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Activity of 3β-hydroxysteroid dehydrogenase associated with progesterone production in bovine granulosa cells cultured under different concentrations of serum, insulin-like growth factor I, and gonadotropin

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Abstract: Three-β-hydroxysteroid dehydrogenase (3β-HSD) is the enzyme responsible for progesterone production. This study aimed to determine whether 3β-HSD activity can be shown to reflect progesterone production by granulosa cells cultured under different serum conditions and follicle stimulating hormone (FSH), luteinising hormone (LH), and insulin-like growth factor I (IGF-I) concentrations. Large bovine follicles were dissected from abattoir ovaries to recover granulosa cells. Cells were washed, stained for viability, and plated for 48 h in basic medium with or without 5% foetal calf serum (FCS). Subsequently, cells were exposed to FSH (1 ng/mL), LH (10 or 100 ng/mL), or FSH (1 ng/mL) + IGF-I (1 or 10 ng/mL) in a serum-free medium for another 96 h to predict degree of luteinisation. Before and after incubation, granulosa cells were stained for 3β-HSD activity. The high dose of IGF-I (10 ng/mL) increased ($P < 0.05$) progesterone secretion over 2.5-fold compared with FSH alone or the low dose of IGF-I (1 ng/mL) in cells preincubated with FCS. This was clearly reflected by darker 3β-HSD staining than in cells exposed to FSH and low dose IGF-I.

Key words: 3β-Hydroxysteroid dehydrogenase, bovine granulosa cells, progesterone, FSH, LH, IGF-I

1. Introduction

Three-β-hydroxysteroid dehydrogenase (3β-HSD) is one of the key enzymes in the steroidogenic pathway responsible for producing progesterone in the ovary (1,2). During this pathway, pregnenolone is converted into progesterone by the action of 3β-HSD in the endoplasmic reticulum. Progesterone-producing ovarian cells were identified by testing the 3β-HSD activity in the luteal cells recovered from caprine or feline corpora lutea (3–5). However, the 3β-HSD activity of luteinising follicular granulosa cells has not yet been investigated or related to their progesterone-producing capacity.

The 2 gonadotropins follicle stimulating hormone (FSH) and luteinising hormone (LH) regulate follicular growth and development. Antral follicles are highly dependent on gonadotropins to successfully achieve ovulatory size (6,7). Within the follicles, the granulosa cells respond to FSH with proliferation, then increasing oestradiol synthesis, and, in ruminants, acquire at large sizes the ability to produce progesterone following induction of 3β-HSD. In addition to gonadotropins, insulin-like growth factor I (IGF-I) plays a key role in follicular development (8–11), modifying FSH activity and thus stimulating proliferation and steroid synthesis. A plethora of information exists on the steroidogenic responses of granulosa cells to gonadotropins and IGF-I under in vitro culture conditions, both in the presence of serum and under serum-free conditions in many species including cattle (12–14). Culture of granulosa cells from large antral follicles generally leads to their luteinisation or at least increasing progesterone production even in serum-free conditions, and this can be induced and enhanced by cyclic adenosine monophosphate (cAMP) and LH, while FSH has less of an effect (compared with granulosa cells from small and medium follicles). The addition of IGF-I to the medium enhances progesterone synthesis from granulosa cells of follicles of all sizes, and it amplifies the response to gonadotropins (15,16). Culture of granulosa cells in medium containing serum immediately and strongly enhances their ability to attach and survive and causes luteinisation both functionally and morphologically (12).

While several studies have related 3β-HSD activity to caprine and feline luteal cell function in vitro, there is no report of 3β-HSD activity of bovine granulosa cells stimulated to synthesise progesterone under different

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hormonal conditions. Therefore, this study aimed to determine whether 3β-HSD activity detected histologically reflects the capacity for progesterone production of bovine granulosa cells recovered from large antral follicles and cultured under different serum, FSH, LH, and IGF-I conditions.

