Evaluation of bovine uterine gland functions in 2D and 3D culture system

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Abstract. In ruminants, uterine glands play key roles in the establishment of pregnancy by secreting various factors into the uterine lumen. Although a three-dimensional (3D) culture system has been used for investigating cellular functions in vitro, the detailed functions of uterine gland have not been fully elucidated. In this study, we examined the benefits of 3D culture system to examine the innate functions of bovine uterine glands. Isolated bovine uterine glands were cultured on Matrigel (2D) or in Matrigel (3D), respectively, and the mRNA levels of secreted proteins (SERPINA14, MEP1B, APOA1, ARSA, CTGF, and SPP1) were measured in isolated and cultured uterine glands. The protein expression of estrogen receptor β (ERβ) and progesterone receptor (PR) and the establishment of apico-basal polarity were examined. In isolated uterine glands, the mRNA levels of secreted proteins changed during the estrous cycle. Although uterine glands cultured in both 2D and 3D expressed ERβ and PR, progesterone did not affect SERPINA14 mRNA expression. The expression of APOA1 mRNA in 2D cultured uterine glands did not respond to estrogen and progesterone. Additionally, the mRNA levels of secreted proteins in the 3D culture system were significantly higher than those in the 2D culture system, which might be attributed to the different cellular morphology between them. The locations of ZO-1 and β-catenin in 2D cultured uterine glands were disordered compared with 3D cultured uterine glands. These results showed that the hormonal responsiveness of secreted factor expression and cellular morphology were different between 2D and 3D cultured bovine uterine glands.

Key words: Bovine, Secreted proteins, Three-dimensional culture, Uterine glands

Embryonic mortality is one of the major factors affecting meat and milk production and economic efficiency of the livestock industry [1, 2]. In cattle, a high frequency of embryonic loss occurs between days 8 and 16 after insemination [2–4]. During this period, the embryo grows rapidly and elongates in the uterus. Blastocysts hatch from the zona pellucida and develop into an ovoid conceptus on day 13, which then elongates into a filamentous shape by implantation [5]. Increasing the weight of the trophectoderm enhances the secretion of IFNτ, which interacts with its receptor on the endometrium and supports pregnancy recognition by suppressing prostaglandin F2α secretion from endometrial cells [5–7]. Therefore, conceptus elongation is essential for the maternal recognition and establishment of pregnancy [8, 9].

Various secreted factors from the endometrial luminal and glandular epithelium are crucial for conceptus elongation [10–12]. In particular, the proteins serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14 (SERPINA14), meprin A beta (MEP1B), apolipoprotein A1 (APOA1), alylosulfatase A (ARSA), connective tissue growth factor (CTGF), and secreted phosphoprotein 1 (SPP1), which have been detected in the endometrium during early pregnancy, are considered to be involved in embryo development [13–15]. SERPINA14 inhibits lymphocyte function in vitro, enabling the uterine immunosuppressive action of P4 on the conceptus [16, 17]. MEP1B is a secreted zinc metalloendopeptidase that induces enzymatic cleavage of components of the uterine luminal fluid [18, 19]. APOA1 is thought to play roles in embryo implantation by inhibiting lipid peroxidation [20] while the precise roles of ARSA are still unclear. CTGF has several functions, including stimulation of cell proliferation, migration, and adhesion [21], which may be important for the development of bovine embryos [22]. SPP1 enhances migration and mediates the attachment of trophodectoderm cells by binding to integrin [5, 23, 24]. In the ovine uterine gland knockout model, the embryo fails to survive and elongate due to the depletion of uterine gland secretion rather than to alternation of adhesive factors on the endometrium or to responsiveness to embryo recognition systems [25, 26]. Although the uterine gland plays vital roles in the establishment of pregnancy, its detailed functions remain unclear.

The establishment of apico-basal polarity is necessary to elicit proper functions in epithelial cells, such as the cellular barrier system and endocytic transport [27, 28]. A three-dimensional (3D) culture system using an extracellular matrix is more suitable for polarization than conventional two-dimensional (2D) culture [29, 30]. In cattle and buffalo, 3D cultured mammary epithelial cells promote the expression of polarized proteins and casein, the lactation-related protein [31, 32]. Furthermore, mouse and human endometrial epithelial cells in 3D culture form an organoid, a spherical aggregate with cavity, and retain apico-basal polarity and hormonal sensitivity [33, 34]. Haeger et al. evaluated the characteristics of the 3D cultured bovine endometrial gland cell line (BEGC), such as the epithelial phenotype and responsiveness to IFNτ [35]. However, the detailed functions of the uterine gland have not been investigated.

