Quantitative analyses of insulin-like peptide 3 and sex steroid hormones in dominant follicles and corpora lutea during the estrus cycle and in follicular cysts in beef heifers

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Abstract. Insulin-like peptide 3 (INSL3) and sex steroids were measured in bovine dominant follicles and corpora lutea during the estrus cycle and in follicular cysts. Paired ovaries from beef heifers (n = 47) were classified, by their morphological features, either into four stages of the estrus cycle (Day 1 = day of ovulation, Day 20 = day of estrus) as Stage I (Days 1–4; n = 8), Stage II (Days 5–10; n = 10), Stage III (Days 11–17; n = 8), and Stage IV (Days 18–20; n = 11) or follicular cystic (n = 10). Cysts (n = 15) were subdivided into estrogen-active (n = 7) and estrogen-inactive (n = 8) cysts. INSL3, testosterone, and estradiol-17β concentrations in the dominant follicular fluid of Stage IV were higher than those in Stages II and III (P < 0.05). INSL3 concentrations in the cystic fluid were similar to those in dominant follicles at Stage IV, whereas testosterone and estradiol-17β concentrations were lower in cysts (P < 0.05). INSL3 content per estrogen-inactive cyst was higher than that of Stage IV (P < 0.05). INSL3 and progesterone concentrations in luteal tissue and contents per corpus luteum were higher in Stages II and III (P < 0.05). In conclusion, INSL3 secretion in bovine dominant follicles increased with maturation. Follicular cysts may retain the production of INSL3 during their formation but tend to lose the capacity for testosterone secretion. Estrogen-inactive cysts subjected to advanced atresia may accumulate more INSL3. INSL3 production in bovine corpora lutea is enhanced during maturation.

Key words: Beef heifers, Corpus luteum, Dominant follicle, Follicular cyst, Insulin-like peptide 3

Insulin-like peptide 3 (INSL3), also known as a relaxin-like factor, is exclusively produced in testicular Leydig cells in males [1–3]. INSL3 plays a role in transabdominal testicular descent in fetal mice [4, 5] and spermatogenesis in sexually mature rats and boars [6, 7]. INSL3 is also expressed in the theca interna cells of antral follicles and luteal tissues in female mammals, including humans [8, 9] and cattle [10, 11]. The INSL3 receptor was identified as relaxin family peptide receptor 2 (RXFP2; formerly known as LGR8), a G protein-coupled receptor [12]. Previous studies have demonstrated impaired fertility associated with a prolonged estrus cycle [4] or higher rates of follicular atresia in INSL3-deficient female mice [13], suggesting that INSL3 plays a role in maintaining normal estrus cycles and fertility. This peptide is also involved in maturation of oocytes in rats [6].

INSL3 is considered a hormone owing to its ability to circulate in the peripheral blood of men [14], which was later confirmed in various male mammals [15–20]. In contrast, INSL3 concentrations in the blood are extremely low in women [14] and female cattle compared to males [21, 22]. Thus, INSL3 secretory changes during the estrus cycle require are yet to be explored. Notably, no previous work attempted to quantify INSL3 concentrations in bovine dominant follicles and corpus luteum during the estrus cycle.

Ovarian follicular cysts are a significant cause of reproductive failure in cattle. They are formed when mature follicles fail to ovulate and enlarge abnormally [23]. Owing to their frequent occurrence and prolonged intervals from calving to conception, bovine follicular cysts cause significant economic losses [23]. Follicular cysts are likely the result of reduced responsiveness of the hypothalamus and/or pituitary gland(s) caused by a deficiency of positive feedback of estrogen, consequently leading to a lack of luteinizing hormone (LH) surge [24]. Concentrations of steroid hormones such as testosterone, estradiol-1β, progesterone, and some peptide hormones have been reported in the follicular fluids of cows with ovarian cysts [25–27]. In women with polycystic ovarian syndrome (PCOS), blood INSL3 concentrations are higher than those in women with normal menstrual cycles [28, 29]. INSL3 is thought to play a role in stimulating folliculogenesis in women with PCOS [29]; however, no information is available regarding the ovarian secretion of INSL3 in cattle with follicular cysts. In the present work, quantitative analyses of INSL3 peptide and sex steroid hormones were performed in dominant follicles and corpora lutea during the estrus cycle and in follicular cysts in beef heifers. The goal was to explore the possible roles of INSL3 in the turnover of dominant follicles and follicular maturation during the bovine estrus cycle, follicular cyst formation, and luteal development and regression.

