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Expression of calbindin-D9k and vitamin D receptor in the uterus of Egyptian buffalo during follicular and luteal phases☆

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ABSTRACT

Uteri of mature Egyptian buffalo cows (5–10 years old) were collected at follicular ($n = 12$) and luteal ($n = 16$) phases of estrous cycle to investigate the expression of calbindin-D9k (CaPB-9k) and vitamin D receptor (VDR). This study was done using avidin-biotin immunohistochemistry method. In addition, blood levels of calcium (Ca), vitamin D3 (Vit D), estrogen (E2) and progesterone (P4) were measured. The immunohistochemical findings restricted the expressions of CaBP-9k and VDR to the luminal and glandular epithelia of the endometrium implicating the importance of CaBP-9K and VDR in the function of endometrial epithelium, especially the glandular one, in order to prepare a receptive uterus. On the other hand, the myometrium did not express CaBP-9k or VDR that denies the potential role of CaBP-9k and VDR in the uterine contractility during the estrous cycle of Egyptian buffalo. All of Ca, Vit D, and P4 blood levels significantly ($P < 0.05$) increased during luteal phase however, blood level of E2 significantly ($P < 0.05$) increased during follicular phase. The expressions of CaBP-9k and VDR in the uterus of Egyptian buffalo were significantly ($P < 0.05$) higher during luteal (P4 dominant) phase than during the follicular (E2 dominant) phase indicating that P4 up-regulates the expressions of CaBP-9k and VDR. In view of these observations, this study represents the first characterization of CaBP-9K and VDR expression in the uterus of Egyptian buffalo and suggests the pivotal role of CaBP-9k and VDR in the uterine receptivity. Furthermore, it demonstrates the regulatory role of P4 for expressions of CaBP-9k and VDR in buffalo uterus.

1. Introduction

Establishment of receptive endometrium is essential for embryo implantation (Nie et al., 2000). It is well known that the function of reproductive organs and its musculature is depending on influx of extracellular calcium or the release of intracellular stores. The calcium binding protein, calbindin-D9K (CaBP-9K) is first known as a vitamin D dependent which is involved in maintaining calcium balance (Christakos et al., 1989). Many investigations reported the expression of CaBP-9k in the female reproductive systems including uterus (Mathieu et al., 1989; An et al., 2003). It can be hypothesized that uterine CaBP-9k may be involved in controlling myometrial activity that may be under the control of vitamin D3 and sex steroid hormones.

However, the primary role of vitamin D3 is to regulate calcium and phosphorus metabolism, recently vitamin D receptors (VDR) have been demonstrated in the uterus (Zarnani et al., 2010; Shahbazi et al., 2011) implicating the importance of vitamin D3 in the female reproduction (Johnson et al., 1996; Reichrath et al., 1998; Kinuta et al., 2000).

To our knowledge, expression of CaBP-9K and VDR in some reproductive organs are well established for rat, mice and cattle but, it is not established for buffalo yet. In Egypt, buffaloes are being reared for meat and milk production (Arefaine and Kashwa, 2015). The age of buffalo heifers at the first estrus varies between breeds from 13 to 33 months. Although buffalo is polyestrous, its reproductive efficiency shows wide variation throughout the year (Barile, 2005).

Therefore, this study expands the investigation of these proteins to include the buffalo's uterus especially during follicular and luteal phases of the estrous cycle. In addition, blood levels of calcium (Ca), vitamin D3 (Vit D), estrogen (E2) and progesterone (P4) were measured during both phases. Overall, this study represents the first characterization of CaBP-9K and VDR expression in the uterus of Egyptian buffalo. We hypothesize involvement of CaBP-9k and/or VDR in the uterine contractility in addition to that the expression of

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CaBP-9k may be under the control of vitamin D and/or sex steroid hormones.

2. Materials and methods

2.1. Animals

A total of 28 non-pregnant, mature, clinically healthy Egyptian buffalo cows (5–10 years old) were slaughtered at a local abattoir, Kalubyia Province, Egypt during winter and spring of 2015. The slaughtering was carried out according to guidelines of animal care committee in Benha University. The ages of animals were determined by their dentition according to Kartha (1975). All animals were examined before slaughtering and the findings on the ovaries were recorded however after slaughtering, the changes in gross appearance of the follicles and corpus luteum on the ovaries determined whether the animals were in the follicular phase ($n = 12$) or luteal phase ($n = 16$).

2.2. Tissue specimens and preparation

A license for animal tissue use was obtained from the director of abattoir who is considered as a representative of veterinary medicine organization in Egypt. Uteri of the 28 Egyptian buffalo cows were collected at the abattoir within 20 min from slaughtering. The uteri were examined to be free from any abnormal signs and embryos. Cross sections (1 cm thick for each) from the mid uterine horns of all animals were cut and collected. The specimens were fixed in 10% neutral formalin for 48 h. They were dehydrated in alcohols, cleared in xylene, and embedded in paraffin. Sections of 5 μm thick were cut and collected on positive charged slides for immunohistochemistry examination.

