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Effects of dbcAMP on progesterone synthesis by cultured goat luteal cell subpopulations isolated from early and late luteal stage corpora lutea

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Abstract

This research aimed to investigate the effects of dbcAMP on steroid accumulation by culturing two distinct luteal cell subpopulations isolated from early and late luteal stage corpora lutea. Cells were isolated from corpora lutea collected from eight Angora goats on either the 5th or 15th days of their estrous cycles. Cell isolation was performed by enzymatic digestion using collagenase and DNase. Isolated cells were separated into two distinct subpopulations enriched with small and large luteal cells by percoll density-gradient centrifugation. Isolated cells were stained in order to detect 3β-hydroxysteroid dehydrogenase (3β-HSD). Cells stained positively for 3β-HSD activity (5 x 10^4 cell/well) were incubated with dbcAMP in the absence or presence of 22(R)-hydroxycholesterol (22R-HC) for periods of up to 7 days. Large luteal cell enriched subpopulations produced more basal progesterone ($P < 0.05$) than did the small luteal cell enriched subpopulations. Treatment of cells with 22R-HC alone induced 4.00 to 11.60 times increase in steroid synthesis depending on type of cells incubated, luteal age and days of incubation. Incubation of cells with 1 mM dbcAMP in the absence or presence of 22R-HC induced in a significant increase ($P < 0.01$) in steroid accumulation in all treated groups. In contrast, when cells are treated with low dose dbcAMP (0.1 mM), treatment induced stimulation failed to reach significant level in most treated groups. In conclusion, although treatment of goat luteal cells with dbcAMP induces an increase in steroid accumulation, a high dose is necessary to reach significant levels. Stimulatory effect of dbcAMP on steroidogenesis maintains during long life culturing.

Keywords: cholesterol, dbcAMP, goat, luteal cells, progesterone.

Introduction

The corpus luteum is known to be a temporary endocrine gland that secretes progesterone during the luteal stage of the estrous cycle (Band *et al*., 1987; Arikan *et al*., 2010) and pregnancy (Arikan and Yigit, 2003) in goats. If fertilization does not occur, the corpus luteum regresses in order to allow larger ovarian follicles to mature. The corpora lutea of the goat comprises a diverse population of cells that differ in capacity of steroid synthesis and size in diameter (O'Shea, 1987; Arikan and Yigit, 2003; Kalender and Arikan, 2007). Size distribution of luteal cells having steroidogenic activity covers a wide spectrum of sizes, ranging from 5 to 37.5 µm and 5 to 45 µm in diameter in cyclic (Kalender and Arikan, 2007) and pregnant goats (Arikan and Yigit, 2003), respectively. We found a linear correlation between mean cell diameter and luteal age both in cyclic and pregnancy corpora lutea (Arikan and Yigit, 2003; Kalender and Arikan, 2007). Steroidogenic luteal cells have been classified into two groups as small luteal cells and large luteal cells, based on the size measurement of cells in diameter (Chegini *et al*., 1984; Lei *et al*., 1991). The cells having steroidogenic activity can be determined by testing 3β-HSD activity. This is due to the fact that the conversion of pregnenolone to progesterone requires 3β-HSD located largely in the endoplasmic reticulum of the luteal cells (Bao *et al*., 1995).

Luteal tissue also comprises non-steroidogenic cells such as macrophages, blood cells, endothelial cells and fibroblasts (O'Shea *et al*., 1989; Fields and Fields, 1996). It is well known that synthesis of luteal steroids requires cholesterol that can be derived either from plasma lipoproteins or *de novo* cellular synthesis (O'Shaughnessy and Wathes, 1985b). The predominant carrier of cholesterol in goat plasma is a high density lipoprotein (Vitic and Stevanovic, 1993). It is reported that incubation of goat and feline luteal cells with 22R-HC, a membrane permeable steroid precursor, induced a dose-dependent increment in steroid synthesis. When cells were treated with 22R-HC alone at a concentration of 10 µg/ml, it resulted in 4 to 11 times increases in steroid synthesis throughout 7 days of incubation (Arikan and Yigit, 2009; Arikan *et al*., 2010). In a study which lasted 24 h, Brannian *et al*. (1995) stated that culturing of porcine luteal cells with 22R-HC (25 g/ml) induces a 3-time increases in steroid synthesis in comparison with control cells. In a second short culturing study, Musicki *et al*. (1994) stated that culturing of luteal cells with 22R-HC induced in a dose-dependent increment in steroid accumulation in rats when cells were incubated with 1 to 10 µg/ml 22R-HC.