To clarify whether a 3D culture system of uterine gland fragments is beneficial for investigating its detailed functions, we evaluated the expression levels of secreted proteins, sex steroid hormone reactivity, and cellular morphology of 2D and 3D cultured bovine uterine gland.

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Materials and Methods

Isolation of bovine uterine glands

Bovine uterine glands were transported on ice from a local abattoir to the laboratory after exsanguination. The samples were carefully evaluated to eliminate infected uteri from the laboratory experiments. Each uterus was classified into four stages (stage I, days 1–4 after ovulation; stage II, days 5–10; stage III, days 11–17; stage IV, days 18–20) following a previous research [36]. Uteri ipsilateral to the corpus luteum or the dominant follicle were used.

The inner part of the uteri was washed with Hank’s balanced salt solution (HBSS) containing 1 mM CaCl₂ (Nacalai Tesque, Inc., Kyoto, Japan) and MgCl₂ (Ishizu Seiyaku, Ltd., Osaka, Japan) and the uteri were cut open along the long axis. The endometrial epithelium and compact layers were peeled, and only the sponge layers containing a lot of uterine glands were retained. Two grams of sponge layers were minced into a 5 mm cube and gently dissociated in HBSS (30 ml) containing 6600 unit/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 8000 units/ml DNase (BBI Solutions, Newport, UK) for 2 h at 38.5°C. The cell suspension was filtered through a 280 µm mesh to remove undigested tissues. Fragments of the uterine glands and single cells were separated using 70 µm mesh filtration. Trapped fragments of the uterine glands were acquired by washing with HBSS and DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation (180 g, 8 min), the supernatant was removed and the uterine gland fragments were resuspended in DMEM/F12 containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.1% bovine serum albumin (BSA, Nacalai Tesque, Inc.), 5 ng/ml sodium selenite (Sigma-Aldrich), 2 µg/ml insulin (Sigma-Aldrich), 5 µg/ml transferrin (BBI Solutions), 0.5 mM ascorbic acid (Wako Pure Chemical Industries, Ltd.), 20 µg/ml gentamycin (Sigma-Aldrich), and 2 µg/ml amphotericin B (Sigma-Aldrich) for 7 days at 38.5°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was replaced every 2–3 days (Fig. 1).

For gene and protein expression analysis, uterine gland fragments were cultured in 48-well plates (Greiner Bio-One). In the 2D culture system, the plates were coated with 1% (V/V) Matrigel (growth factor-reduced phenol-red free, Corning, NY, USA) in the culture medium, and 100 fragments in 500 µl of the medium were seeded onto each well of the coated plates. In the 3D culture system, 100 fragments were embedded in 200 µl culture medium with 50% (V/V) growth factor-reduced phenol-red free Matrigel, and 300 µl medium was added on the Matrigel after polymerizing (30 min at 38.5°C).

For immunohistochemistry, 24-transwell inserts (Corning) were coated with Matrigel, as described in the 2D culture system, and each insert, which was seeded with 100 fragments in 100 µl culture medium, was set in 24-well plates (Greiner Bio-One) containing 400 µl of the medium.

To investigate the effects of steroid hormones on the gene expression of uterine glands in 2D and 3D cultures, uterine gland fragments were treated with 0.002% ethanol (Control, Nacalai Tesque, Inc.), 5 µg/ml estradiol-17β (E2, Sigma-Aldrich), 10 ng/ml progesterone (P4, Sigma-Aldrich), or E2 and P4. The concentrations of E2 and P4 were determined based on their blood levels in cows at stage III [37, 38].

