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PROLIFERATIVE PROCESSES WITHIN THE EQUINE CORPUS LUTEUM MAY DEPEND ON PARACRINE PROGESTERONE ACTIONS

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Soon after ovulation, the corpus luteum (CL) starts secreting progesterone (P_4), a hormone necessary for implantation. The aim of the study was to evaluate whether P_4 exerts an autocrine/paracrine action on luteal angiogenic activity and P_4, prostaglandin E_2 (PGE_2) and NO production in the mare. Corpora hemorrhagica (CH) and mid-luteal phase CL (MCL) were cultured with (i) no hormone (Control); (ii) P_4; (iii) a P_4 precursor - pregnenolone; or (iv) a P_4 antagonist - onapristone [10^{-4}M;10^{-5}M; all steroids]. NO production decreased in MCL, with respect to CH, when treated with P_4 [10^{-4}M] and pregnenolone [10^{-5}M]. PGE_2 increased from CH to MCL, and showed a tendency to rise in pregnenolone treated luteal tissues (10^{-4}M; p=0.06). In the CH, P_4 decreased with pregnenolone [10^{-4}M] compared to control, P_4 [10^{-5}M], onapristone [10^{-4}M;10^{-5}M] and pregnenolone [10^{-5}M]($p<0.05$). In the MCL, pregnenolone [10^{-5}M] decreased ($p<0.05$) and P_4 tended to decrease (p=0.06) bovine aortic endothelial cell (BAEC) mitogenesis. Onapristone [10^{-4}M] increased BAEC proliferation with respect to P_4 (p=0.01). Since there was no direct effect of treatments on BAEC, these data suggest that long-lasting effects of P_4 and its precursor may inhibit angiogenic factor(s) production by equine MCL, preparing for CL functional and structural regression.

Key words: *corpus luteum, mare, nitric oxide, onapristone, progesterone, pregnenolone, prostaglandins.*

INTRODUCTION

Although the temporal pattern of pulsatile progesterone (P_4) is well established in the mare (1), the factors regulating P_4 synthesis and its actions on the corpus

lutem (CL) itself are not fully understood. In the mare, endocrine and vascular changes occur throughout physiologic growth and regression of the corpus luteum (2, 3). In this species, synthesis of P_4 starts early in the luteal structure, with a simultaneous increase in P_4 receptors (PR) in large luteal cells, luteal microvascularization, proliferating cell nuclear antigen (PCNA) expression, and large luteal cell count, until the mid-luteal phase (3, 4). The hormone P_4 has been shown to act as a luteotropin (5), maintaining the synthesis of this steroid in the ovary (6 - 8). Acting as an autocrine factor by a PR-dependent mechanism, P_4 suppresses the onset of apoptosis in the CL in several species (7 - 12).

Ovarian steroids and angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), endothelial growth factor and epidermal growth factor, are important modulators of nitric oxide (NO) synthesis (13 - 18). This important signalling molecule is a powerful vasodilator that has been known to play a role in the reproductive system (14, 19, 20). In the mare, NO appears to be involved in follicular growth and ovulation (20). Nitric oxide has also been shown to increase the production of prostaglandins in the bovine CL (21), and to decrease P_4 in the rat (22). Impaired steroidogenesis by NO has been also reported in the rabbit (23), human (24), and cow (25). Actually, NO may be involved in the autocrine/paracrine luteolytic cascade induced by PGF2α in the cow (25). However, in the ewe, NO may be antiluteolytic and prevent luteolysis (26), while in the rat it has a dual role, being protective in the mid luteal phase and pro-oxidant/luteolytic in the late luteal phase (27).

Besides being the major hormone of the luteal tissue, P_4 also regulates prostaglandin (PG) synthesis by the CL. The synthesis of prostaglandins in the early CL may have numerous physiological functions such as intercellular communication, regulation of blood flow or cellular differentiation (28), that have not yet been clearly understood, especially in the mare.

Since the role of P_4 on luteal function in the mare is still not clear, the main aim of the study was to evaluate whether P_4 exerts an autocrine/paracrine action on luteal angiogenic activity and in the production of P_4, prostaglandin E_2 (PGE_2) and NO.