It has been previously demonstrated that incubation of dispersed luteal cells with dbcAMP, which is a membrane-permeable cAMP analogue, stimulates progesterone synthesis in bovine (O'Shaughnessy and Wathes, 1985a; Grazul-Bilska *et al*., 1996), rat (Tekpetey and Armstrong 1991), human (Carrascol *et al*., 1996), ovine (Borowczyk *et al*., 2007) and cats

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(Arikan and Yigit, 2009). It is also reported that incubation of rabbit ovarian fragments with dbcAMP stimulates steroid synthesis (Sirotkin *et al*., 2014). However, response of the cells to the treatment varies depending on both the breed studied and the dose of dbcAMP used. It is not known how goat luteal cells respond to the cAMP treatment. Thus, it is important to examine the effects of dbcAMP on steroidogenesis in goat luteal cell subpopulations isolated from early and late luteal phase corpora lutea. Therefore, present study is aimed to examine interaction among luteal age, luteal cell type, cAMP, and steroid synthesis.

Materials and Methods

Animals and tissue collection

Animals used in the present study were purchased from an Angora goat breeder. The research proposal has been reviewed and approved by the local ethics committee. Eight healthy adult female goats (3-4 years old and 35-40 kg) were randomly selected into two groups in separate pens throughout breeding season (the period between August to March) in Kırıkkale (latitude 39°52′45.6″N, longitude 33°26′45.1″E). A fertile billy goat was also accommodated in a third pen located between two pens of female goats for monitoring estrus cycle. To avoid mating, the abdominal region of the male goat was covered. Corpora lutea were surgically removed from the animals following laparoscopy on early (5th day) and late (15th day) luteal phase of the estrous cycle. Immediately (within 15 min) after surgical operation, the luteal tissues were transported to the laboratory in an ice-cold sterile medium containing 100 IU⁄ml penicillin, 100 µg⁄ml streptomycin, and 2.5 µg⁄ml fungizone (Arikan *et al*., 2010).

Luteal cell preparation

All chemical compounds used in preparation of cells were purchased from Sigma Chemical Company (Sigma-Aldrich, Co., Munich, Germany). All processes carried out during cell preparation were performed on laminar flow hood under sterile conditions. Luteal tissue was dispersed by collagenase digestion as previously described (Kalender and Arikan, 2007; Arikan *et al*., 2010) with minor modifications. Briefly, the luteal tissue was sliced into very small pieces after decapsulating corpora lutea. Sliced tissues were then transferred to an erlenmeyer flask for cell dissociation. The cells were then dissociated by four consecutive 1 h incubations at 37°C in a shaking water bath (90 cycles/min) in the aerated (with oxygen for 2 min) nutrient culture medium comprising 0.2% collagenase, 0.005% DNase, 0.5% bovine serum albumin (BSA), 100 IU⁄ml penicillin, 100 µg⁄ml streptomycin, and 2.5 µg⁄ml fungizone. Undigested tissue fragments were removed from the dissociated cells by filtering the pooled supernatants from four incubations through a cell strainer (100 µm, BD Biosciences, San Jose, CA, USA). Cells were then resuspended in phosphate buffered saline (PBS).

Cell fractionation

Percoll (Sigma-Aldrich, Co.) was used for the purpose of purification and fractionation of luteal cells by density gradient centrifugation. Cell fractionation was performed as previously described (Brannian, 1997; Arikan *et al*., 2010). Briefly, percoll gradients of 10, 20 and 40% (densities = 1.0188, 1.0318 and 1.0578 g⁄ml, respectively) were prepared in 15 ml conical centrifuge tubes. Cells suspended in PBS (4 ml) were gently stratified on top of the discontinuous percoll gradient. Tubes were centrifuged on a fixed angle rotor at 400 g for 20 min. Blood cells were located at the bottom of the tubes. In contrast, cell debris was fractionated on top of 10% interphase. Two luteal cell fractions were recovered from the 10⁄20% (small luteal cell enriched) and 20⁄40% (large luteal cell enriched) interphases. Finally, in order to remove percoll, pooled cells were washed with culture medium. Trypan blue was used to determine cell viability.

