Effects of forskolin and PGE_2 on progesterone secretion by goat luteal cells at early and late stages of corpus luteum

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ABSTRACT

The aim of this research was to examine the effects of forskolin and PGE_2 on steroid synthesis in goat luteal cells, cultured at early and late corpus luteum. Therefore, the luteal cells removed from both stages of the corpus luteum were cultured with newborn calf serum for the first 18 h. Then the media was changed and different concentrations of forskolin (10, 100 ng/ml) or PGE_2 (10, 100 ng/ml) were added to the fresh media for another 96 h. The culture media was replaced every 48 h and the retrieval media was kept frozen at -20 °C, until hormone analysis. Luteal cells treated with forskolin produced between 1.87-13.17 times higher production of progesterone, in a dose-dependent manner, compared to the control at early and late stages of corpus luteum ($P<0.05$). Lower dose of PGE_2 increased the progesterone secretion between 2.19-3.28 times in luteal cells compared to the control groups at the late stage of corpus luteum ($P<0.05$), but not at early stage. The cells treated with a higher dose of PGE_2 had no significant effect ($P>0.05$) on progesterone synthesis at the early and late phases of goat corpus luteum, in comparison to control groups. As a result, this study in goat luteal cells shows that forskolin promotes progesterone synthesis at the early and late corpus luteum, but PGE_2 is only effective in cells treated with a low dose at the late stage.

Introduction

Female goats in temperate northern hemisphere parallel have repeated estrous cycles at 21-day intervals from mid-September to the beginning of February. The mating season begins when the day length decreases. Progesterone levels change during the estrous cycle. While plasma progesterone levels in the luteal phase are between 4 and 8 ng/ml, they decline to under 1 ng/ml throughout the estrous and seasonal anoestrus (10, 28).

In goats, as in other mammals, the corpus luteum (CL) has a key role in the control of the estrous cycle by synthesizing progesterone. Luteal tissue, is formed by steroidogenic and other cell types, such as endothelial cells, fibroblasts and blood cells (4, 22, 26). 3β-Hydroxysteroid dehydrogenase (3β-HSD) enzyme transforms pregnenolone to progesterone in the endoplasmic reticulum (18, 20, 24). Thus, steroidogenic cells were stained to identify 3β-HSD enzyme activity throughout the cell counting procedure in the luteal and granulosa cell studies (3, 6, 27).

Forskolin is a complex natural product derived from the *Indian Plectranthus barbatus* plant. Forskolin resensitizes cell receptors by activating adenylylcyclase and raising intracellular cyclic AMP (cAMP) levels (1). Cyclic AMP is a key signal carrier required for cells to have a proper response to hormones and other extracellular signals. It acts by activating protein kinase A, which has several functions, such as regulation of glycogen, sugar and lipid metabolism in the cells. Forskolin was widely used in the cell studies because of this steroidogenic pathways (1, 16). Previous studies have been shown that forskolin treatments may affect steroid synthesis in luteal cells cultured from bovine (19), human (29), pig (12) and rats (9).

Prostoglandins are involved in tissue remodeling, steroid synthesis, and the regulation of neovascularization of luteinized follicles. Prostoglandins, especially PGE_2, play a key role in the ovulatory process (11). Fitz et al. (14) observed that prostoglandin receptors in ovine are much more effective on large luteal cells than small luteal cells. Consistent with this data, they found that the large luteal cells treated with PGE_2 produced more progesterone than the small ones (13). This result is similiar to a study found in porcine (25). In other study, Wiesak et al. (31) showed that culture of mixed porcine luteal cells with PGE_2 enhanced progesteron secretion. None of these studies on forskolin and PGE_2 provide in-vitro effects on steroid production in luteal cells at the early and latest ages of goat corpus luteum.

The objective of this study was to investigate the effects of forskolin and PGE_2 on progesterone synthesis in goat luteal cells cultured from early (day 5) and late (day 15) stages of corpus luteum.

Materials and Methods

Animals: In this study, one male and eight female Angora goats were housed at Kırıkkale University following the animal experiments local ethics committee approval (2008/05). Female goats were placed into two groups. In addition, male goat was also housed separately in a cage next to females to induce estrous. Female goats were monitored for estrous activity twice a day by using the male goat. To prevent mating during this procedure, the male goat's waist was wrapped with a cloth. Following the estrous behaviour, the corpus luteum was surgically removed at an early (day 5) and a late stage (day 15) of the reproductive cycle.