Total RNA extraction and cDNA synthesis

Total RNA was extracted from isolated (stages I–IV) and cultured (stage II) uterine glands using RNAiso Plus (Takara Bio Inc., Shiga, Japan) following the manufacturer’s instructions to determine the mRNA expression of SERPINA14, MEP1B, APOA1, ARSA, CTGF, and SPP1. Total RNA (2 µg) was treated with DNase (Promega Corporation, Madison, WI, USA) to remove genomic DNA and reverse-transcribed to cDNA using oligo dT primers in the PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc.) according to the manufacturer’s instructions.

Gene expression analysis by quantitative RT-PCR

Quantitative RT-PCR analysis with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was performed using the AriaMx Real-Time PCR System (MY15245343, Agilent Technologies, Santa Clara, CA, US). Each reaction was performed in a total volume of 8 µl containing 8 ng template cDNA, 500 nM of each forward and reverse primers, 4 µl of SsoAdvanced Universal SYBR Green Supermix, and dH₂O. Standard curves were prepared from 7-point serial dilutions of PCR products of target genes. The linear dynamic range, defined as the lowest to highest quantifiable copy number, ranged from 2 × 10¹ to 2 × 10⁷ copies. The amplification was conducted with an initial hot start at
95°C for 30 sec, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at the temperature indicated in Table 1 for 10 sec, and extension at 72°C for 15 sec, followed by dissociation program (95°C for 1 min, temperature indicated in Table 1 to 95°C at 0.5°C intervals, 5 sec per interval). Primers sequences were determined using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), and the specificity of each primer was analyzed by 1.5% agarose gel electrophoresis of PCR products. The sequences of each primer are shown in Table 1.

Three housekeeping genes, GAPDH, β-actin, and H2A, were tested to determine the optimal reference gene for quantitative RT-PCR data from isolated and cultured bovine uterine glands using the Normfinder software (http://moma.dk/normfinder-software). GAPDH was selected as a reference gene because the expression of GAPDH (0.039) was the most stable among the samples compared to those β-actin (0.093) and H2A (0.080).

Western blotting

Isolated and cultured uterine glands were directly homogenized in SDS gel-loading buffer (50 mM Tris-HCl (Nacalai Tesque, Inc.), 2% SDS (Nacalai Tesque, Inc.), 10% glycerol (Nacalai Tesque, Inc.), and 1% β-mercaptoethanol (pH 6.8, Wako Pure Chemical Industries Ltd.]). Protein samples were separated by 12% (V/V) SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene (PVDF) membranes (0.45 μm, GE Healthcare, Buckinghamshire, UK). After blocking with the PVDF Blocking Reagent (Toyobo, Osaka, Japan), the membranes were incubated with primary antibodies [PR (SABA4502185, Sigma, 1:10000 dilution) and ERβ (sc-8974, Santa Cruz Biotechnology, Heidelberg, Germany, 1:200 dilution) in Can Get Signal Immunoreaction Enhancer Solution for primary antibody (Toyobo), and GAPDH (NB300-221, Novus Biologicals, Centennial, CO, USA, 1:80000 dilution) in TBS-T] for at least 20 h at 4°C. After incubation, the membranes were incubated again with secondary antibodies [anti-rabbit, horseradish peroxidase (HRP)-linked whole antibody produced in donkey (HRP) (ab9726, Abcam, Cambridge, UK, 1:100000 dilution) for GAPDH] for 60 min at room temperature. Finally, the sections were covered with 4', 6- diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for nuclear staining. Immunoreactions were observed using a fluorescence microscope (FSX100, Olympus, Tokyo, Japan).

Table 1. Sequences of primers used for quantitative RT-PCR

| Genes | Forward and reverse primers | Accession no. | Product size (bp) | Annealing temperature (°C) |
|-------|------------------------------|---------------|------------------|--------------------------|
| SERPINA14 | 5'-GCTTCCAAGATTGACCCCA 5'-TGACCAAGATGGGTGTGTCGA | NM_174797.3 | 154 | 57 |
| MIP1 | 5'-TGCCAAAGCTGAGTGCATGTGTGGG 5'-GGACCGATGGAAGATGGGCCGAT | NM_001144098.1 | 116 | 65 |
| APOA1 | 5'-AGACTGCTGGCATTAGGAGTC 5'-GCCACATAGTCCTGCGACACT | NM_174242.3 | 199 | 58 |
| ARSA | 5'-AGCTTGCGTGTCTGCTGTGTG 5'-CACCTGCGCATCCTGCAGTG | NM_001075205.1 | 184 | 60 |
| SPP1 | 5'-CCTGACCCCATCTCCAAGACGAG 5'-AGCGTCTGCGGAGTCCATTAG | NM_174187.2 | 183 | 59 |
| CTGF | 5'-GCTTCCTGCTGCCTCAGCTC 5'-GAGCAGCGAATGCGCGAAGAG | NM_174030.2 | 224 | 55 |
| GAPDH | 5'-ACCCCTCAAAGATTGTCAGCA 5'-GGTCATAAGTCTCCACGA | NM_001034034.2 | 103 | 60 |

