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The effects of LH on progesterone production by cell subpopulations isolated from early and late luteal phase goat corpora lutea

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Abstract: The objective of this study was to investigate the complementary effects of luteinizing hormone (LH) on cell subpopulations. Corpora lutea were collected on days 5 and 15 of the cycle. The luteal tissues were treated with collagenase for cell dissociation. Percoll density-gradient centrifugation was performed to separate cells into 2 distinct subpopulations. Luteal cells (5 × 104 cells/well) were cultured with and without 22(R)-hydroxycholesterol (22R-HC, 10 µg/mL) and LH (10 and 100 ng/mL) alone, and also with 22R-HC (10 µg/mL) plus LH (10 and 100 ng/mL), in a serum-free culture medium for 6 days of incubation. Untreated cells isolated from luteal tissue collected on day 15 of the estrous cycle produced more steroids than those collected on day 5. Treatment of the cells with LH resulted in a significant ($P < 0.05$) increase in steroid synthesis on both days 5 and 15 of the cycle. Throughout the 7 days of incubation, 22R-HC caused a significant increase in progesterone production compared to untreated controls and to the LH treatments ($P < 0.01$). As a result, it appears that LH is mainly effective on small luteal cells in both the early and late luteal stages in goats.

Key words: Luteal cells, goat, progesterone, cholesterol, luteinizing hormone, ovary

1. Introduction

The corpus luteum plays an important role in the regulation of the goat estrous cycle. This role is performed largely by progesterone synthesized by this temporary endocrine gland. If the ovum is not fertilized, the corpus luteum regresses and allows a new estrous cycle to proceed. The mature corpus luteum is composed of heterogeneous cell populations that differ in steroidogenic capability, cell size, and appearance of cell organelles. Luteal tissue consists of steroidogenic and nonsteroidogenic cell populations including blood cells, fibroblasts, and endothelial cells (1,2). Steriodogenic cells in the mammalian corpus luteum are classified into 2 categories: large luteal cells and small luteal cells, each with different morphologies and functions (1,3,4). 3β-Hydroxysteroid dehydrogenase (3β-HSD) is an enzyme that catalyzes the synthesis of progesterone from pregnenolone (5). Thus, steroidogenic cells can be identified by the determination of 3β-HSD activity during the cell counting process (6). In goats, as in other mammals, the luteal cell size progressively increases as more and more small-sized cells achieve the process of cell differentiation to form large luteal cells during the progress of the luteal phase (2,3).

Percoll is a substance commonly used for luteal cell fractionation and enrichment in pigs (7), humans (8,9), and goats (10). It was reported that there were no significant differences between cells isolated from corpora lutea collected on days 5 and 15 of the estrous cycle in terms of progesterone accumulation. In contrast, cell fractions having mostly large cells produced more progesterone in comparison to cell fractions having mostly small cells (10).

As cholesterol is the precursor for the steroid hormones, supplementation of the culture media with cholesterol is obviously a factor in the control of the rate of progesterone synthesis. Cholesterol can be provided from either plasma lipoproteins or de novo cellular synthesis (11). The cholesterol that arrives at the ovary via the blood stream is transported by either high-density lipoprotein (HDL) or low-density lipoprotein (LDL), depending on the animal species. LDL cholesterol accounts for the majority of blood cholesterol in pigs (12), whereas HDL cholesterol predominates in goats (13) and bovines (14).

There are a number of factors that contribute to the development and maintenance of the corpus luteum. Among them, luteinizing hormone (LH) is the most common agent studied in vitro in many species, including bovine (15), ovine (16), porcine (17), and rat (18) species. It is well established that the in vivo function of LH is primarily to stimulate the maturation and ovulation of antral follicles, and secondly to stimulate the development

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of luteal cells. Small luteal cells are derived from theca cells while large luteal cells are derived from granulosa cells (19). Both cell types produce progesterone, but they differ in their ability to secrete progesterone in the presence of LH. Although basal progesterone production is lower in small luteal cells than in large luteal cells, small luteal cells are more responsive to stimulation by LH than larger luteal cells (20,21).

