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Expression and localization of ghrelin and its functional receptor in corpus luteum during different stages of estrous cycle and the modulatory role of ghrelin on progesterone production in cultured luteal cells in buffalo

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ABSTRACT

Evidence obtained during recent years provided has insight into the regulation of corpus luteum (CL) development, function, and regression by locally produced ghrelin. The present study was carried out to evaluate the expression and localization of ghrelin and its receptor (GHS-R1a) in bubaline CL during different stages of the estrous cycle and investigate the role of ghrelin on progesterone (P4) production along with *messenger RNA* (*mRNA*) expression of P4 synthesis intermediates. The mRNA and protein expression of ghrelin and GHS-R1a was significantly greater in mid- and late luteal phases. Both factors were localized in luteal cells, exclusively in the cytoplasm. Immunoreactivity of ghrelin and GHS-R1a was greater during mid- and late luteal phases. Luteal cells were cultured in vitro and treated with ghrelin each at 1, 10, and 100 ng/mL concentrations for 48 h after obtaining 75% to 80% confluence. At a dose of 1 ng/mL, there was no significant difference in P4 secretion between control and treatment group. At 10 and 100 ng/mL, there was a decrease ($P < 0.05$) in P4 concentration, cytochrome P45011A1 (CYP11A1), and 3-beta-hydroxysteroid dehydrogenase *mRNA* expression and localization. There was no difference in *mRNA* expression of steroidogenic acute regulatory protein between control and treatment group. In summary, the present study provided evidence that ghrelin and its receptor are expressed in bubaline CL and are localized exclusively in the cell cytoplasm and ghrelin has an inhibitory effect on P4 production in buffalo.

1. Introduction

Buffalo (*Bubalus bubalis*) is an important livestock species, concentrated mostly in the tropical and subtropical regions of the world. Asian buffaloes, which represent 97.2% of the world's buffalo, lead the worldwide population of 189.8 million [1]. In India, the population of buffalo is half compared with the cattle population but contributes 55% of the total milk production (62.8 MT in 2010) [1]. A large number of buffaloes (30%–40%) remain unproductive as the result of reproductive problems such as subestrus, anestrus, and infertility, all of which have hormonal etiologies [2,3], incurring an estimated loss of 19 to 20 million tons of milk each year. Infertility may result from poor development and

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incomplete vascularization of the corpus luteum (CL), leading to a deranged luteal function. A thorough understanding of the endocrine, autocrine, and paracrine factors that regulate luteal development and function would be helpful in elucidating the mechanisms underlying luteal insufficiency, thereby facilitating development of approaches for alleviation of infertility resulting from luteal dysfunction.

Reproductive cycles in mammals represent a process of chronological proliferation, differentiation, and transformation of ovarian follicular cells followed by formation and regression of the CL in a cyclic manner. The CL is a remarkable, transiently functioning organ and plays a central role in the regulation of the estrous cycle and in the maintenance of pregnancy. This function is carried out largely by progesterone (P4), which is the main steroid hormone synthesized by this gland [4]. This process is well regulated by pituitary gonadotropins. However, recent reports also provide evidence of an essential modulatory role on luteal dynamics by locally produced factors [5].

Data obtained in recent years have demonstrated a close relationship between ghrelin and reproductive functions in females. Ghrelin is a novel growth hormone–releasing acylated peptide, isolated from the stomach. It is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a), a 7-transmembrane G protein–coupled receptor [6]. Activity of ghrelin is dependent on a unique n-octanoylation at Ser-3 [6]. Acylation of ghrelin is necessary for binding and activating the GHS-R1a [7]. A striking feature of ghrelin is its widespread pattern of expression [8,9]. Expression of ghrelin has been demonstrated in an array of tissues and cell types, including the stomach, small intestine, pancreas, lymphocytes, placenta, kidney, lung, pituitary, and brain [9]. Although the general view has been that the principal effects of ghrelin are on the neuroendocrine component of reproduction [10–13], evidence has emerged to indicate direct involvement of ghrelin in ovarian function. Expression of ghrelin and its cognate receptor has been demonstrated in the ovary of human [14], rat [15], sheep [16], goat [17], pig [18], and cattle [19]. In human and pigs, it is reported that expression of ghrelin and its active receptor GHS-R1a paralleled follicular development [14,20]. In pig, sheep, and goat ovaries, it has been demonstrated that ghrelin and its receptor expression is estrous-cycle dependent, with maximal expression during the development of the CL [16–18]. Plasma ghrelin concentration ranges from 100 to 150 pg/mL in fed cattle to 600 to 1,000 pg/mL in fasted cows [21], whereas ovarian follicular fluid (FF) ghrelin concentrations vary from 109 to 148 pg/mL [20].