MATERIAL AND METHODS

Mares

Starting on the Spring equinox until the end of August, luteal tissue and venous blood were collected *post mortem* at an abattoir from randomly designated cycling mares. Reproductive and clinical histories of all mares were unknown, but as assessed by veterinary inspection, they were in good physical condition for human consumption.

Collection and preparation of luteal tissues

Immediately after slaughter, ovaries were collected from 8 mares and luteal tissue structures were classified according to their macroscopic aspect as: early structures (corpora hemorrhagica, CH; n=4) and mid-luteal phase CL (MCL; corpora lutea associated with follicles 15 to 20 mm in diameter;

n=4). Small samples of corpora lutea were cut, rinsed thrice in sterile PBS with gentamycin [50μg/ml], and transported, on ice, to the laboratory in culture medium for further incubation. Culture medium consisted of Dulbecco´s Modified Eagle´s Medium and Ham´s F-12 medium (1:1, v:v), 0.1% BSA and gentamycin [50μg/ml] (all reagents from Sigma, St. Louis, MO, USA).

Treatment with Steroids

To determine whether endogenous P_4 exerts an autocrine/paracrine action on the CL angiogenic activity, exogenous P_4 and its precursor pregnenolone were tested *in vitro*. Besides, in order to evaluate if P_4 acts at the classical PR level, the action of the PR antagonist onapristone was also studied. In order to accomplish this, luteal tissue (CH=4; MCL=4), was minced into small explants (approximately $1mm^3$), weighed, rinsed in sterile PBS with gentamycin [50μg/ml], and 60mg of tissue were placed in 2ml of culture medium in polystyrene culture tubes (Sarstedt, Numbrecht, Germany). The assays were run in triplicate. Luteal tissues were pre-incubated for 1h in medium, on a shaker (Titertek; Huntsville, AL, USA; 150 rpm), in a tissue incubator (Biosafe Eco-Integra Biosciences, Chur, Switzerland; 37°C, 5%CO_2, 95% air). Under the same experimental conditions, 24h incubation was then carried out with either (i) no exogenous hormone (Control); or with (ii) progesterone (P_4); (iii) pregnenolone; or (iv) onapristone added to the culture media [10^{-4}M;10^{-5}M; all steroids]. These experiments were carried out in the presence of endogenous P_4. Negative controls consisted of culture media, without or with exogenous steroid hormones added, incubated in the absence of luteal tissue. Conditioned media by luteal tissues and from negative controls were stored at -70°C to be later analyzed for their ability to stimulate bovine aortic endothelial cell (BAEC) proliferation *in vitro* and for nitric oxide (NO), P_4 and prostaglandin E_2 (PGE_2) production.

Endothelial cell proliferation assay
Preliminary studies

The ability of media conditioned by equine luteal tissue to stimulate proliferation of BAEC was assessed by using Alamarblue™ reagent (Serotec Ltd, Oxford, UK). This reagent is designed to measure quantitatively the proliferation of various animal cell lines based on metabolic activity in response to a chemical reduction of a growth substrate and detection of cellular incorporation of a fluorometric/colorimetric growth indicator (29).

Since no specific information concerning the use of this cell proliferation indicator on BAEC was available, preliminary assays were carried out to determine the optimum incubation time of BAEC in 30% conditioned media. Briefly, BAEC were cultured in 30% conditioned medium in a 24-well culture plate (Nuclon-Nunc®) and incubated at 37°C, 5% CO_2 , 95% air, at $1x10^4$ cells/ml to $7x10^4$ cells/ml for 24, 48 and 72h. Then, culture medium was replaced by the same volume of DMEM (without phenol) containing 10% Alamarblue™. Absorbance was read at 570 and 600nm (SpectrMax 340 PC, Molecular Devices) every hour for 7h for determination of the percentage of indicator reduction. These preliminary results established the optimum BAEC incubation time to be 48h, since it was the time that showed a greater percentage of reduction of Alamarblue™ among the various cell concentrations and final cell count. Optimal reading time was after a 5h incubation, since it corresponded to the value where linear correlation between the percentage of reduction of the indicator and cell density was the highest (R^2=0.9507). This value was calculated according to Alamarblue™ manufacturer's instructions.