Cell dissociation: Corpus luteum was transported to the laboratory immediately under cold chain and sterile conditions following the removal from the ovary. Firstly, the capsule of the CL was removed. Then luteal cells were separated by using the collagenase enzyme as outlined previously (3). Briefly, luteal tissues were chopped in HAM'S F-12 media including antibiotic mixture. Tissue pieces were taken into an Erlenmeyer flask and then added to HAM'S F-12 medium, which was aerated with O_2 for 3 min, containing 0.005% DNase I Type IV, 0.2% collagenase Type I, 0.5% bovine serum albumin (BSA) and 1% antibiotic/antimycotic mixture. Tissues in the flask were kept in a shaking machine (90 rpm, 37 °C) water bath for 1 h (Julabo, Labortechnik GmbH, Seelbach, Germany). The supernatant was taken into a 50 ml falcon tube after incubation. This dissociation process was repeated four times to completely detach the cells. Finally, the undigested tissue pieces were eliminated by filtering the extract collected from four cultures through a disposable strainer suitable for cells. All chemicals used in the cell dissociation were purchased from Sigma Chemical Company (Sigma-Aldrich, Co., Munich, Germany).

Steroidogenic cell identification: To identify the steroidogenic activity, luteal cells were stained for 3β-HSD enzyme according to the method reported by Arikan et al. (3). Shortly, luteal cells were fixed in 1% paraformaldehyde at 37 °C for 20 min. After that, cell suspension was centrifuged for 5 min at 400 g to remove the paraformaldehyde. Finally, the luteal cells were kept in staining solution (0.1 M phosphate buffered saline including 0.25 mM nitro blue tetrazolium, 0.1% BSA, 1.5 mM nikotinamide adenine dinucleotide hydrate, and 0.2 mM 5β-androstene-3β-ol-17 one) in the dark for 4 h at 37 °C.

Cell incubation: Luteal cells dispersed from corpus luteum were incubated as previously stated (2). Briefly, steroidogenic cells ($5x10^4$ live cells⁄well) having a positive 3β-HSD staining were incubated in culture plates with a six-well (Corning Life Sciences, Netherlands) in CO_2 incubator (Binder GmbH, CB150, Germany). Each well was filled with 2 ml medium (DMEM/F-12), including 10% newborn calf serum, 1% antibiotic/antimycotic mixture. The cells were firstly incubated for 18 h without treatment. After the first incubation the media was replaced with serum-free media, including ITS mixture (5.5 mg⁄ml transferring, 5 ng⁄ml sodium selenite, 1 mg⁄ml insulin), and plus the specific forskolin (10, 100 ng/ml) and PGE_2 (10, 100 ng/ml) concentrations for another 96 h. The doses of forskolin and PGE_2 used in the study were determined based on previous studies (1, 15). During treatment incubation, the media was replaced every 48 h and the retrieved media was stored at -20 °C until progesterone analysis. The same process was applied for the four independent cell cultures.

Monitoring cell growth: Apart from the treatment groups, an extra culture plate was incubated to check cell growth throughout the incubating period. Luteal cells on the culture dish were stained for 3β-HSD enzyme on days 3 and 5 of the culture as described before (5). After staining, culture dish was inspected by an inverted microscope (Olympus, Tokyo, Japan) to assess the attachment and growth of luteal cells incubated.

Progesterone assay and statistical analysis: The concentrations of progesterone in collected medium from all groups were analyzed by radioimmunoassay (RIA) using a kit specified for progesterone (Biosource Europe SA, Nivelles, Belgium). The manufacturer's instruction was followed. Coefficients of variation for the intra- and inter- assay were 4.2% and 8.5%, respectively. Assay sensitivity was 0.05 ng/ml. The recovery rate ranged from 92% to 103%.

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In this study, SPSS (version 14.0) was used for data analysis. The results were shown as mean ± standard error of the mean (± SEM) of four independent experiments and were considered significant at 5% ($P<0.05$). The progesterone levels produced by luteal cells were reported as ng⁄50.000 cells. The statistically significance of the differences among groups was investigated by using ANOVA followed by Duncan multiple range test.

Results

Cell staining: The cells having steroidogenic activity were stained before and after the incubation as blue/black dye following the reaction with 3β-HSD enzyme (Figure 1). The nucleus of cells and cell borders were monitored easily with this staining. Additionally, this staining procedure also allowed us to keep track of the cell attachment and the growth during the culture. The round shape of the cells was transformed into an elliptical shape, as cell membranes extended to the closest cells throughout the growth on the culture plate (Figure 2). In the case of any destruction or attachment problems of the cells during incubation could be monitored with this staining procedure.