Materials and Methods

Bovine ovaries

Paired ovaries were collected from crossbred beef heifers (F1 between Japanese Black beef and Holstein cattle; n = 47) in a local abattoir. The samples were transported to the laboratory in an insulated box with a cooling agent. Paired ovaries were classified into four
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stages of the estrus cycle as follows: (Day 1 = day of ovulation, Day 20 = day of estrus) as Stage I (Days 1–4; n = 8), Stage II (Days 5–10; n = 10), Stage III (Days 11–17; n = 8), and Stage IV (Days 18–20; n = 11), based on the morphological features of the ovaries by the method previously described by Ireland et al. [30]. When the paired ovaries have a cyst or cysts > 20 mm diameter and no corpora lutea, the heifers were classified as follicular cysts (n = 10) [23–25, 31]. Among the collected samples, three heifers had single cysts, and the other seven had coexisting cyst(s) > 20 mm in diameter or large follicle(s) > 10 mm (Supplementary Table 1). Follicular cysts were considered non-luteinized by visual inspection and did not include any luteal tissue in the cyst wall [32]. The non-luteinization of the cysts was further confirmed by the ratio of estradiol-17β/progesterone concentration in the cystic fluid, according to previous studies [26, 33–37]. Follicular cysts (n = 15) were subdivided into estrogen-active (n = 7) and estrogen-inactive (n = 8) cysts according to the ratio of estradiol-17β to progesterone concentration in follicular fluid (estrogen-active > 1, estrogen-inactive < 1), as previously described [27].

Dominant follicles, cysts, and corpora lutea were excised from the ovaries. The diameters of the follicles and cysts were measured in two directions using a Vernier caliper, and their average was used as the diameter. Follicular fluid was aspirated from the dominant follicles and cysts using a 10-ml syringe attached to an 18-G needle. The isolated corpus luteum was weighed, and its central part (approximately 0.3 g) was excised. Follicular fluid and luteal tissue were measured using a Polytron PT-10 (Kinematica AG, Littau/Luzern, Switzerland) until the tissue fragments became invisible. The tissue was homogenized using a Polytron PT-10 (Kinematica AG, Littau/Luzern, Switzerland) until the tissue fragments became invisible. The homogenate was then centrifuged at 1,300 × g for 10 min at 4°C, after which the supernatant was centrifuged at 12,000 × g for 10 min at 4°C. The resultant supernatant was filtered through a cotton-plugged funnel, and the filtrate was stored at –30°C until further analysis.

Hormone analysis

INSL3 assay: INSL3 concentrations were measured using a time-resolved fluorescence immunoassay (TRFIA) [38–40]. Briefly, microtitration plates (1244-550, PerkinElmer, Boston, MA, USA) were coated with 100 µl of anti-mouse IgG goat polyclonal antibody (01-18-06, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) per well for 2 h at room temperature. The wells were washed thrice with 300 µl of 0.15 M sodium chloride. Approximately 150 µl of Delfia assay buffer (1244-111, PerkinElmer) was then added, and the plates were incubated at 4°C overnight. Immediately before the assay, the wells were drained, to which either 50 µl of synthetic bovine INSL3 [41] for the standards or 50 µl of diluted follicular fluid plus 50 µl of anti-bovine INSL3 mouse monoclonal antibody (2-8F) [41] was added to each well and incubated for 2 h. Subsequently, 50 µl of biotin-labeled canine INSL3 [17] was added, and the plates were incubated for 1 h at room temperature. The wells were then washed thrice with 300 µl TRFIA wash buffer (0.05 M Tris-HCl buffer containing 0.15 M sodium chloride and 0.05% Tween 20, pH 7.8), after which 100 µl of Eu-labelled streptavidin (1244-360, PerkinElmer) was added. The plates were then incubated for 30 min at room temperature. After washing thrice with 300 µl of wash buffer, 100 µl of enhancement solution (1244-105, PerkinElmer) was dispensed into each well, followed by shaking at 500 rpm for 15 min at room temperature. Finally, time-resolved fluorescence was measured using an ARVO X multilabel counter (PerkinElmer). The minimum detection limit of INSL3 TRFIA was 0.15 ng/ml. The intra- and inter-assay coefficients of variation (CVs) for follicular fluid samples were 5.6% (n = 4) and 10.2% (n = 12), respectively.