Samples testing for BAEC proliferation capacity

Bovine aortic endothelial cells ($2x10^4$cells/ml) were allowed to adhere to the bottom of a 24-well culture plate (Nuclon-Nunc®), in a tissue incubator at 37°C, 5% CO_2, 95% air for 14h. Afterwards, 30% conditioned samples were added to each well in triplicate, and incubated for

another 48h. Conditioned media was then replaced by fresh DMEM without phenol and 10% Alamarblue™. Two internal negative controls were used as follows: culture media was pippetted into two extra wells without BAEC, and 10% Alamarblue™ was added to only one of them. Absorbance was read at 570 and 600nm after a 5h incubation and amount of reduced reagent was calculated. Proliferative response of BAEC to luteal tissue conditioned media was evaluated by comparing the percentage of reduction of this media with media from negative controls (with no luteal tissue), which were considered 100% of cell mitogenesis (3, 30).

Determination of nitric oxide

The *in vitro* production of NO by early and mid luteal phase corpora lutea was assessed according to the protocol for Griess reagent as previously reported (31). The amount of NO produced was determined, against a standard graph of sodium nitrite, at 540nm (Labsystems iEMS Reader MF). Nitric oxide production (ng) per mg of tissue was calculated and data subjected to statistical analyses.

Progesterone and PGE_2 determination

Since luteal tissue was collected from mares of unknown reproductive history, their estrous cycle determination was based on *post-mortem* observation of the internal genitalia and further P_4 analysis. The presence of *corpora lutea* in the ovary indicated that the mares were in the luteal phase of the estrous cycle, which was always confirmed by plasma P_4 concentration >1 ng/ml. Jugular blood (10ml) was collected from all mares into heparinized tubes (monovettes®- Sarstedt) and transported on ice to the laboratory. After blood centrifugation, plasma was kept at –20°C until P_4 determinations were performed.

A solid-phase radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Product Corp., Los Angeles, U.S.A.) was used for P_4 analyses in both plasma and luteal tissue conditioned media. Plasma P_4 intra-assay coefficient of variation for 6.4nmol/L (2 ng/ml) was 9.5%. For the determinations of P_4 in the culture medium, respective intra- and inter-assay coefficients of variation for 15.9 nmol/L (5 ng/ml) were 1.9 % and 5.7%.

Concentrations of PGE_2 in the culture medium were determined with an enzyme immunoassay as described previously (32). The PGE_2 standard curve ranged from 0.39 ng/mL to 100 ng/mL, and the ED_{50} of the assay was 6.25 ng/mL. The intra- and inter-assay coefficients of variation were 1.6% and 11.0%, respectively.

Statistical Analysis

All data, concerning BAEC proliferation assays, plasma P_4 concentrations, and *in vitro* NO, P_4 and PGE_2 concentrations in luteal tissue culture media were analyzed by use of a one-way ANOVA. Significance was defined as values of $P<0.05$. Post-hoc comparison tests (LSD test) were performed whenever a significant difference was detected (Statistic for Windows, Statsoft, Inc., 1995, Tulsa, OK, U.S.A.).

RESULTS

Endothelial cell proliferation assay

Equine luteal tissue alone showed the capability to increase BAEC proliferation (*Fig. 1*), when compared to negative controls ($p<0.05$). No difference was

observed on BAEC proliferation among treatments for negative controls (no tissue added) (data not presented). Since no hormonal treatment had any direct effect on BAEC proliferation ($p>0.05$), these data show that cell proliferation depended on angiogenic activity of the luteal structures submitted to the various exogenous steroids tested and not to the treatments themselves.

In the early luteal structures, angiogenic activity was not affected by any treatment tested, since BAEC proliferation was similar for all groups. However, in the MCL, exogenous P_4 [10^{-4}M;10^{-5}M] had a tendency ($p=0.06$) to reduce BAEC mitogenesis, with respect to controls (*Fig. 1*). Also, these corpora lutea, whenever in the presence of pregnenolone [10^{-5}M], lowered BAEC proliferation when compared to other treatments ($p<0.05$), except with P_4 [10^{-4}M;10^{-5}M]. When the P_4 receptor inhibitor onapristone [10^{-4}M] was used in the MCL culture, BAEC proliferation increased with respect to P_4 ($p=0.01$; *Fig. 1*). The lowest concentration of onapristone [10^{-5}M] also showed a tendency ($p=0.06$) to stimulate endothelial cells multiplication.