HE stains and immunohistochemistry

Isolated and 3D cultured uterine glands were fixed in 4% (W/V) paraformaldehyde (Nacalai Tesque, Inc.) in PBS for 60 min at 4°C, dehydrated step-wise using gradient ethanol (from 70% to 100%, Nacalai Tesque, Inc.), and embedded in paraffin (Sigma-Aldrich). 2D cultured uterine glands were separated from a 24-well transwell by cutting the membrane of the transwell where cells adhered, and then fixed and paraffin-embedded in the same way as described above. Blocks were sliced at 6 and 4 μm thickness for HE stains and immunohistochemistry, respectively.

The sections were deparaffinized and rehydrated with xylene (Nacalai Tesque, Inc.) and gradient ethanol (from 100% to 70%). Antigen was retrieved by microwaving in 0.1 M Tris-EDTA buffer (pH 9.0) for 15 min at 600 W. After being washed with PBS, the sections were incubated with 5% (W/V) BSA in PBS for 30 min to block non-specific binding, followed by overnight incubation with ZO-1 (sc-33725, Santa Cruz Biotechnology, 1:100 dilution) and β-catenin (ab32572, Abcam, 1:100 dilution) at 4°C in a humidified chamber. Rat and rabbit serum were used as negative controls. Sections were then incubated with Alexa Fluor goat Anti-rat 594 (A-11007, Thermo Fisher Scientific, 1:500 dilution) and Alexa Fluor goat anti rabbit 488 (A-11008, Thermo Fisher Scientific, 1:500 dilution) for 60 min at room temperature. Finally, the sections were covered with 4', 6- diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for nuclear staining. Immunoreactions were observed using a fluorescence microscope (FSX100, Olympus, Tokyo, Japan).

Statistical analysis

The experimental data for the analysis of gene expression are shown as mean ± standard error of the mean (SEM). All data were confirmed to be normal and homoscedastic using the Shapiro-Wilk and Brown-Forsythe tests in R (Ihaka, R., and R. Gentleman. 1996). In Experiment 1, data were evaluated using non-parametric one-way ANOVA and non-parametric Tukey’s multiple comparisons test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). In Experiment 2, data were analyzed using three-way ANOVA followed by Tukey’s multiple comparison test after logarithmic transformation using R. A post hoc comparison was conducted.
when there was a significant interaction among all factors (culture condition, E2, and P4). P values less than 0.05 were considered to be statistically significant.

**Results**

Experiment 1: Gene expressions in isolated bovine uterine glands during estrous cycles.

The mRNA expression of SERPINA14 gradually increased from stage I to IV, and its expression level in stage IV was significantly higher than that in stage I (P < 0.05; Fig. 2A). MEP1B, APOA1, and ARSA genes were most upregulated at stage III, whereas there was no significant difference among stages I, II, and IV (Fig. 2B, C, D). The mRNA expression of CTGF at stage II was significantly lower than that in stages I and IV (P < 0.05; Fig. 2E). SPP1 gene expression was significantly higher in stages III and IV than that in stage I (P < 0.05; Fig. 2F).

Experiment 2: Relationship between culture conditions (2D or 3D) and ovarian steroid hormones in terms of gene expression levels in bovine uterine gland.