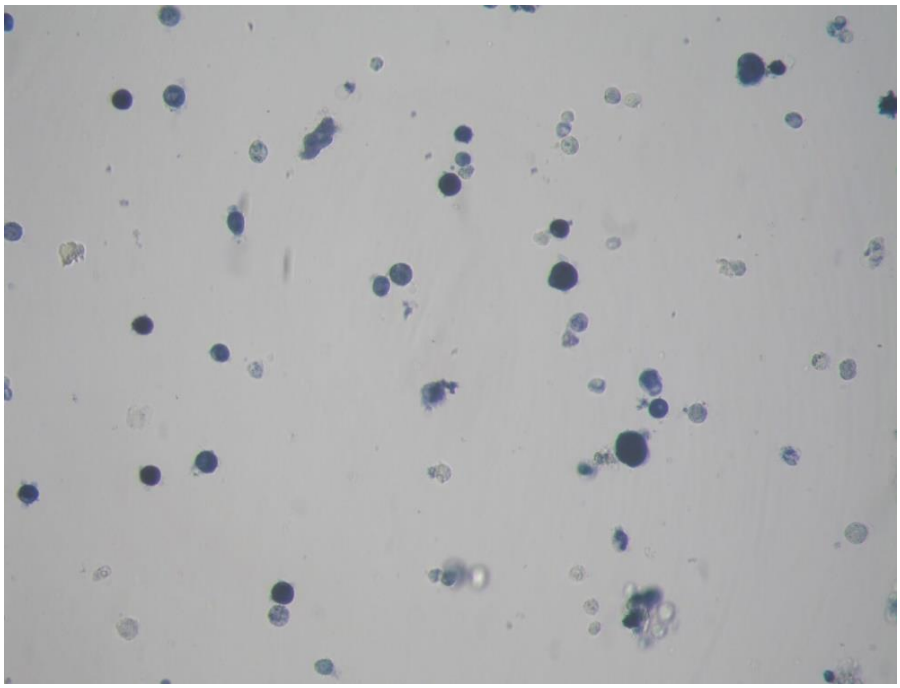

Figure 1. Image of cells stained for 3β-HSD activity in a cell suspension before incubation (magnification x200).

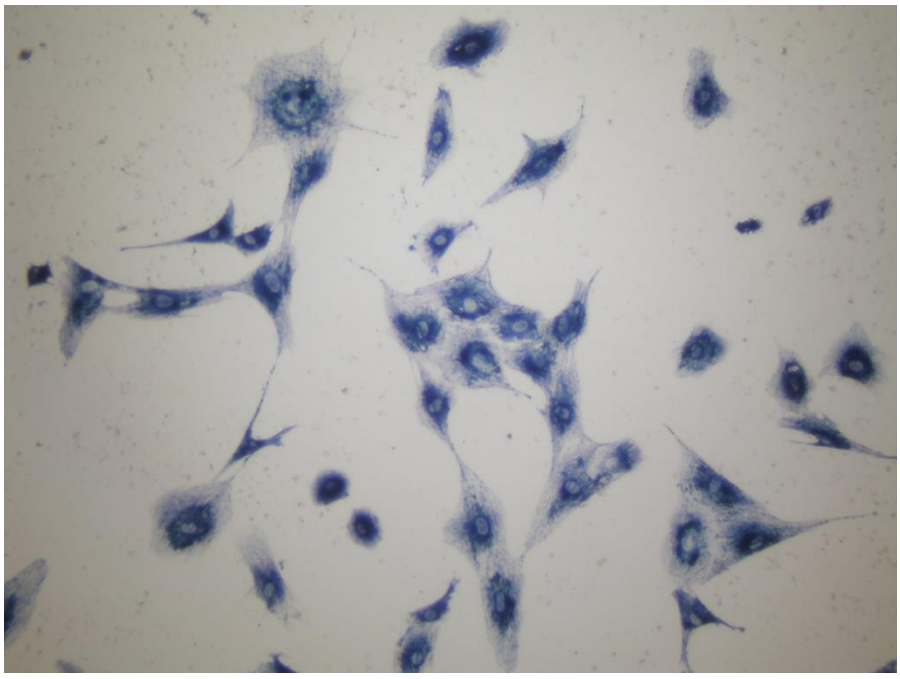

Figure 2. Image of cells stained for 3β-HSD activity on the bottom of the culture plate on day 5 of incubation (magnification x200).