The aim of the present study was to evaluate the effects of luteal age and cell fractionation on 22(R)-hydroxycholesterol (22R-HC), a membrane-permeable cholesterol analogue, and LH-stimulated progesterone production, and to examine the interaction between 22R-HC and LH in terms of stimulating progesterone biosynthesis in goat luteal cells.

2. Materials and methods

2.1. Animals

Eight healthy adult female goats were randomly divided into 2 groups in separate pens during the breeding season after the approval of the local ethics committee (Kırıkkale University, Animal Ethics Committee, 18.04.2008, No: 08/05). A fertile male goat was also kept in a third pen, located between the others, in order to induce estrus behavior. Female animals were checked for estrus by means of the male goat twice a day. Corpora lutea were collected from the animals following surgical operations in the early (day 5) and late (day 15) luteal stages of the estrous cycle of the animals kept in pens 1 and 2, respectively. Luteal tissues were transported to the laboratory immediately after removal from the ovary. Cold chain was maintained during processing of the tissue.

2.2. Preparation of cells for incubation

All chemicals used in cell dissociation were obtained from Sigma (Sigma-Aldrich Co., Munich, Germany). After dissecting the capsule of the corpus luteum, the luteal tissue was cut into small pieces. Cells were isolated by collagenase digestion as previously described (3). In brief, luteal tissues were dissociated in an aerated (with O_2 for 3 min) culture medium (Ham's F-12) containing bovine serum albumin (BSA, 0.5%), DNase (0.005%), collagenase (0.2%), and antibiotics. Dissociation of the luteal cells was performed by 4 successive 1 h incubations (90 rpm at 37 °C) in a shaking water bath (Julabo, Labortechnik GmbH, Seelbach, Germany). The pooled supernatants from 4 incubations were filtered through a 100-µm falcon cell strainer (BD Biosciences, San Jose, CA, USA) to separate undigested tissue debris.

Fractionations of luteal cells were carried out by Percoll density gradient centrifugation as previously described (7,10). In brief, Percoll gradients of 40% (v/v), 20% (v/v), and 10% (v/v) were prepared in 15-mL conical centrifuge tubes. The suspended cells (4 mL) were then transferred on the top of the discontinuous Percoll gradient and centrifuged in a fixed-angle centrifuge at 400 × *g* for 20 min. Cell debris, the first luteal cell fraction (having more than 85% small luteal cells), the second luteal cell fraction (having more than 85% large luteal cells), and blood cells were located at the top 10%, next 20% (10%/20% interphase), next 40% (20%/40% interphase), and bottom of the tubes, respectively. Cells of the first and second luteal cell fractions will be presented as smaller and larger luteal cells throughout this paper, respectively. After recovering the cells, pooled cells were then washed with culture medium to remove the Percoll.

Cell viability was determined by trypan blue. Cell counting was based on the number of steroidogenic cells, identified by staining cells for 3β-HSD activity as described previously (2,22). In brief, a small quantity (200 µL) of cell suspension was incubated in paraformaldehyde (1%) for 20 min. After centrifugation (400 × *g* for 5 min), the cell pellet was resuspended with 200 µL of staining solution (0.1 M PBS containing 1.5 mM NAD, 0.25 mM nitroblue tetrazolium, 0.1% BSA, and 0.2 mM 5β-androstene-3β-ol-17-one) and incubated in the dark, in a shaking water bath at 37 °C for 4 h.