Culturing of luteal cell subpopulations

Cell counting was based on steroidogenic lutal cells that were identified by staining cells for 3β-HSD activity (Payne *et al*., 1980; Arikan and Yigit, 2002). Then, the cells were incubated as previously described by Arikan *et al*., 2010. Briefly, after counting steroidogenic cells on a hemocytometer, the cells (5×10^4 cells⁄well) were cultured in a humidified incubator (Binder GmbH, CB150, Tuttlingen, Germany), containing a mixture of 5% CO_2 and 95% air. Incubation of cells was carried out in plastic culture dishes (six-well) that include 2 ml medium [DMEM/F-12 with 15 mM HEPES containing 10% foetal bovine serum, penicillin (100 IU⁄ml), streptomycin (100 µg⁄ml) and fungizone (2.5 µg⁄ml)] in each well. Cells were permitted to attach to tissue culture plate without any treatment for the first 18 h. Thereafter, cells were cultured in a serum-free medium supplemented with ITS premix (10 µg⁄ml insulin, 5.5 µg⁄ml transferrin and 5 ng⁄ml sodium selenite) throughout culture for periods of up to 7 days.

Luteal cells, which were isolated from early luteal phase corpora lutea, were treated with dbcAMP (0.1 mM and 1 mM) in the absence or presence of 22R-HC for 7 days. Media were collected every 48 h and stored at -20°C. Progesterone levels in the conditioned medium were 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

All results are reported as mean ± SEM of four independent experiments for each treatment group. Steroid synthesis was expressed as ng⁄50.000 cells. All statistical analyses were carried out with SPSS 14.0 (SPSS, Chicago, IL, USA). Different treatments were assessed by ANOVA followed by Duncan test for

multiple comparisons when appropriate.

Results

Monitoring cell morphology

Determining 3β-HSD activity in fresh cell suspension allows identifying the steroidogenic luteal cells. Occurring mostly in clumps, non-steroidogenic cells were stained negative for 3β-HSD activity. In contrast, stained steroidogenic luteal cells usually lined individually (Fig. 1). This process allowed us to exclude non-steroidogenic cells during the cell counting process.

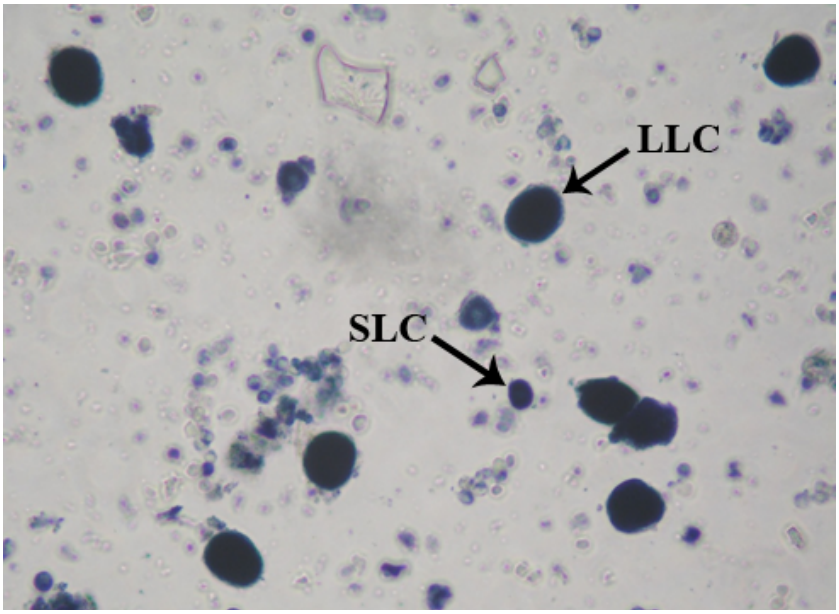

Figure 1. Picture of stained cells for 3β-HSD activity in cell suspension before incubation. (200X). LLC: Large luteal cell, SLC: Small luteal cell.