Nitric oxide production

In this study, the concentration of NO in luteal tissue conditioned media showed a significant decrease in the mid-luteal stage with respect to the early luteal stage ($p<0.05$) when luteal tissues were treated with P_4 [10^{-4}M] and pregnenolone [10^{-5}M] (*Fig. 2*).

Progesterone and PGE_2 production

Progesterone concentrations in the plasma from the mares used in this study showed differences in the synthesis of this hormone, depending on the nature of

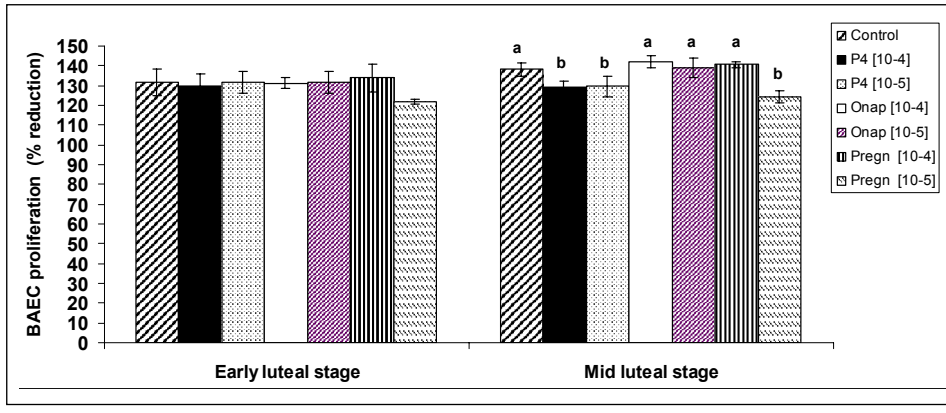

Fig. 1. Mitogenic capacity of early and mid luteal stage CL on bovine aortic endothelial cells (BAEC) in the presence or absence (control) of different steroid hormones. Values reported are means±SEM of reagent reduction. Values (a and b) differ significantly ($p<0.05$; LSD test).

the luteal structures present (*i.e.* CH, MCL). There was a significant rise in P_4 from CH stage (7.5ng/ml±0.8) to mid luteal phase CL (14.1ng/ml±0.9; $p<0.001$).

In the culture medium conditioned by CH treated with pregnenolone [10^{-4}M], P_4 concentration decreased with respect to control, P_4 [10^{-5}M], onapristone [10^{-4}M;10^{-5}M] and pregnenolone [10^{-5}M] groups ($p<0.05$; *Fig. 3*). However, the release of P_4 in culture media conditioned by MCL revealed no difference among treatments ($p>0.05$; *Fig. 3*). In addition, for each treatment, no difference in P_4 levels was observed between CH and MCL.

The analysis of medium PGE_2 from luteal tissue culture revealed differences in the synthesis of this eicosanoid, depending on the luteal stage (*i.e.* CH, MCL).

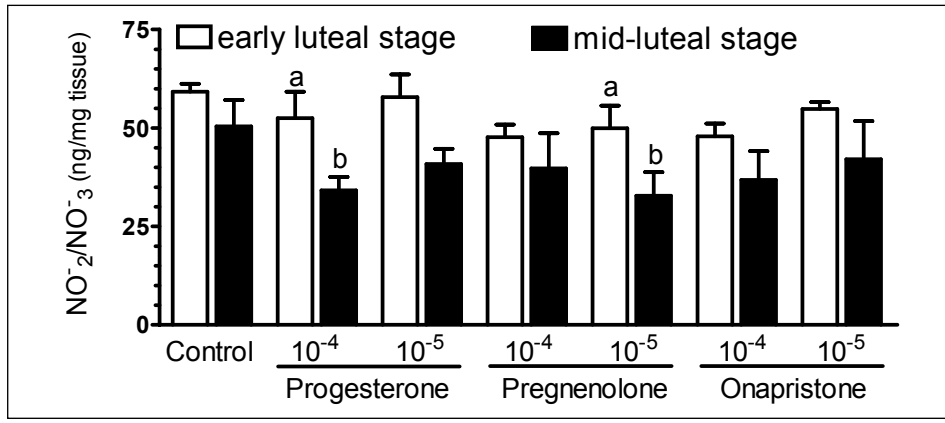

Fig. 2. In vitro NO production by early and mid luteal stage CL, in the presence or absence (control) of different steroid hormones. Values reported are means±SEM. Values (a and b) differ significantly ($p<0.05$; LSD test).