2.3. Incubation of luteal cells

Luteal cells were incubated as previously described by Arikan et al. (10). In brief, cells (5×10^4 live cells/well) with a positive stain for 3β-HSD were cultured in 6-well tissue culture dishes in an incubator (CB150, Binder GmbH, Tuttlingen, Germany) containing 95% air and 5% CO_2. Each well included culture medium (2 mL), Dulbecco's modified eagle medium and Ham's F-12 at 1:1 (v/v), with fetal bovine serum (10%), HEPES (15 mM), antibiotics, and antimycotics. Cells used in the experiment were dissociated from corpora lutea collected from goats on days 5 and 15 of their estrous cycle. Cells were incubated without any treatment for 18 h. Thereafter, cells were treated with and without 22R-HC (10 µg/mL) and with LH (10 and 100 ng/mL) alone, and also with 22R-HC (10 µg/mL) plus LH (10 and 100 ng/mL), in serum-free culture media supplemented with ITS premix, which consists of insulin (10 µg/mL), transferrin (5.5 µg/mL), and sodium selenite (5 ng/mL). Each treatment consisted of 4 separate cell wells. Thereafter, the medium was changed every 48 h. Cells were incubated for up to 7 days. Used medium was kept frozen at –20 °C until assay for progesterone by radioimmunoassay (RIA).

During each incubation period, another identical group of steroidogenic cells was incubated in a separate culture dish to monitor cell growth. These cells were also stained for 3β-HSD activity on days 1, 3, 5, and 7 of incubation in culture dishes. Stained cells were monitored throughout the incubation on an inverted microscope (Olympus, Tokyo, Japan).

2.4. Progesterone assay and statistical analysis

Progesterone content in used medium was determined by using commercial progesterone RIA kits (BioSource Europe SA, Nivelles, Belgium). The limit of assay sensitivity was 0.06 ng/mL and the intra- and interassay coefficients of variation were 4.4% and 8.8%, respectively. The recovery varied between 93% and 105%.

Statistical analysis was conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Interactions between treatments were assessed by 2-way ANOVA followed by a Duncan test for multiple comparisons where appropriate. All results were expressed as mean ± SEM of 4 independent experiments for each cell group. Significance was defined as $P < 0.05$. Progesterone content of used medium was expressed as ng/50,000 cells. Cell counting was performed at the beginning of each of the experiments.

3. Results

3.1. Activity of 3β-HSD in luteal cells

Determination of 3β-HSD activity allowed the identification of steroidogenic luteal cells in freshly prepared cell suspensions, which contained both nonsteroidogenic and steroidogenic cells. Although most steroidogenic luteal cells were lined individually, nonsteroidogenic cells mostly occurred in clumps and stained negative for 3β-HSD activity (Figure 1). This method included only steroidogenic cells in the cell numbers, which are necessary for calculation of progesterone content synthesized by steroidogenic luteal cells.

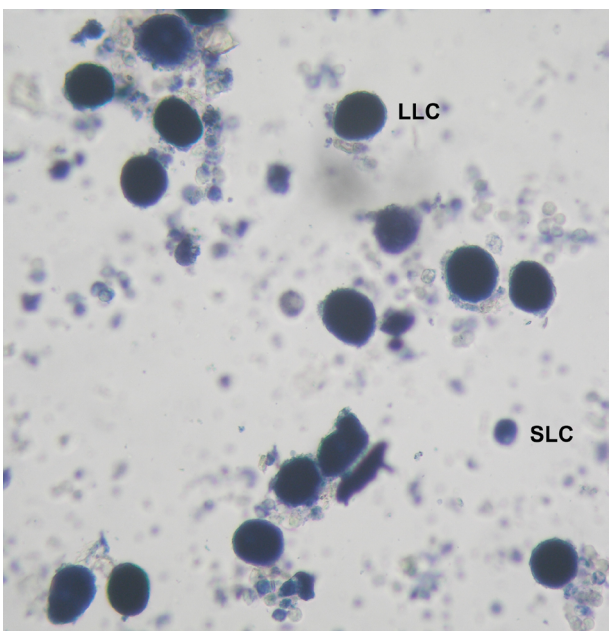

Figure 1. Cells stained for 3β-HSD activity in fresh prepared cell suspensions recovered from the 20%/40% interphase. LLC: Large luteal cell, SLC: Small luteal cell.