Testosterone assay: Follicular testosterone concentrations were measured using an enzyme immunoassay (EIA), which was established in the laboratory [22]. Eight-well strips (2580; Corning Inc. Life Sciences, Lowell, MA, USA) were coated with 100 µl of anti-rabbit IgG goat polyclonal antibody (5210-0174, Kirkegaard & Perry Laboratories) per well for 2 h at room temperature. The wells were then washed thrice with 300 µl of 0.15 M sodium chloride and 200 µl of assay buffer (0.01 M phosphate buffer containing 0.15 M sodium chloride, pH 7.4) supplemented with 0.1% bovine serum albumin (A7888-50G, Fraction V, Sigma-Aldrich). Subsequently, 0.02% Proclin 950 (46885-U, Sigma-Aldrich) was added, and the plates were incubated at 4°C overnight. After draining the wells, 50 µl of each testosterone standard (T1500, Sigma-Aldrich) or diluted follicular fluid samples were added, in addition to 50 µl of HRP-labeled testosterone (FKA-101, Cosmo Bio, Tokyo, Japan), and 50 µl of anti-testosterone rabbit polyclonal antibody (FKA-102-E, Cosmo Bio). The mixture was then incubated for 2 h at room temperature. After the reaction, the wells were washed thrice with 300 µl of EIA wash buffer (0.15 M sodium chloride containing 0.05% Tween 20) and 100 µl of the substrate containing 3, 5, 7, 9-tetramethylbenzidine (T8665, Sigma-Aldrich) for 30 min at room temperature. The reaction was stopped by adding 100 µl of 2 M sulfuric acid. The optical density was then measured at 450 nm using an xMark microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA). The minimum detection limit for testosterone EIA was 0.078 ng/ml. The intra- and inter-assay CVs for follicular fluid samples were 5.7% (n = 4) and 8.6% (n = 7), respectively.

Estradiol-17β assay: Follicular estradiol-17β concentration was measured using the previously described TRFIA method [42]. The microtitration plates were coated with 100 µl of anti-sheep IgG rabbit polyclonal antibody (01-23-06, Kirkegaard & Perry Laboratories), and blocking was performed as described for INSL3 TRFIA. Fifty microtiter plates of estradiol-17β for the standards (E8750, Sigma-Aldrich), or 50 µl of diluted follicular fluid samples, and 50 µl of anti-estradiol-17β rabbit polyclonal antibody (GDN no. 244) were added to the wells and incubated for 2 h with shaking (500 rpm). Subsequently, 50 µl of biotinylated-estradiol-17β (E1360-100, Steraloids, Newport, RI, USA) was added and incubated for 1 h at room temperature. The remaining procedures were performed as described for INSL3 TRFIA. The minimum detection limit of estradiol time-resolved fluorescence immunoassay was 3.9 pg/ml. The intra- and inter-assay CVs for follicular fluid samples were 4.9% (n = 4) and 15.7% (n = 7), respectively.

Progesterone assay: Follicular progesterone concentrations were measured using an enzyme immunoassay (EIA), which was established in the laboratory [43]. Fifty microtiter plates of progesterone standards (P8783, Sigma-Aldrich) or diluted follicular fluid samples, 50 µl of HRP-labeled progesterone (FKA-301, Cosmo Bio), and 50 µl of anti-progesterone rabbit polyclonal antibody (FKA-302-E, Cosmo Bio) were added to the wells which were coated with anti-rabbit IgG goat polyclonal antibody. The remaining procedures were performed as previously described for testosterone EIA. The minimum detection limit of progesterone EIA was 0.1 ng/ml. The intra- and inter-assay CVs for follicular fluid samples were 5.4% (n = 4) and 11.2% (n = 7), respectively.
Data analysis

The analysis of variance (ANOVA) using the Generalized Linear Models procedure of the SPSS version 24 software (IBM, Somers, Tokyo, Japan) was adopted to examine the differences among the stages in the estrus cycle or estrogen-active and estrogen-inactive follicular cysts and Stage IV in follicular fluid volume, hormonal concentrations, and contents per follicle or cyst. Follicular cysts are considered to be formed by anovulation of the dominant follicle in Stage IV [23]. Thus, the parameters of estrogen-active and estrogen-inactive follicular cysts were compared with those of dominant follicles at Stage IV. Bonferroni’s method was used to examine significant differences between the two groups. Data are expressed as mean ± SEM. Differences were considered statistically significant at P < 0.05.