As isolated uterine gland fragments from the endometrium, estrogen receptor β (ERβ) and progesterone receptor (PR) proteins were expressed in both 2D and 3D cultured uterine glands (Fig. 3). The difference between the 2D and 3D culture conditions had a significant effect on the mRNA expression levels of the secreted proteins (SERPINA14, MEP1B, APOA1, ARSA, CTGF, and SPP1) in uterine glands (P < 0.001; Table 2). The expression levels of these genes in 2D cultured uterine glands were significantly lower than those in 3D cultured uterine glands, regardless of steroid hormone administration (P < 0.05; Fig. 4A–F). The expression of all target genes in cultured uterine glands was affected by E2 (P < 0.05; Table 2). Administration of E2 downregulated the expression levels of SERPINA14, MEP1B, APOA1, and ARSA, while upregulated CTGF and SPP1 mRNA expressions compared to the non-treated condition (P < 0.05; Table 2 and Fig. 4). Except for SERPINA14, the expression levels of the other target genes were affected by P4 administration (P < 0.05; Table 2), particularly CTGF and SPP1 gene expressions

![Fig. 2. Gene expressions (A: SERPINA14, B: MEP1B, C: APOA1, D: ARSA, E: CTGF, F: SPP1) relative to GAPDH in isolated uterine glands among the estrous stages. Expression values are presented as means ± SEM. Different superscripts indicate significant differences among the estrous stages, as determined using non-parametric one-way ANOVA followed by non-parametric Tukey’s multiple comparison. Different superscripts (a–c) show significant differences within each estrous stage (P < 0.05).](image-url)
There was a strong interaction between culture conditions and E2 administration in the gene expression of MEP1B, APOA1, ARSA, and CTGF (P < 0.001; Table 2), and culture conditions were significantly correlated with P4 administration in ARSA and CTGF gene expressions (P < 0.05; Table 2). In MEP1B, APOA1, ARSA, and CTGF mRNA expression levels, E2 strongly interacted with P4 (P < 0.001; Table 2). Moreover, there was an interaction between culture conditions, E2, and P4 in MEP1B, APOA1, ARSA, and CTGF mRNA expression levels (P < 0.05; Table 2). In contrast, there were no interactions between culture conditions, E2, and P4 in SERPINA14 and SPP1 gene expression levels (Table 2).

Discussion

In ruminants, various factors are secreted into the endometrial lumen under the regulation of sex steroid hormones involved in conceptus development and establishment of successful implantation [39–41]. This is the first study to manifest gene expression of secreted proteins in isolated bovine uterine glands during the estrous cycle. (Fig. 2). The gene expression of SERPINA14 continuously increased from stages I to IV (Fig. 2A). This result corresponds with previous reports in which the gene expression of the bovine endometrium was investigated [13]. The expression of SERPINA14 before ovulation may contribute to sperm survival by suppressing the immunoactivity in the uterus [42]. The expression of CTGF in bovine uterine glands was upregulated during the luteal phase (Fig. 2B–D, F). These results are consistent with those of previous studies indicating that the gene expressions of these are regulated by P4 [5, 14, 15, 43]. The upregulation of these genes is considered to play roles in the development of the conceptus in ruminants [5, 14, 19, 24]. Conversely, the mRNA expression of CTGF in bovine uterine glands was lower during the luteal phase than the peri-ovulation phase (Fig. 2E). This result might be related to the increased ratio of proliferative cells in the uterine glands during the peri-ovulation phase [44], as the expression of CTGF was regulated by E2 [45] and CTGF induces cell proliferation cooperating with other growth factors [21]. As opposed to the present results for bovine uterine glands, P4 stimulates the gene and protein expression of CTGF in the endometrium [15, 45, 46]. Considering that CTGF is also expressed in the luminal epithelium and caruncle matrix in the endometrium [45], the expression of CTGF in response to P4 may be largely attributed to its expression in luminal epithelial and stromal cells rather than in uterine glands, although the detailed mechanism is unclear. To better understand this mechanism, the expression level of CTGF in various cell types in the bovine endometrium during the estrous cycle should be investigated.