Staining the luteal cells attached to the bottom of culture dishes during the incubation period allowed us to monitor the border and nucleus of the steroidogenic cells. Cell monitoring showed that cell shape changed from round to elliptical during cell growth on the plate surface. We also observed that cell membranes protruded through the neighboring cells during cell development. In the case of cell damage, poor cell growth could easily be monitored on the bottom surface of the tissue plate (Figure 2).

3.2. Effect of LH on luteal progesterone biosynthesis

Cells isolated from early and late luteal phase corpora lutea were incubated with LH (10 and 100 ng/mL) in the absence or presence of 22R-HC (10 μg/mL) for 7 days. When cells were incubated without 22R-HC, progesterone biosynthesis decreased as incubation time advanced in all groups. By day 7 of incubation, the basal progesterone production of cells that were isolated from early and late luteal phase corpora lutea decreased to 42% and 44% of the starting value, respectively. In contrast, incubation of cells with 22R-HC led to significant increase ($P < 0.01$) in progesterone production during the 7 days of incubation in all groups. In comparison to the control groups, cholesterol-treated cells that were isolated on days 5 and 15 of the estrous cycle resulted in 1.8–3.98- and 1.65–2.95-fold increases in progesterone production, respectively. Cells collected from the 20%/40% interphase of Percoll layers produced 2.1-fold more progesterone in comparison to cells collected from 10%/20% interphase of Percoll layers in untreated cells on day 3 of incubation (Figures 3a, 3b, 4a, and 4b).

Incubations of the cells with LH or 22R-HC + LH both resulted in a higher progesterone production ($P < 0.01$) in comparison to the untreated groups on days 5 and 15 of the estrous cycle. In contrast, steroid synthesis in cells treated

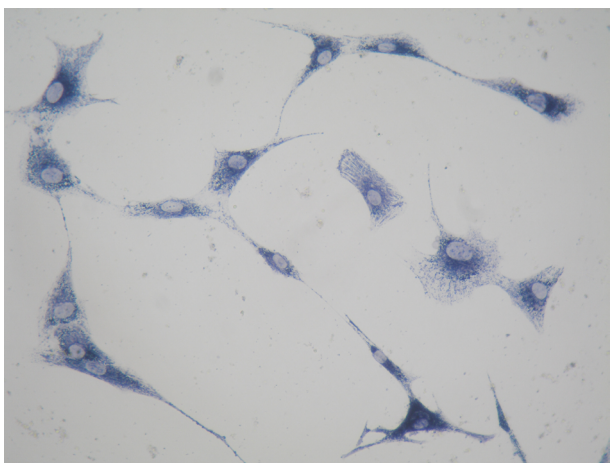

Figure 2. 3β-HSD activity of luteal cells growing on the culture dishes bottom. Picture was taken on day 3 of incubation. Cell-to-cell contacts and cell nuclei are easily recognized.