Results

The fluid volume of the dominant follicles was significantly different among the estrus cycle stages (P < 0.0001) (Fig. 1A). The fluid volume of the dominant follicles did not differ significantly among Stages I, II, and III. However, it was higher in Stage IV than in Stage III (P < 0.005). The fluid volumes of estrogen-active and estrogen-inactive follicular cysts were greater than those of Stage IV (P < 0.0001) (Fig. 1A). However, the diameter of the dominant follicles was significantly different among the stages of the estrus cycle (P < 0.0001). The diameter of the dominant follicles in Stage IV (18.09 ± 0.56 mm; mean ± SEM) was higher (P < 0.05) than that in Stages I (11.25 ± 1.06 mm), II (14.17 ± 0.73 mm), and III (15.16 ± 0.74 mm). The diameters of estrogen-active (22.14 ± 0.43 mm) and estrogen-inactive (22.50 ± 0.61 mm) follicular cysts were greater than that of Stage IV (P < 0.0001).

Fluid INSL3, testosterone, and estradiol-17β concentrations in the dominant follicles were significantly different among the stages (P < 0.0001) (Figs. 1B, C, and D), but no significant differences among the stages were found in the fluid progesterone concentrations (Fig. 1E). No significant difference was observed in the fluid INSL3 and estradiol-17β concentrations in the dominant follicles among Stages I, II, and III but these concentrations were greater in Stage IV than in Stage III (P < 0.05). In addition, the fluid testosterone concentrations in the dominant follicles were higher in Stages I and IV than those in Stages II and III (P < 0.05).

No significant difference was observed between estrogen-active, estrogen-inactive follicular cysts, and Stage IV dominant follicles on fluid INSL3 concentrations by ANOVA (Fig. 1B). Notably, no noticeable differences were observed between estrogen-active, estrogen-inactive follicular cysts, and Stage IV dominant follicles in fluid testosterone, estradiol-17β, and progesterone concentrations by ANOVA (P < 0.05) (Figs. 1C, D, and E). However, the fluid testosterone and estradiol-17β concentrations were lower in the estrogen-active and estrogen-inactive follicular cysts compared to those in the dominant follicles in Stage IV (P < 0.001 and P < 0.0001, respectively). Fluid progesterone was observed at elevated concentrations in the estrogen-inactive follicular cysts than in the dominant follicles of Stage IV and estrogen-active follicular cysts (P < 0.001). Ratios of estradiol-17β to progesterone concentrations in dominant follicles and follicular cysts were as follow: 1.93 (Stage I), 0.69 (Stage II), 1.66 (Stage III), 23.99 (Stage IV), 13.86 (estrogen-active cysts) and 0.197 (estrogen-inactive cysts).

When comparing the different stages, fluid INSL3, testosterone, and estradiol-17β levels per dominant follicle were found to be significantly different (P < 0.0001) (Figs. 2A, B, and C), but no significant differences among the stages were found in the fluid
progesterone levels per dominant follicle (Fig. 2D). The fluid INSL3, testosterone, and estradiol-17β contents per dominant follicle were greater in Stage IV than in the other stages (P < 0.05).

We detected effects of difference among estrogen-active, estrogen-inactive follicular cysts, and Stage IV dominant follicles on fluid INSL3, testosterone, estradiol-17β, and progesterone contents by ANOVA (P < 0.05) (Figs. 2A, B, C, and D). The fluid INSL3 and progesterone contents per estrogen-inactive follicular cyst were higher than those per dominant follicle at Stage IV (P < 0.001). In contrast, the fluid testosterone and estradiol-17β contents per estrogen-inactive follicular cyst were lower than those per dominant follicle in Stage IV (P < 0.05) and (P < 0.0001), respectively.

All data on cystic or follicular diameter and hormonal concentrations in each heifer with follicular cysts are shown in Supplementary Table 1.

The weight of the corpus luteum was significantly different among stages (P < 0.0001) (Fig. 3A). The corpus luteum weights were larger in Stages II and III than in Stages I and IV (P < 0.005). Luteal INSL3, progesterone concentrations (P < 0.05), and progesterone content per corpus luteum were found to be significantly different among the stages (P < 0.01) (Figs. 3B, C, D, and E). Furthermore, the luteal INSL3 and progesterone concentrations and contents per corpus luteum were both greater in Stages II and III than those in Stages I and IV (P < 0.05).