2. Materials and methods

2.1. Cell isolation and in vitro culture

All chemicals used in cell preparation and incubation were obtained from Sigma Chemical Company (Sigma-Aldrich, Co., Munich, Germany). Ovaries were obtained at a nearby abattoir from mostly beef heifers and cows after slaughter, transported to the laboratory in an ice-cold sterile medium (M-199) containing penicillin (100 IU/mL), streptomycin (100 µg/mL), and fungizone (2.5 µg/mL), and processed for granulosa cell recovery as previously described (17). Briefly, after being immersed in ethanol (70% v/v) for 30 s, selected ovaries were transferred to fresh M-199 and large antral follicles from 8 mm were quickly dissected and placed in a petri dish containing phosphate buffered saline (PBS). After aspirating follicular fluid using a syringe with a 19-gauge needle, a slit was made in the follicle wall and the granulosa cell layer was gently scraped with an inoculation loop. Cells harvested from the follicles were pooled, pelleted by centrifugation (300 × *g* for 10 min), and resuspended in 2 mL of PBS. Double-distilled water (2 mL) was added and the cells were agitated for 10 s to lyse any red blood cells present, followed by restoration of isotonicity using 15 mL of PBS. The cells were then pelleted by centrifugation and resuspended in a small volume of culture medium consisting of McCoy 5A modified medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL fungizone, 10 ng/mL bovine insulin, 2 mM L-glutamine, 10 mM Hepes, 5 µg/mL apotransferrin, 5 ng/mL sodium selenite, 0.1% BSA, and 10^{-7} M androstenedione. Cells were counted using a haemocytometer, and the viability estimated by trypan blue dye exclusion was 20%–30%. Between 2 and 3 × 10^5 viable cells in 50 µL were seeded into 24 well plates, with each well containing 450 µL of pre-equilibrated basic medium with or without 5% foetal calf serum (FCS) for 48 h at 37.5 °C in 5% CO_2 in humidified air (17). Subsequently, cells in 2 different media were exposed to FSH (1 ng/mL), LH (10 and 100 ng/mL), or FSH (1 ng/mL) + IGF-I (1 or 10 ng/mL). The medium was changed every 48 h. After the first 48 h of incubation, cells were incubated in a serum-free medium for the remaining 96 h. The protocol was repeated in 3 independent experiments, in which each treatment was performed in duplicate wells.

2.2. 3β-HSD activity staining of granulosa cells

Cells with 3β-HSD activity were detected as previously described (18,19) before and after hormonal treatments in vitro. The cell suspension (200 µL) was incubated in paraformaldehyde (1%) for cell fixation for 20 min. Thereafter, the suspension was centrifuged (400 × *g*) for 5 min. The cell pellet was then resuspended in 200 µL of staining solution (0.1 M PBS containing 0.1% BSA, 1.5 mM NAD, 0.25 mM nitro blue tetrazolium, and 0.2 mM 5-androstene-3-ol-17-one, prepared from an 8 mM stock solution in ethanol). Finally, the suspended cells were incubated for 4 h in the dark, in a metabolic shaker at 37 °C. This assay is based on the production of progesterone and black/blue dye formation following a colour reaction with the 3β-HSD enzyme (Figure 1). Staining for 3β-HSD activity was carried out on culture dishes at the end of culture, and 3β-HSD activity was detected using the objective (200×) on a dissection microscope with a mounted camera. The degree of staining (size and darkness of brown deposits) was subjectively evaluated across treatments under exactly the same conditions.

2.3. Progesterone assay

Progesterone concentrations in the used media were measured by radioimmunoassay using a progesterone RIA kit (Biosource Europe SA, Nivelles, Belgium) as previously described (20). The limit of assay sensitivity was 0.05 ng/mL and the intra- and inter-assay coefficients of variation were 7.5% and 10.5%, respectively.

2.4. Statistical analysis

All results were reported as means ± SEM of 3 cultures for each group and were considered statistically significant at $P < 0.05$. Progesterone synthesis was expressed as ng/10,000 cells. The number of cells was based on the cell numbers added to the culture at the beginning of each experiment. Statistical analysis was carried out using the General Linear Model (Proc GLM) and Fisher's least significant difference (LSD) within SAS, with serum presence in medium and hormone treatments as the 2 main factors.

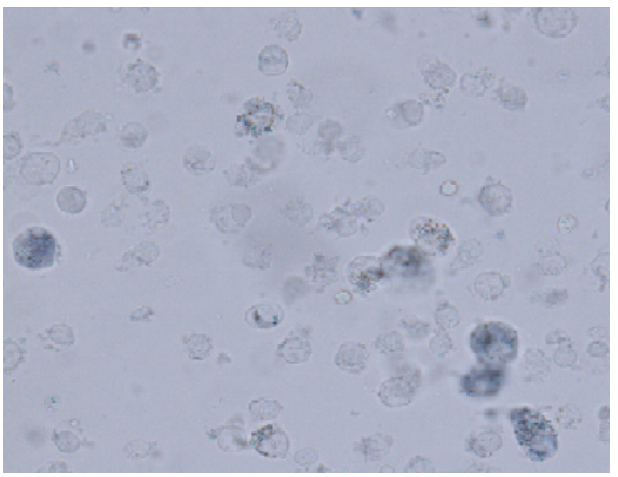

Figure 1. Cells stained for 3β-HSD activity before the first 48 h of incubation.