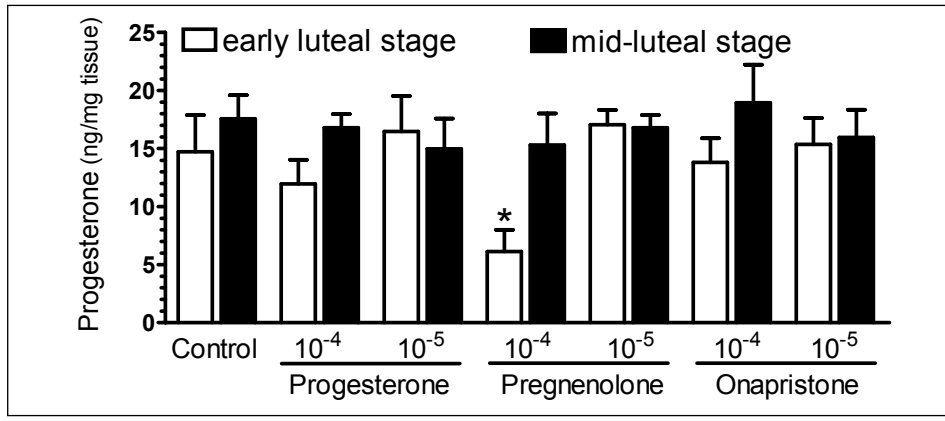

Fig. 3. In vitro progesterone production by early and mid luteal stage CL, in the presence or absence (control) of different steroid hormones. Values reported are means±SEM. Values (*) differ significantly ($p<0.05$; LSD test).

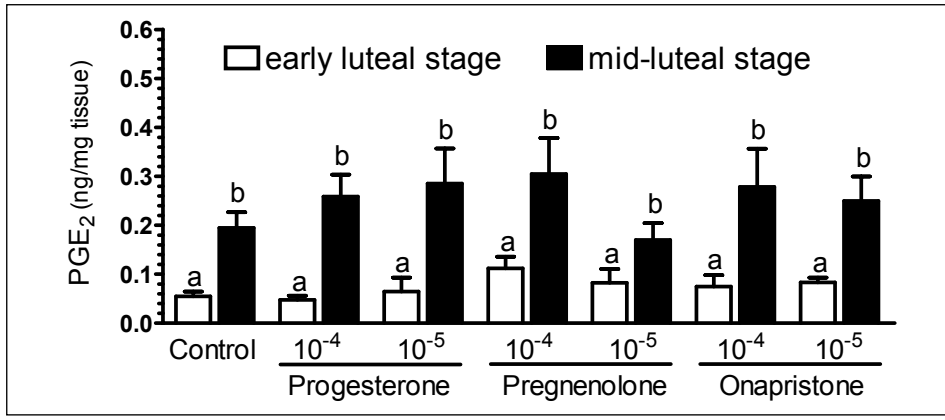

Fig. 4. In vitro prostaglandin E_2 production by early and mid luteal stage CL, in the presence or absence (control) of different steroid hormones. Values reported are means±SEM. Values (a and b) differ significantly ($p<0.05$; LSD test).

There was a significant rise in PGE_2 from CH stage (0.055ng/mg tissue ± 0.010) to mid luteal phase CL (0.164ng/mg tissue ± 0.032; $p<0.01$; *Fig. 4*). Although there was a tendency for PGE_2 secretion to increase in luteal tissues treated with pregnenolone (10^{-4}M; $p=0.06$), there were no significant differences in PGE_2 secretion among all treatments ($p>0.05$; *Fig. 4*).