In order to monitor cell attachment and growth, cells on the bottom of the culture plate were also stained during incubation. Apart from the cell nucleus, during the cell growth we observed that the round shape of the cells change to elliptical. In addition to this, cell membrane protruded between the nearest cells during the cell growth (Fig. 2). Any cell damage or poor cell development could easily be monitored on the bottom surface of the plate after staining cells for 3β-HSD activity.

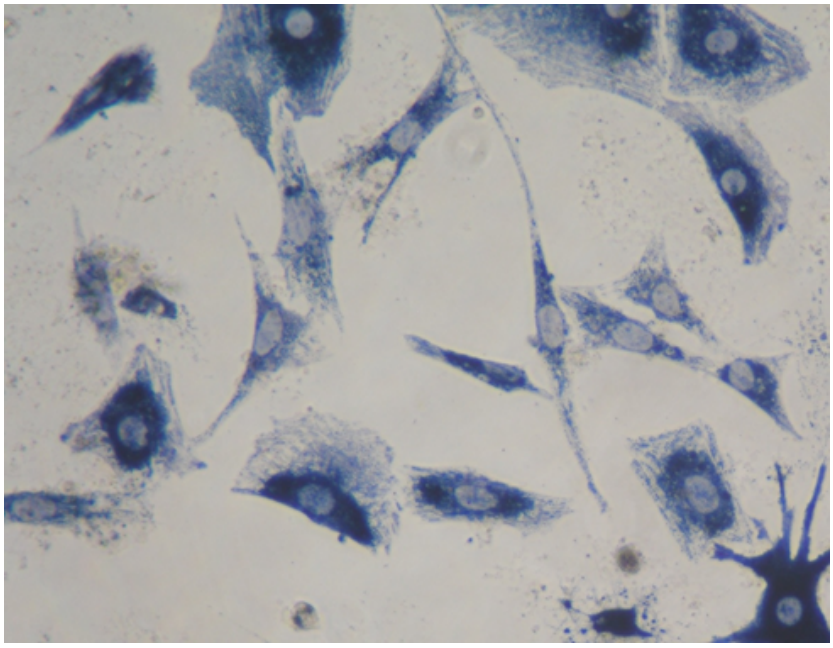

Fig. 2. Picture of stained cells for 3β-HSD activity on the bottom surface of the plastic tissue culture plate on day 7 of incubation (x400).

Effect of dbcAMP on progesterone accumulation by cells isolated from early luteal phase corpora lutea

Luteal cells maintained steroidogenic capacity in all groups of 22R-HC treated cells throughout culture periods. In contrast, as incubation time increased, steroid synthesis decreased in all groups of untreated cells. The cells treated with 22R-HC alone resulted in significant stimulation ($P < 0.01$) on progesterone synthesis in all treatment.

Treating cells with dbcAMP without 22R-HC resulted in a higher progesterone synthesis ($P < 0.05$) in comparison with the untreated groups on the 5th day of estrous cycle (Fig. 3). The stimulation varied between 1.50 to 2.50 times. In contrast, increase in progesterone synthesis in one of the groups treated with 0.1 mM dbcAMP failed to reach a significant level ($P > 0.05$) on the 3rd day of the incubation of cells collected from 20/40% of percoll layers (Fig. 3b).

Treating the cells with cholesterol showed a 4 to 8.50 times increase in progesterone accumulation in comparison to the control cells. Significant stimulation in steroid synthesis was seen in cells treated with 1 mM dbcAMP + 22R-HC during every treatment day. However, treatment of cells with 0.1 mM dbcAMP + 22R-HC failed to cause a significant effect on progesterone synthesis except on the 5th day of the incubation of cells collected from 20/40% of percoll layers (Fig. 3b). By the 7th day, basal progesterone production, in cells collected from 10/20% and 20/40% of percoll layer, decreased to 50 and 59% of starting value, respectively (Fig. 3).

