and a tonic uterus with the presence or absence of mucous vaginal discharge).

#### 2.1.3. Corpus luteum development and blood flow

Ovarian ultrasonography examinations were performed using a portable SonoAce PICO Ultrasound unit (Medison, Seoul, South Korea) equipped with a 10-MHz linear transducer adapted for transrectal examination in large animals. Characteristics of the CL (size and blood flow parameters) were examined daily from Days 5 to 10 after TAI. Once the ovary was visualized, the image was adjusted to give an optimal definition of the CL and then frozen to measure the long and short axes. The color Doppler mode was then activated to display signals for blood flow in the CL, and the spectral mode was applied to calculate the resistive index (RI), pulsatile index (PI), and time average medium velocity (TAMV). All Doppler scans were performed at a constant color gain setting, velocity setting, and a color-flow filter setting. The entire CL was scanned in a slow continuous motion. Real-time B-mode/color Doppler images of the continuous scans were recorded with a digital video-recording system for subsequent analysis.

#### 2.1.4. Progesterone

The function of the CL was evaluated by measuring circulating concentrations of P<sub>4</sub> by RIA from Days 5 to 10 after TAI [25,26]. Blood samples obtained from the jugular vein were centrifuged at ×800 g for 15 minutes, and the serum was stored at –20 °C until analyzed for P<sub>4</sub>. The minimum detectable amount of P<sub>4</sub> was 2.1 ± 0.08 pg and the intra-assay and interassay coefficients of variation were 6.2% and 11.8%, respectively.

#### 2.1.5. Embryonic development and pregnancy

Buffaloes were assessed for embryonic development by ultrasonography on Day 45 after TAI, and pregnancies were confirmed on Day 70 by rectal palpation.

## 2.2. Experiment 2

#### 2.2.1. Blood flow kinetics

Stage of the estrous cycle was synchronized in Italian Mediterranean buffalo cows (n = 20) as described in experiment 1. On Day 5 of the synchronized cycle, three buffaloes with a highly vascularized CL (HVCL) and three buffaloes with a lowly vascularized CL (LVCL) were selected

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