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Cell treatments: The cells at early and late corpus luteum were cultured with low and high dose (10 and 100 ng/ml) of forskolin or PGE_2 for 4 days after the first incubation of 18 h. By day 5, the basal progesterone synthesis in untreated cells on days 5 and 15 of the reproductive cycle declined to 50% and 61% of the beginning value, respectively (Figure 3, 4).

The luteal cells incubated with different doses of forskolin resulted in the increase of progesterone synthesis ($P<0.05$) compared to the control on day 5 and 15 of the reproductive cycle (Figure 3, 4). This progesterone increase in cells treated with forskolin was between 1.87-4.54 and 3.26-13.17 fold on days 5 and 15 of estrous cycle, respectively. The cells incubated with high dose of forskolin produced significantly more progesterone than lower dose in both early and late stages of corpus luteum ($P<0.05$). This significant increase in cells treated with high dose forskolin was between 1.90-4.04 fold compared to the lower dose forskolin.

Lower dose of PGE_2 treatments resulted in 2.19-3.28 times more progesterone production compared to control groups at late stage of corpus luteum ($P<0.05$). The cells treated with higher dose of PGE_2 had no effect on progesterone synthesis at early or late phase of corpus luteum in comparison to the control groups (Figure 3, 4). Additionally, there was no significant difference between different doses of PGE_2 according to luteal steroid synthesis in the both groups of corpus luteum ($P>0.05$). When forskolin and PGE_2 treatments in luteal cells were compared, higher dose of forskolin had more progesterone secretion ($P<0.05$) than both doses of PGE_2 on day 5 and 15 of the reproductive cycle (Figure 3, 4).

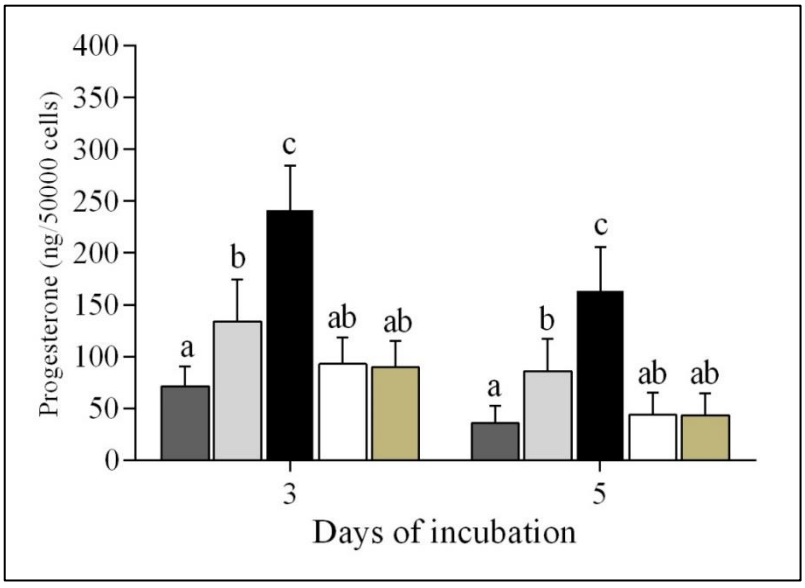

Figure 3. Effects of forskolin and PGE_2 on progesterone secretion by goat luteal cells collected at early stage of corpus luteum. Control (■), 10 ng/ml forskolin (■), 100 ng/ml forskolin (■), 10 ng/ml PGE_2 (□), 100 ng/ml PGE_2 (■). Letters indicate the difference significantly ($P<0.05$).