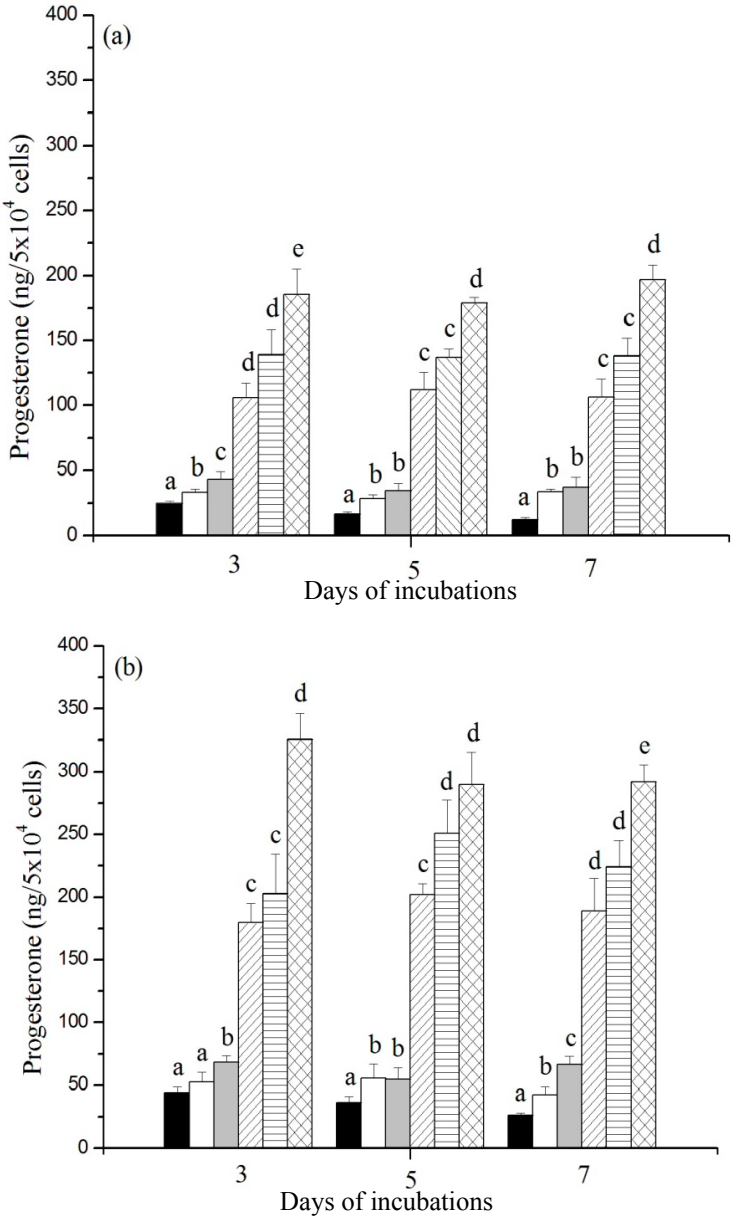

Figure 3. The effect of dbcAMP on progesterone synthesis by cells isolated from early luteal phase corpora lutea. (a) Incubated cells were harvested from 10/20% of percoll layers (b) Incubated cells were harv ested from 20/40% of percoll layers. Control (■), 0.1 mM dbcAMP (□), 1 mM dbcAMP (▩), 10 µg/ml 22R-HC (▨), 10 µg/ml 22R-HC + 0.1 mM dbcAMP (▤), 10 µg/ml 22R-HC + 1 mM dbcAMP (▧). Results are the mean ± SEM of 4 separate experiments. Groups with different letters above standard error bars are significantly different within each day ($P < 0.05$).

Effect of dbcAMP on progesterone accumulation by cells isolated from late luteal phase corpora lutea

Two different doses of dbcAMP (0.1 and 1 mM) were used to treat the cells. Treating cells with 1 mM dbcAMP in the absence or presence of 22R-HC increased ($P < 0.01$) progesterone accumulation significantly. In contrast, treatment of cells with 0.1 mM dbcAMP did not have a significant effect ($P > 0.05$) on progesterone synthesis in any treated group (Fig. 4).