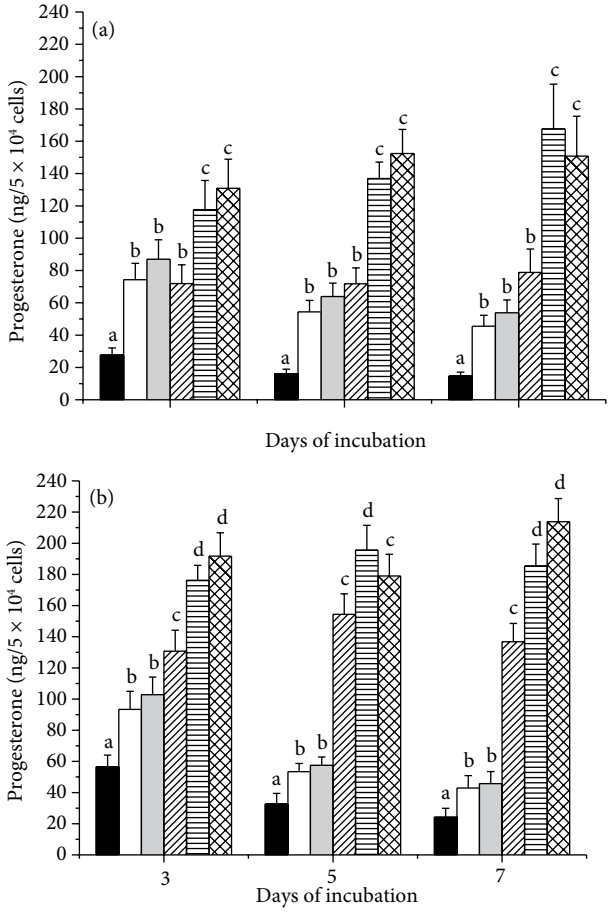

Figure 3. Effect of LH on steroidogenesis by 2 luteal cell subpopulations isolated on day 5 of goat estrous cycle. The first and second subpopulations of cells were collected from the 10%/20% interphase of Percoll layers (a) and 20%/40% interphase of Percoll layers (b), respectively. Control (■); 10 ng/mL LH (□); 100 ng/mL LH (▒); 10 µg/mL 22R-HC (▨); 10 µg/mL 22R-HC + 10 ng/mL LH (▤); 10 µg/mL 22R-HC + 100 ng/mL LH (▩). Results shown are the means ± SEM of data from 4 independent experiments. Within each day, groups labeled with different letters are significantly different from each other ($P < 0.05$).

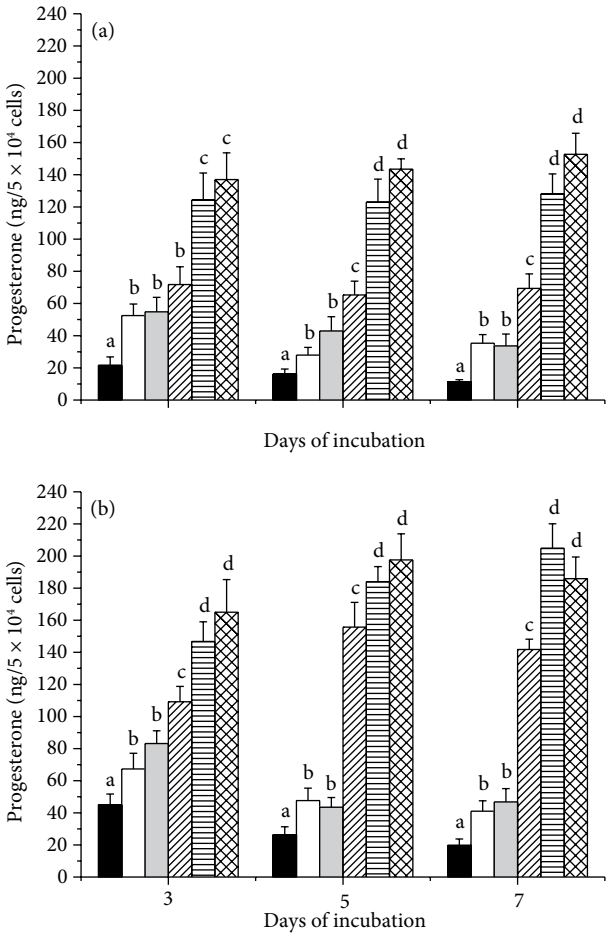

Figure 4. Effect of LH on steroidogenesis by 2 luteal cell subpopulations isolated on day 15 of goat estrous cycle. The first subpopulations of cells were collected from the 10%/20% interphase of Percoll layers (a). The second subpopulations of cells were collected from the 20%/40% interphase of Percoll layers (b). Control (■); 10 ng/mL LH (□); 100 ng/mL LH (▒); 10 µg/mL 22R-HC (▨); 10 µg/mL 22R-HC + 10 ng/mL LH (▤); 10 µg/mL 22R-HC + 100 ng/mL LH (▩). Results shown are the means ± SEM of data from 4 independent experiments. Within each day, groups labeled with different letters are significantly different from each other ($P < 0.05$).