3. Results

3.1. Effect of FCS

Before the first 48 h of incubation, the granulosa cell suspensions harvested from large antral follicles were stained for 3β-HSD activity. Steroidogenic granulosa cells stained positive for 3β-HSD and usually lined individually (Figure 1). Granulosa cells treated in the absence of FCS gathered in loose clumps that contained some cells with 3β-HSD activity at the end of the culture (Figure 2A). However, cells incubated with FCS in the first 48 h were firmly attached with the typical phenotype of luteinised granulosa cells and a high proportion showed 3β-HSD activity staining at the end of the culture (Figure 2B).

3.2. Effect of IGF-I

The high dose of IGF-I increased ($P < 0.05$) progesterone secretion into the medium over 2.5-fold compared with FSH alone or the low dose of IGF-I in granulosa cells preincubated with FCS (Figure 5). This was mirrored by the degree of 3β-HSD staining in these cells at 144 h of culture (Figures 3A and 3B). No significant effect was seen ($P > 0.05$) following culture under serum-free conditions (Figure 4).

3.3. Effect of FSH and LH

None of the doses of LH and FSH had any effect on progesterone production by granulosa cells on day 6 of incubation in serum-free conditions or following preincubation with FCS (Figures 4 and 5).

4. Discussion

This is the first study that showed the activity of 3β-HSD associated with progesterone production in bovine granulosa cells by 3β-HSD staining. Similarly, 3β-HSD activity has been shown in ovine (3), caprine (5), and feline (4) luteal cell culture studies using the same method.

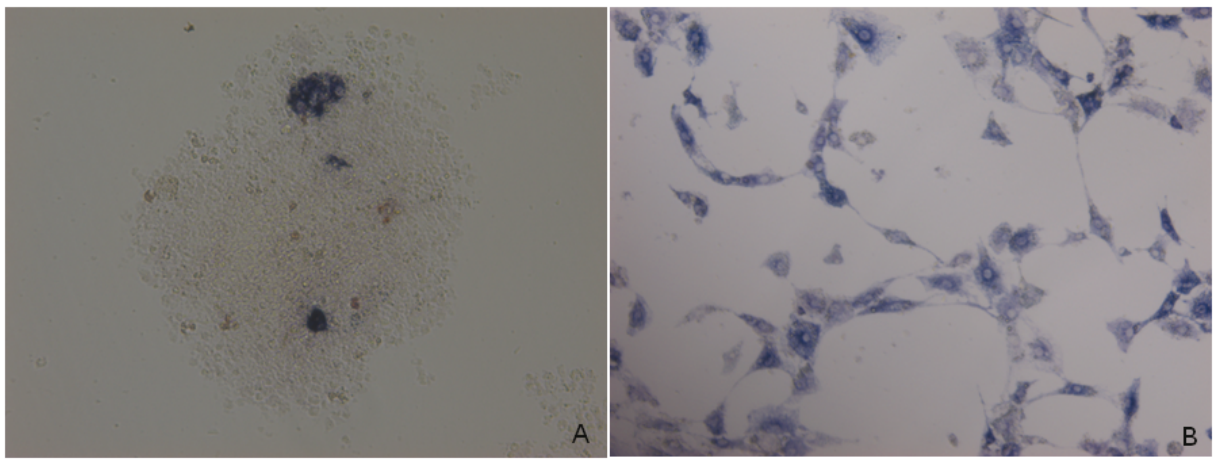

Figure 2. Cells stained for 3β-HSD on a culture plate on day 6 of incubation. A: in the absence of FCS. B: in the presence of FCS.

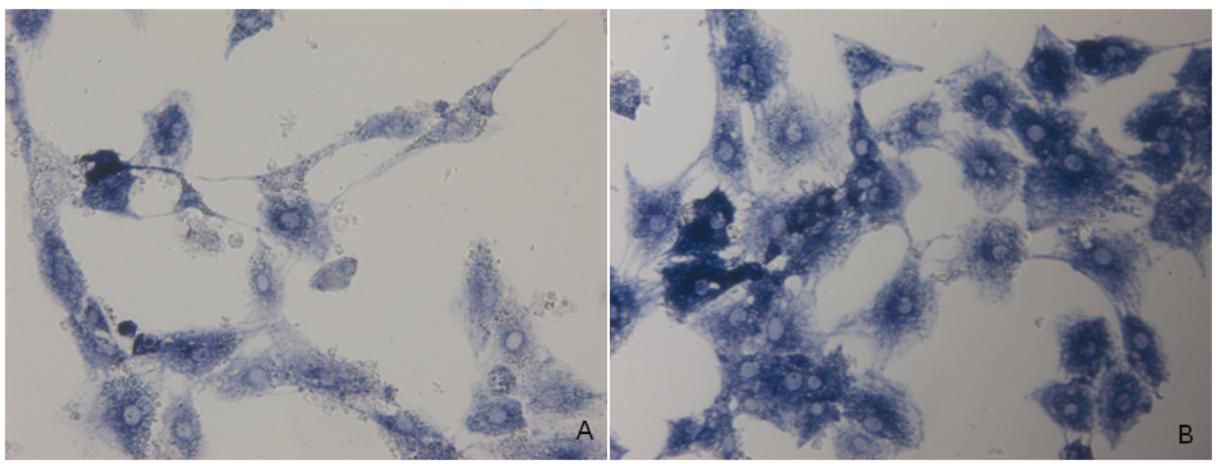

Figure 3. Cells stained for 3β-HSD on a culture plate on day 6 of incubation. A: treated with 1 ng/mL FSH + 1 ng/mL IGF-I. B: treated with 1 ng/mL FSH + 10 ng/mL IGF-I.