Additionally, the mRNA expression of SERPINA14 in cultured uterine glands was not affected by P4 (Table 2), even though PR was expressed in cultured uterine glands (Fig. 3), and the main regulator

\[ \text{Table 2. Result of three-way analysis of variance for experiment 2} \]

| F-value | SERPINA14 | MEP1B | APOA1 | ARSA | CTGF | SPP1 |
|---------|-----------|-------|-------|------|------|------|
| Culture condition (2D or 3D) | 510.49 *** | 1117.44 *** | 147.74 *** | 2037.83 *** | 2497.76 *** | 17.35 *** |
| E2 | 213.98 *** | 277.63 *** | 22.51 *** | 369.89 *** | 770.82 *** | 4.25 * |
| P4 | 0.83 | 4.36 * | 4.70 * | 6.96 * | 81.83 *** | 41.21 *** |
| Culture condition × E2 | 1.68 | 22.99 *** | 14.61 *** | 161.53 *** | 31.06 *** | 0.03 |
| Culture condition × P4 | 0.20 | 0.04 | 3.65 | 4.67 * | 18.06 *** | 4.08 |
| E2 × P4 | 2.03 | 13.53 *** | 47.10 *** | 574.59 *** | 125.97 *** | 0 |
| Culture condition × E2 × P4 | 2.16 | 6.66 * | 23.84 *** | 249.97 *** | 5.22 * | 3.56 |

* P < 0.05, ** P < 0.01, *** P < 0.001.

\[ (P < 0.01; \text{Table 2}). \text{There was a strong interaction between culture conditions and E2 administration in the gene expression of MEP1B, APOA1, ARSA, and CTGF (P < 0.001; Table 2), and culture conditions were significantly correlated with P4 administration in ARSA and CTGF gene expressions (P < 0.05; Table 2). In MEP1B, APOA1, ARSA, and CTGF mRNA expression levels, E2 strongly interacted with P4 (P < 0.001; Table 2). Moreover, there was an interaction between culture conditions, E2, and P4 in MEP1B, APOA1, ARSA, and CTGF mRNA expression levels (P < 0.05; Table 2). In contrast, there were no interactions between culture conditions, E2, and P4 in SERPINA14 and SPP1 gene expression levels (Table 2).} \]
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of SERPINA14 expression in ovine and bovine endometria was reported to be P4 \([47, 48]\). A possible reason for this difference is that the original expression of SERPINA14 may not be responsive to P4, as indicated in this study. A previous study showed that the gene expression of SERPINA14 in 3D cultured bovine endometrial epithelial (BEE) cells, including uterine glands, did not respond to P4. However, when BEE cells were co-cultured with stromal cells, they were responsive to P4 \([49]\). Further investigation of the culture system, including the interaction between the uterine glands and stromal cells, is needed to imitate the innate hormonal response of bovine uterine glands.

In 3D culture, the gene expression levels of the secreted proteins were significantly higher than those in 2D culture \(\text{Table 2, Fig. 4}\). Although ER\(\beta\) and PR proteins were expressed in both 2D and 3D cultured uterine glands \(\text{Fig. 3}\), the expression of APOA1 mRNA in 2D cultured bovine uterine glands did not react to E2 and P4 administration, whereas the 3D cultured uterine glands responded \(\text{Fig. 4C}\). In this study, 3D cultured uterine glands better established apico-basal polarity than 2D cultured uterine glands \(\text{Fig. 5}\). A study by Arévalo \textit{et al.} reported that 3D cultured mammary epithelial cells maintained apico-basal polarity and expressed proteins that are responsible for polarization, including CDH1 and occludin, rather than a conventional 2D culture system \([50]\). Moreover, the expression level of a lactation-related gene, cascin, was upregulated more in 3D cultures than in 2D cultures \([31, 32]\). Therefore, we suggest that the difference in cellular morphology between 2D and 3D cultured uterine glands affect their gene expression and hormonal responsiveness.

In summary, this study suggests that the 3D culture system is more beneficial for imitating the functions of bovine uterine glands than the 2D culture system. However, further investigation of hormonal responsiveness and gene expression of secreted proteins in the uterine gland \textit{in vitro} may be needed.

\textbf{Conflict of interest:} The authors declare that they have no conflict of interest.

\textbf{Acknowledgement}

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Fig. 5. Morphology of isolated and cultured bovine uterine glands. A–C) Isolated and cultured bovine uterine glands stained with hematoxylin and eosin (H&E). D–F) Immunohistochemistry for apico-basal marker (ZO-1: red, β-catenin: green) in isolated and cultured uterine glands. The cell nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

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