with 10 ng/mL and 100 ng/mL LH in all groups was similar ($P > 0.05$). Treatment of the cells with LH (100 ng/mL) + 22R-HC induced a 13-fold increase in progesterone synthesis compared to control groups (Figures 3a, 3b, 4a, and 4b).

4. Discussion

This is the first study of effects of LH on progesterone biosynthesis by dispersed luteal cells isolated from early and late luteal phase goat corpora lutea. There is a paucity of studies investigating luteal cell activity in cultured goat luteal cell culture. Thus, we have very restricted knowledge about cell cultivation and steroidogenesis in caprine luteal cell cultures (10,23,24). We have therefore studied the effects of cell separation and luteal age on LH-stimulated steroidogenesis in cell cultures that were incubated with or without 22R-HC.

Two distinct strips of steroidogenic luteal cells were separated from corpora lutea in both the early and late luteal phase in the present study. Since the cells of these 2 bands differed in cell size and density, the 2 bands represent small and large luteal cell enriched fractions. A similar relationship between cell size and density has been reported by previous studies carried out on humans (9,25) and pigs (7).

Basal progesterone synthesis diminished as incubation time advanced in all groups of untreated cells (Figures 3a, 3b, 4a, and 4b). A similar decline in steroid synthesis has been reported in previous culture studies performed in bovines (26), ovines (27), felines (28), and goats (10). It is unlikely that the decline in progesterone production throughout the culture observed in this study could be related to the low capacity of de novo synthesis of cholesterol in culture. In the present study, larger cells produced basal progesterone levels 1.6- to 2.1-fold greater than smaller cells. These results are supported by several previous studies performed on human (25), goat (10), and bovine cells (21). These studies suggest that in mature sheep corpora lutea, large luteal cells contain 1.8-fold more mitochondria per unit volume of cytoplasm (29). This may explain why large luteal cells produce more progesterone than small luteal cells.

As luteal synthesis of progesterone requires cholesterol that can be derived from either plasma lipoproteins or de novo cellular synthesis (11), luteal cells were treated with 22R-HC to maintain a high level of progesterone synthesis throughout 7 days of culture. Thus, when cells were supplemented with 22R-HC, progesterone accumulation remained constant as incubation progressed. We have previously studied dose-dependent effects of 22R-HC on luteal steroidogenesis in cats (28) and goats (10). In light of those 2 studies, we made a decision to use a dose of 10 µg/mL of 22R-HC in this present study.

In this study, although LH treatments induced increased progesterone production in all treated luteal cells, the smaller luteal cells showed a greater response to LH stimulation than the larger cells in both in the early and late luteal phases. It is reported that small luteal cells have many more receptors for LH than larger luteal cells (4). This may explain why small luteal cells are more responsive to LH than larger luteal cells. LH receptors were not quantified in the present study, but based on previous research (30), the effects of LH are limited by the number of receptors for LH on the luteal cells, which might be the reason for the similarity of the effect of LH on steroid synthesis of cells treated with 10 ng/mL and 100 ng/mL LH.

In conclusion, this study indicates that 22R-HC supplementation of luteal cell cultures is important not only to maintain progesterone production during longer periods of incubation but also to augment the effects of LH. It would appear that LH is mainly effective on small luteal cells in both the early and late luteal stages in goats. These results also indicate that sufficient cholesterol, LH, and LH receptors on cells are necessary for optimal synthesis of luteal progesterone.

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