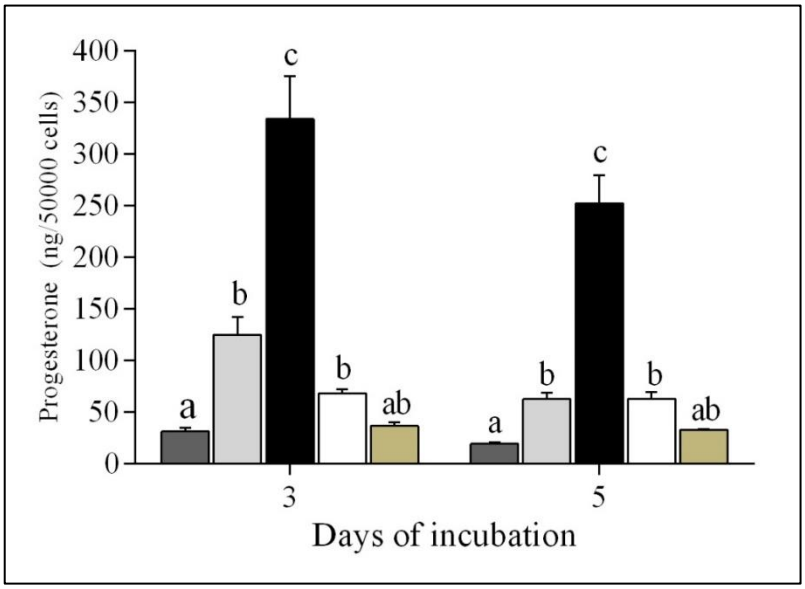

Figure 4. Effects of forskolin and PGE_2 on progesterone synthesis by goat luteal cells collected at late stage of corpus luteum. Control (■), 10 ng/ml forskolin (■), 100 ng/ml forskolin (■), 10 ng/ml PGE_2 (□), 100 ng/ml PGE_2 (■). Letters indicate the difference significantly ($P<0.05$).

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Discussion and Conclusion

This is the first report that focused on the effect of forskolin and PGE_2 on steroid production in early (day 5) and late (day 15) luteal stage of corpus luteum in goats. Although there are some studies in goat luteal cells, they are limited (3, 7, 8, 17, 23). None of them investigated the effects of forskolin and PGE_2 on progesterone synthesis in goats at the early and late luteal stages of the estrous cycle. Because of the paucity of literature, in this research, we examined the effect of forskolin and PGE_2 on progesterone synthesis in goat luteal cells on days 5 and 15 of reproductive cycle.

Steroid production in the cells isolated from goat corpus luteum was maintained for 5 days. However, basal progesterone secretion was decreased according to the incubation time in the control groups. Previous studies in luteal cells reported similar decrease in goats (17) and other species, such as bovine (2, 21) and cat (5). This decline might be due to the limited capacity in de novo cholesterol synthesis in goat luteal cells. Comparing the luteal stages of corpus luteum according to basal progesterone, in this work, luteal cells at early stage of corpus luteum produced more basal progesterone than the late stage, unlike our previous study in goats (17). This difference in our studies could be explained by incubating separately subpopulations of luteal cells in our previous study.

Forskolin is a natural product derived from the *Indian Plectranthus barbatus* plant. Alasbahi and Melzig (1) reported that forskolin resensitizes cell receptors by activating adenylylcyclase and increasing intracellular cAMP levels. Previous studies showed that forskolin induces the steroid production by using this steroidogenic pathway (1, 16). This effect was shown in luteal cells collected from human (29), pig (12), rats (9) and bovine (19). Similarly, in this study, forskolin treatments increased the progesterone synthesis between 1.87-13.17 fold compared to the control groups in the cells incubated from early and late corpus luteum. The cells treated with high dose of forskolin produced 1.90-4.04 times more progesterone than lower dose in both group of corpus luteum. These increases in steroid production could be explained that forskolin works as a cAMP pathway activator in luteal cells in goats like other species.

Prostoglandin E_2 play a key role in ovulation and work as a luteotrophic agent (11, 30). It has been shown in previous studies that PGE_2 induced more progesterone synthesis in large luteal cells than in small luteal cells from ovine (13) and pig (25). Additionally, Wiesak et al. (31) showed that incubation of mixed porcine luteal cells with PGE_2 enhanced progesterone secretion. Unlike these studies, in this research, lower dose of PGE_2 increased the progesterone synthesis in comparison to control group in the mixed luteal cells only at the late stage of goat corpus luteum. Prostoglandin E_2 had no effect on steroid production compared to untreated cells at the early phase of corpus luteum. Gregoraszczuk and Michas (15) have found similar results with our study in porcine luteal cells treated PGE_2 at early luteal stage. This could be explained that the late phase of corpus luteum includes more large luteal cells, which are rich with prostoglandin receptors, than small luteal cells.

In conclusion, this is the first time it has been shown by comparing the effects of forskolin and PGE_2 on progesterone production in early and late luteal stage of corpus luteum in goats. Culture of luteal cells with low and high doses of forskolin increased the progesterone production during five days incubation compared to the control groups at the both luteal stages. Incubation of cells with PGE_2 had no significant effect on steroid synthesis in comparison to untreated cells apart from lower dose PGE_2 treatments at the late stage of corpus luteum. These findings bring new insights to the understanding of the luteotropic effects of forskolin and PGE_2 in early and late stages of estrous cycle in goats.

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Conflicts of Interest

The authors declared that there is no conflict of interest.

Author Contributions

In general, all authors have partly contributed to all the aspects of research and analysis. Additionally, all authors provided critical feedback and assisted in the formation of this manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Kırıkkale University Animal Experiments Local Ethics Committee (Decision number: 2008/05).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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