Treating cells only with cholesterol resulted in a 4.40 to 11.60 times increase in progesterone accumulation in comparison to the untreated cells. In comparison to the cells collected from 10/20% of percoll layers, the cells collected from 20/40% of percoll layers produced more progesterone, in 22R-HC untreated cell groups. In addition, progesterone synthesis was decreased as incubation time increased in all the groups of untreated cells throughout culture (Fig. 4).

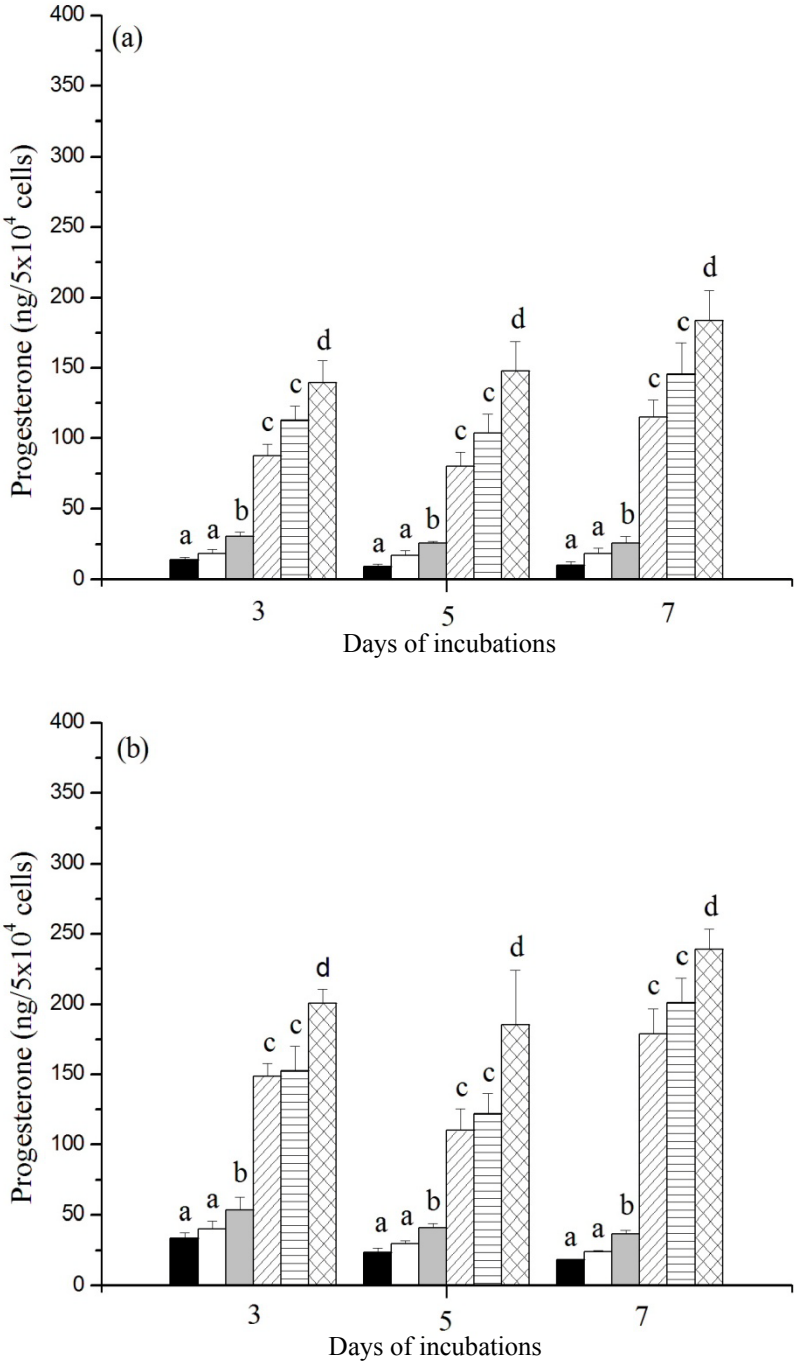

Figure 4. The effect of dbcAMP on progesterone synthesis by cells isolated from late luteal phase corpora lutea. (a) Incubated cells were harvested from 10/20% of percoll layers (b) Incubated cells were harvested from 20/40% of percoll layers. Control (■), 0.1 mM dbcAMP (□), 1 mM dbcAMP (▩), 10 µg/ml 22R-HC (▨), 10 µg/ml 22R-HC + 0.1 mM dbcAMP (▤), 10 µg/ml 22R-HC + 1 mM dbcAMP (▦). Results are the mean ± SEM of 4 separate experiments. Groups with different letters above standard error bars are significantly different within each day ($P < 0.05$).