DISCUSSION

Vascularization of the female reproductive system during the cyclic period is influenced by the ovarian steroid hormones progesterone (P_4) and estradiol (33). In the process of luteal regression, degeneration of steroidogenic and endothelial cells and blood vessels is essential (34). Actually, endothelial cells in bovine corpora lutea may be strongly involved in the luteolytic process, since large luteal cells undergo luteolysis when stimulated by $PGF_{2\alpha}$ only in the presence of endothelial cells (35). Besides, microvascular endothelial cells are the first ones to undergo apoptosis in the CL (36). In the mare, during the late luteal phase, overall apoptosis increases (2), and $PGF_{2\alpha}$ may play a role on luteal vascular regression by decreasing endothelial cell proliferation (37). Actually, a significant increase is observed in the expression of active caspase-3 - the effector enzyme of apoptosis - in large luteal cells, in all equine luteal structures when compared to early luteal tissue (37). In the present study, there was no direct effect of treatments on BAEC proliferation, showing that the steroids themselves are not influencing BAEC proliferation but most likely the angiogenic factors produced by steroid treated luteal tissue. The decrease in endothelial cell proliferation observed in mare´s mid luteal phase CL, when in the presence of progesterone or

its precursor, could be due not only to a lack of synthesis of angiogenic factors, but also to a rise in anti-angiogenic factors, such as thrombospondins (38), angiostatin and endostatin (38 - 40). These factors may be modulating angiogenesis and luteal regression (39, 40). Some anti-angiogenic factors might be involved in luteal formation to avoid excessive vascular development and to mediate endothelial cells apoptosis during regression (41). Long-lasting effects of P_4 and its precursor pregnenolone may inhibit angiogenic factor(s) or stimulate anti-angiogenic factor(s) production by equine MCL. However, the PR antagonist onapristone did not allow for this fall in BAEC proliferation. This may suggest that in the equine mid-luteal phase CL, inhibition of angiogenesis by progestagens is preparing for CL functional and structural regression. Besides this decrease in BAEC proliferation in the mid-luteal phase CL, incubated in the presence of P_4 or its precursor pregnenolone, NO production was also lower. Therefore, it appears that inhibition of the angiogenic activity in the equine CL by exposure to long term progestagens might be a NO mediated process. However, NO anti-angiogenic properties as previously suggested for porcine granulosa cells (18), need to be further investigated.

Even though the highest plasma P_4 production was observed in the mid luteal phase, as previously reported for the mare (3), *in vitro* release of this hormone did not increase in the MCL with respect to CH, when luteal tissue was not treated with any exogenous hormone (Control group). In the present study, onapristone, a P_4 receptor antagonist, did not decrease *in vitro* P_4 concentration at any stages of the luteal phase studied. Other reports on the *in vitro* use of onapristone showed it was able to reduce P_4 release to the culture media in the early CL, but not in the MCL (7). Also, after short incubation times, P_4 [10^{-5} M] stimulated *in vitro* P_4 content in early luteal tissue, while onapristone [10^{-4} M] did not reduce it, with respect to controls (8). However, similar doses to the ones used in the present study have been effective on reducing *in vitro* P_4 release by bovine corpora lutea cells at shorter incubation times (42). Bovine luteal cells obtained from an early pregnancy CL, when in the presence of PR antagonists (onapristone and RU-486) for 48h, also showed no difference in P_4 concentrations in the culture media, when compared to controls (10). The reason why no change in P_4 concentrations was observed in the present study is not clear. Since the doses of onapristone used were based on previous *in vitro* studies on bovine luteal cells (9, 11), this lack of responsiveness might be due to species differences or to methodological aspects (luteal tissue vs. luteal cells). Such paracrine regulation of luteal cells by endothelial cells may be mediated by factors produced by endothelial cells such as prostaglandin I_2 (PGI_2) and endothelin-1 (ET-1) (35), which exert luteotropic and luteolytic effects on luteal cells, respectively (43, 44).

In an early study, mean P_4 concentration determined in the equine luteal tissue in six cycling mares was 7µg/g (45). Therefore, the relatively low concentrations of the PR antagonist used in this study might explain this lack of P_4 inhibition. However, other explanations should not be discarded. This observation may be

due to the fact that this steroid hormone may act via nonclassical receptor target sites by binding to the cell membrane (46). However, in the mare this mechanism of P_4 action in the corpus lutem seems unlikely, since PR have been detected in increasing intensity in large luteal cells, from early CL to MCL (4). In addition, the lack of response to onapristone could also be ascribed to P_4 receptor desensitization to continuous stimulation. This biological adaptation of desensitization of receptors is characterized not only by loss of receptor responsiveness but also of receptor number, affinity, and ability to activate second messengers systems (7, 47, 48).