2.3. Blood collection and measurements

Blood samples were collected, in plain vacutainer tubes, from jugular vein of the animals immediately before slaughtering. The blood samples were kept in an ice box and transported within 30 min to the laboratory. After centrifugation, the separated sera were stored in deep freeze (−20 °C) till analysis. The sera were biochemically analyzed to determine levels of Ca, vit D, P4 and E2 within 10 days from the blood collection. The determination of serum Ca level was done as described by Gindler and King (1972) using colorimetric assay of spectrophotometer at wavelength 575 nm. The linear range of detection for this kit was between 0.4–2.0 mg and the serum sample size was 5 μl. However, the serum levels of vit D, E2, and P4 were done using competitive ELISA as described by Makin and Gower (2010), Hall (1988) and Hubl et al. (1982) respectively. The used serum sample size was 50 μl for all, meanwhile the sensitivity for vit D, E2, and P4 was 0.5 ng/ml, 8.68 pg/ml, and 0.05 ng/ml respectively. The intensity of the produced color was determined using spectrophotometer at wavelength 412 nm for vit D and wavelength 450 nm for both E2 and P4.

2.4. Immunohistochemistry

To detect the presence and distribution of CaBP-9k and VDR in the uteri of Egyptian buffalo, the avidin-biotin immunoperoxidase technique (Vectastain®ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) was used. After deparaffinization in xylene and rehydration in ethanol gradient, sections were heated with citrate buffer pH 6.0 (Dako Cytomation, Carpinteria, CA, USA) in a steamer for 40 min. Sections were then treated with 3% H202 in absolute methanol for 20 min. Then, sections were treated with 10% normal goat serum for 30 min. Sections were incubated overnight at 4 °C with the primary antibodies of dilution 1:150. Primary antibodies were rabbit polyclonal anti-CaBP-9k (catalogue number, sc-28532) and anti-VDR (catalogue number, sc-9164) from Santa Cruz Biotech., CA, USA. Both antibodies are recommended for detection of CaBP-9k and VDR in bovine according to their data sheets. For colorization, sections were incubated with diaminobenzidine-HCI for 5 min. As immuno-negative controls, sections were incubated with rabbit non-immune serum instead of the primary antibody.

Table 1
Summary of CaBP-9k and VDR immunostainings in the uterus of Egyptian buffalo during the follicular and luteal phases.

	CaBP-9k		VDR	
	FP	LP	FP	LP
Luminal epithelium	–	+	–	+
Superficial gland	+	++	–	++
Deep gland	++	+++	+	+++
Stroma	–	–	–	–
Myometrium	–	–	–	–

FP, follicular phase; LP, luteal phase; –, negative; +, slightly positive; ++, moderately positive; +++, strongly positive.

2.5. Qualitative and quantitative evaluation of immunostainings

The intensity of the immunostainings was scored according to Spencer and Bazer (1995). Intensities were classified as follows: negative (−) when the cells had no any detectable immunostaining, weak (+), moderate (++), and strong (+++). In order to quantify the intensity of the immunostainings, digital color images were obtained using light microscope Leica DM3000 LED with Leica Application Suite Version 4.0 (LAS V4.0). The immunohistochemical images were captured using 40× objective. Image analyses were performed using the public domain Image J software 7. The immunostaining intensity was expressed as relative optical density (ROD) of diaminobenzidine brown reaction product as described by Smolen (1990). It was calculated using the following formula:

$$\mathrm{ROD} = \frac{\mathrm{ODspecimen}}{\mathrm{ODbackground}} = \frac{\log(\mathrm{GLblank}/\mathrm{GLspecimen})}{\log(\mathrm{GLblank}/\mathrm{GLback})}$$

where GL = Gy level, respectively, for specimen (stained area), back (background) and blank (gray level measured after the slide was removed from the light path). A total of 36 various images were used for image analysis. These images were obtained from 36 different microscopic fields of 12 stained uterine sections (3 different sections/antibody/phase).

2.6. Statistical analysis

The data, during both follicular and luteal phases, were statistically analyzed using student's *t*-test. Results are expressed as the mean ± standard deviation (SD). *P*-value of <0.05 was considered significant.

3. Results

3.1. CaBP-9k

The specificity of all immunoreactivities in the uteri was confirmed by negative control sections. Immunostaining of CaBP-9K in the uteri of buffalo cows during both follicular and luteal phases were generally viewed in (Fig. 1A, B) and summarized in Table 1. Immunoreactive CaBP-9k was detected predominantly in the cytoplasm of positive cells and often in the nuclei. Some cells of the