Several studies have shown that ghrelin plays a crucial role in steroidogenesis. Key factors involved in P4 synthesis in the CL of various species include steroidogenic acute regulatory protein (StAR), cholesterol side chain cleavage enzyme complex P450scc or cytochrome P45011A1 (CYP11A1), and 3-beta-hydroxysteroid dehydrogenase (3β-HSD) [22–24]. Both stimulatory and inhibitory effects of ghrelin on the secretion of P4 by cultured rat, porcine, rabbit, human, and chicken ovarian cells have been reported [18,25,26], although such actions have not been found consistently [27,28]. Given their role in controlling ovarian function, ghrelin and its receptor are hypothesized to be involved in CL formation, function, development, and steroidogenesis during the estrous cycle in an autocrine/paracrine manner in buffalo. To test this hypothesis, we evaluated (a) *messenger RNA* (*mRNA*) and protein expression and localization of ghrelin and its receptor in bubaline CL during different stages of estrous cycle and (b) the in vitro effects of ghrelin on P4 secretion, StAR, CYP11A1, and 3β-HSD *mRNA* expression and localization in luteal cell culture.

2. Materials and methods

2.1. Collection of CL during the estrous cycle

Entire reproductive tracts from buffalo cows were collected at a local slaughterhouse within 10 to 20 min after slaughter and were transported on ice to the laboratory. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (color, consistency, CL stage, number, and size of follicles) and the uterus (color, consistency, and mucus) as described previously [29]. Forty ovaries, each with CL, were used to extract 10 CL per group for RNA extraction, Western blotting, and immunohistochemistry studies. The CL was assigned to the following stages: early luteal phase (days 1–4), mid-luteal phase (days 5–10), late luteal phase (days 11–16), and regressed CL (days >17) of estrous cycle. Luteal tissue was frozen in liquid nitrogen and stored at −80°C until RNA and protein isolation.

2.2. Collection of follicles during final follicular growth

Only follicles ($n = 10$) which appeared healthy (ie, well vascularized and having transparent follicular wall and fluid) and whose diameters were >13 mm were used. Large follicles (>13 mm) were collected only after CL regression, with signs of mucus production in the uterus and cervix and were assumed to be preovulatory. For RNA extraction, follicles were dissected from the ovary. The surrounding tissue (theca externa) was removed with forceps under a stereomicroscope as previously described [29]. FF aspirated from follicles was stored at −20°C until determination of P4. As healthy follicles have relatively constant P4 levels in FF, only follicles with P4 <100 ng/mL FF were used for the evaluation to exclude atretic follicles [29]. Follicles were snap-frozen in liquid nitrogen and stored at −80°C until RNA and protein isolation. The preovulatory follicles (PF) were used as a control/calibrator in the quantitative real-time–polymerase chain reaction (qRT-PCR) experiments.

2.3. Hormone determination

Concentrations of P4, determined in the FF and in the spent media of luteal cell culture, were estimated by P4^{125}I RIA kit (IM1188) supplied by Immunotech, Czech Republic as per manufacturer's instruction. The FF was diluted 1:5 with phosphate-buffered saline (PBS). The measurable range was 0.05 to 50 ng/mL. The intra- and interassay coefficients of variation were 6.5% and 7.2%, respectively.

2.4. Primers

A primer for ghrelin was designed using the Fast PCR (Version: 6.2.73) software. Published primers were used for