Discussion

Changes in INSL3 synthesis in bovine ovaries during estrus cycles are yet to be identified. To the best of our knowledge, this study is the first to quantify the INSL3 content in bovine dominant and cystic follicles and the corpus luteum during the estrus cycle. The results revealed that INSL3 concentrations and contents increased in preovulatory follicles, suggesting that INSL3 secretion is enhanced during follicular maturation. To the best of our knowledge, this is also the first study to show quantitative alterations of the INSL3 peptide in bovine dominant follicles during the estrus cycle. The increase in INSL3 content in preovulatory follicles may be attributed to the increased LH pulse frequency immediately after luteal regression in the estrus cycle, eventually stimulating follicular maturation [44]. It has been previously reported that INSL3 increased testosterone levels in cultured rat preantral thecal cells [45] in addition to androstenedione levels in cultured bovine thecal cells [46] through cyclic AMP [47]. Therefore, the increased secretion of INSL3 in the preovulatory follicle in the present study may be partly attributed to the enhancement of testosterone secretion. In addition, the current results showed that while testosterone concentrations in the dominant follicular fluid in the early luteal phase were higher than those in the mid-luteal phase, they were similar to those in the follicular phase. Considering that the LH pulse frequency decreases towards the mid-luteal phase in heifers, the high testosterone concentration in the dominant follicles at the early luteal phase may be linked to the accumulation of steroids caused by low aromatase activity in granulosa cells at this stage [48].

This study revealed that concentrations of INSL3 in follicular cysts were similar to preovulatory follicles, whereas concentrations in estrogen-inactive cysts were higher. However, lower levels of testosterone and estradiol-17β in cysts were observed. These results suggest that although the secretion of INSL3 is transiently maintained by theca interna cells (even after follicular cyst formation), testosterone secretion suddenly ceased. Treating male goats with the GnRH antagonist degarelix acetate decreased the blood INSL3 concentrations to the lowest value approximately three days after treatment; in contrast, testosterone concentrations rapidly dropped the next day [49]. Moreover, adding LH to cultured bovine thecal cells increased androstenedione secretion but did not affect INSL3

![Fig. 2. Changes in follicular fluid contents of INSL3 (A), testosterone (B), estradiol-17β (C), and progesterone (D) during the estrus cycle (Stages I–IV) and comparison between the Stage IV and estrogen-active (E active) and estrogen-inactive (E inactive) follicular cysts (Cyst). Data are expressed as mean ± standard error of the mean. Probability values above brackets indicate significant differences among the stages of the estrus cycle or E active, E inactive follicular cysts, and Stage IV by ANOVA. Differing superscripts indicate significant differences between stages (P < 0.05). The asterisk indicates significant differences compared with Stage IV (P < 0.05). The dagger indicates significant differences compared with E active and E inactive cysts (P < 0.05).](image-url)
secretion [46]. Thus, differential regulation of INSL3 and testosterone secretion from theca interna cells might occur during bovine cyst formation. In women with polycystic ovarian syndrome (PCOS), serum INSL3 concentrations were higher than those in women with normal menstrual cycles [28, 29]. However, no information regarding INSL3 concentration in peripheral blood is available in cattle with follicular cysts. Higher INSL3 content in estrogen-inactive cysts may reflect a more accumulation of INSL3 than in estrogen-active cysts because estrogen-inactive cysts are considered more atretic [25].

The expression of INSL3 mRNA has been reported in the bovine corpus luteum during the estrus cycle [21, 50], and the peptide has been detected in pregnant caprines [51]. In the current work, INSL3 content was measured in the bovine corpus luteum during the estrus cycle. Our results revealed for the first time that higher peptide expression in luteal tissues was linked to elevated progesterone levels, which suggested enhanced INSL3 secretion in the functional corpus luteum. The addition of INSL3 to cultured bovine luteal cells reportedly promotes progesterone secretions [50]. Thus, higher amounts of INSL3 secreted from functional luteal cells could play a role in facilitating progesterone production through autocrine and paracrine mechanisms, as previously suggested in Leydig [52] and theca interna [45, 46] cells.

In conclusion, bovine dominant follicles are likely to secrete higher amounts of INSL3 during their functional maturation. Bovine follicular cysts may transiently maintain INSL3 secretion during their formation, whereas they would rapidly halt testosterone production. Estrogen-inactive cysts that have undergone advanced atresia may accumulate more INSL3. Furthermore, the bovine corpus luteum might produce more INSL3 upon full maturation.

Conflict of interests: None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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