Discussion

This is the first study regarding cAMP effects on luteal steroidogenesis in goats. Since response of luteal cells to the exogenous treatments might be affected by age and type of luteal cells, we used small and large luteal enriched subpopulations from early and late luteal corpora lutea as cell sources in this study.

In the present study, progesterone production was stable in the cholesterol treated group during the 7 days of culture period. However, contrary to this, the cholesterol levels were decreased over time in the control groups. A similar decrease in luteal progesterone synthesis was reported in various cell culture studies performed without cholesterol treatment on sheep (Grazul-Bilska *et al*., 1996), bovine (O'Shaughnessy and Wathes, 1985b; Arikan and Rodway, 2000) and goats (Arikan *et al*., 2010). On the 3rd day, no significant difference was found in terms of steroid synthesis between cells isolated from corpora lutea collected on early and late luteal stages. *In vivo* goat studies, in which similarities in terms of plasma progesterone contents were reported on the 5th and 15th days of the oestrus cycle, support this result (Medan *et al*., 2003; Gaafar *et al*., 2005). As expected, the effect of cholesterol was stimulatory in all cases. Cholesterol supply is an obvious factor in the control of the steroidogenesis rate as it is the precursor of progesterone (O'Shaughnessy and Wathes, 1985b). Depending on the type of incubated cells, luteal age and days of incubation, a 4.00 to 11.60 times increase in progesterone production is seen in the incubation of luteal cells with 22R-HC. Studies on ovine (Fitz *et al*., 1993), bovine (Arikan and Rodway, 2000), goats (Arikan *et al*., 2010), porcine (Brannian *et al*., 1995) and feline (Arikan and Yigit, 2009) support this result.

In this study, significant increase in luteal progesterone synthesis was induced with cells being treated with dbcAMP. Incubation of the cells with cAMP induced up to 2.98 times increase in progesterone release depending on used doses, cell types, luteal stage and days of incubation. Earlier studies also demonstrated that dbcAMP stimulated progesterone release in cultured cells isolated from corpora lute of rat (Tekpetey and Armstrong, 1991), bovine (O'Shaughnessy and Wathes, 1985a; Grazul-Bilska *et al*., 1996), ovine (Borowczyk *et al*., 2007), cats (Arikan and Yigit, 2009) and human (Carrascol *et al*., 1996). However, response of the cells to the treatment varies depending both on the dbcAMP dose used and on the breed studied. A 3.8 times increase in the progesterone synthesis has been reported in the incubation of midluteal bovine luteal cells with dbcAMP (1 mM; Grazul-Bilska *et al*., 1996). In another study, a 2 time increase in progesterone release was reported in which the same dose of 1 mM dbcAMP was used for the incubation of ovine luteal cells collected on the 5th day of the estrus cycle (Borowczyk *et al*., 2007). It is also reported that, in a culture of bovine luteal cells, dbcAMP (1 mM) might act to maintain progesterone synthesis at least for 7 days (O'Shaughnessy and Wathes, 1985a). This study supports our present results, in which goat luteal cells continued to produce progesterone for the 7 day incubation under the dbcAMP effect.

In conclusion, large luteal cell enriched subpopulations produced more basal progesterone than did the cells of small luteal cell enriched subpopulations. As expected, incubation of cells with 22R-HC induced a significant positive effect on progesterone synthesis. Both in the early and late luteal cell subpopulations, steroid synthesis was correlated with doses of cAMP. However, cAMP stimulated progesterone levels did not reach significant levels when cell is treated with 0.1 mM cAMP plus cholesterol.

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