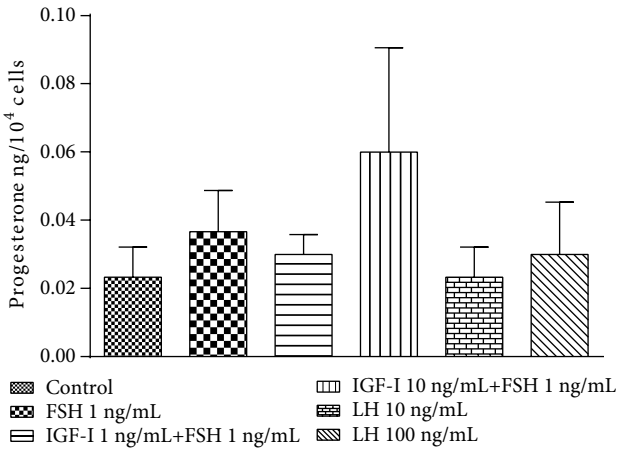

Figure 4. The effect of FSH, IGF-I, and LH on progesterone production of bovine granulosa cells on day 6 of culture in a serum-free medium.

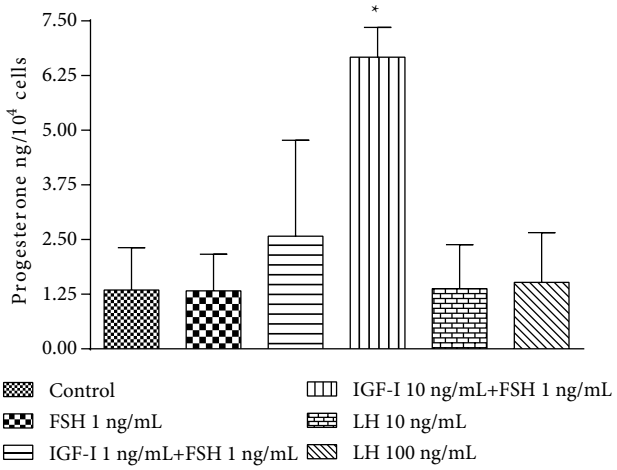

Figure 5. The effect of FSH, IGF-I, and LH on progesterone production of bovine granulosa cells on day 6 of culture after preincubation with FCS. An asterisk indicates a significant difference.

In the present study, the morphological appearance of bovine granulosa cells shown under different serum conditions was similar to that of a previous study published by Gutierrez et al. (12). Granulosa cells treated in the absence of FCS were grouped in clumps that contained some cells with 3β-HSD activity. However, 3β-HSD staining showed that cells preincubated with FCS were firmly covered on the cell culture plate surface with the typical phenotype of luteinised granulosa cells.

A high dose of IGF-I increased progesterone production over 2.5-fold by bovine granulosa cells from large follicles in the present study. This is in concurrence with previous studies that found IGF-I stimulates progesterone production in bovine granulosa cells cultured in vitro (15,21,22). In addition, this increase in progesterone production was reflected in the degree of 3β-HSD staining, with cells exposed to high IGF-I stained much darker compared with the granulosa cells exposed to the low dose of IGF-I and preincubated with FCS in this study.

None of the doses of LH and FSH had any effect on progesterone production of bovine granulosa cells obtained from large follicles on day 6 of incubation. Similar results related to the effect of FSH were reported for bovine granulosa cells by Langhout et al. (22), whereas Jimenez-Krassel and Ireland (23) observed increased progesterone production in response to FSH at dose levels of 1, 10, and 100 ng/mL by bovine granulosa cells from all follicle size categories. In contrast to Xu et al. (24) and Ginther et al. (25), who reported that granulosa cells from follicles >8 mm in size express LH receptors, in this study there was no response in the progesterone production of large bovine granulosa cells exposed to different doses of LH.

In conclusion, measuring 3β-HSD activity before and after in vitro culture appears to be an accurate indicator of the progesterone-producing capacity of live bovine granulosa cells. If quantifiable, this method has the potential to detect the in situ luteinisation status of granulosa cells, as well as the luteinising effect of hormones administered during in vitro cell culture.

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