In the present study, a fall in P_4 was observed in culture media of the CH incubated in the presence of pregnenolone [10^{-4}M] compared to control, P_4 [10^{-5}M], onapristone [10^{-4}M;10^{-5}M] and pregnenolone [10^{-5}M]($p<0.05$). The dose of the P_4 precursor pregnenolone used in this experiment might be crucial for stimulation of P_4 synthesis. Although treatment of sheep large and small luteal cells with pregnenolone caused an increase in P_4 *in vitro* production (49), during pregnancy P_4 did not affect its own secretion by bovine (50) and ovine luteal slices (51). In the cow and sheep, high doses of P_4 given chronically (twice per day) during the early luteal phase shortens the estrous cycle (52, 53). No difference on P_4 release was observed for any treatment for the CLM, or between CH and MCL. Additional experiments will be required to determine the effects and mechanisms of P_4 on equine luteal endocrine function and/or involution. Besides, in the present experiment BAEC death throughout the incubation period should be considered as a potential explanation for some of the effects on P_4 production, and not solely direct regulation of luteal steroidogenesis (54).

It has been shown that the luteotropic effect of P_4 on the bovine CL is mediated by oxytocin (OT) and/or prostaglandins (7, 8). PGE_2 has been found to be the most potent luteotropin in the ovine and bovine CL (55, 56). Therefore, we examined whether P_4 may affect luteotropic PGE_2 production in the equine CL tissue. Equine luteal tissue from the early and mid luteal phases showed the ability to release PGE_2 *in vitro,* as previously reported for equine dispersed luteal cells (57). However, there were no significant differences in PGE_2 secretion among all treatments. Lack of P_4 effect on prostaglandin secretion is also one possible explanation of why P_4 did not regulate its own secretion in the equine CL, as it has been shown in bovine CL cells and tissue (7, 8). Moreover, it has been shown that the enzyme PGE_2-9-ketoreductase (9k-PGR) (58 - 60) or other newly found PG synthases (*i.e.*: aldoketoreductase – AKR1B5) (61) can convert $PGF_{2\alpha}$ to PGE_2 or *vice versa*. Thus, modulation of PG-converting enzymes activity may be the mechanism by which P_4 switches prostanoid metabolism in equine CL from production of luteotropic PGE_2 to luteolytic $PGF_{2\alpha}$. Moreover, since 9k-PGR and AKR1B5 possess 20α-HSD activity (61), it is possible that these enzymes also inter-convert prostaglandins and concomitantly inactivate P_4 within the CL. In addition, steroids - mainly P_4, are suggested to play a mandatory role in regulation of both 9k-PGR and AKR1B5 activity and expression (59, 61).

Although P_4 inhibits 20α-HSD expression in the rat corpus luteum (62), it has been shown that the expression and activity of PG converting enzymes (9k-PGR and AKR1B5) increase after long- lasting P_4 exposure in the ovine and bovine female reproductive tract (59 - 61). Therefore, the high activity of such multifunctional enzymes (9k-PGR and AKR1B5) that combine two converting functions (inactivation of P_4 and generation of luteolytic $PGF_{2\alpha}$ from luteotropic PGE_2) may be a possible reason for the lack of the luteotropic effects of P_4 observed in this study in equine luteal tissue. Further studies are needed to clarify this hypothesis.

In conclusion, long-lasting effects of P_4 and its precursor may inhibit angiogenic factor(s) production by equine mid luteal phase CL, preparing for luteal functional and structural regression. Besides, the simultaneous decrease in *in vitro* NO production and BAEC proliferation in mid luteal phase corpora lutea submitted to progestagens may suggest that inhibition of angiogenic activity in the equine CL might be mediated by NO. The apparent discrepancy among our findings on NO, P_4 and PGE synthesis, might be due to differences in cell-to-cell contact, differences in cell composition, namely steroidogenic and non-steroidogenic cells, that might affect the synthesis of these hormones in the equine CL. Therefore, luteal angiogenesis and endocrine functions in the mare are very complex processes that might be regulated by many different factors and needs further investigation.

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