Effects of LH and PGF_{2α} in Equine Dominant Follicles Observed by MDS

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ABSTRACT. Microdialysis System (MDS) is a novel technique used for investigation of molecule secretion between different cell populations. Local hormonal secretion at follicular wall has been still unclear. This MDS study was used to determine progesterone (P₄), androstenedione (A₄), estradiol-17β (E₂) and Prostaglandin F_{2α} (PGF_{2α}) release in mare pre-ovulatory follicles. Follicles larger than 30 mm were isolated from the ovary and follicular fluid aspirated for hormone assay. Follicular fluid collected from small, middle and large follicles were analyzed by EIA. The concentrations of P₄ and PGF_{2α} were similar among the different sizes of follicles. The release of A₄ was observed in middle and large follicles. E₂ concentration was observed in middle follicles and was higher in large follicles compared with middle follicles. Follicular wall was cut and incubated for MDS and when LH was infused, there was an increase in P₄ and A₄ release. PGF_{2α} release was considerably high after LH infusion compared to the control group. Infusion of PGF_{2α} increased P₄ and A₄ release but there was no change in E₂ release. This results suggest that in pre-ovulatory follicles, LH stimulates theca interna cells and also PGF_{2α} seemed to have a mediator role to induce steroid hormone production and luteinization of follicular cells. The nature of the mechanisms involved in selection of large follicles is still a perplexing research problem in reproduction.

KEY WORDS: follicle, mare, MDS, ovulation, steroid-hormone.

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Mares are seasonal polyestrous animals and the estrous cycle have several unique characteristics, including a long period of estrus, a considerable larger size of preovulatory follicles and the absence of a typical LH surge compared with other species [2, 7, 9]. However, endocrinological changes that occur in the different follicular compartments (theca, granulosa cells and follicular fluid) during follicular development, maturation, and ovulation still remain unclear.

Follicles isolated from mare ovaries maintained the ability to secrete steroid hormones and to respond to hormonal stimulation *in vitro* [18]. The same study demonstrated a variable effect of LH on progesterone (P₄), androstenedione (A₄), and estradiol-17β (E₂) production depending on the stage of the estrous cycle. A₄ is one of the main secretory products of the theca layer that serve as substrate for the synthesis of estradiol in the granulosa cells [4, 19]. These secretory products (steroids and/or prostaglandins) are accumulated in the follicular fluid and may be used as indicators of the follicular development, atretic stage or the proximity of ovulation [21, 22]. Moreover, other report suggests that progesterone can modulate ovarian activity by altering FSH and LH receptor in the follicular wall [11]. In addition, prostaglandins have been considered as an essential mediator for the LH action on the ovulatory processes in several species, including the mares [13].

Prominent structural and secretory changes take place in the follicular wall during the interval of time between the onset of the LH surge and ovulation. Therefore, this study aimed to determine a possible interaction between LH and

prostaglandin F_{2α} (PGF_{2α}) and their effects on the local release of P₄, A₄, E₂, and PGF_{2α} from the mare dominant follicles *in vitro*.

MATERIALS AND METHODS

Follicular collection and classification: Mare follicles used in this study were obtained from ovaries collected at slaughterhouse. The ovaries were transported to the laboratory in sterile saline solution (0.9% NaCl) containing 100,000 IU penicillin and 100 mg/l streptomycin on ice.

Ovaries were visually inspected and classified according to their size as previously described by Kenney *et al.* [14]. For the determination of the content of steroids and PGF_{2α} in the follicular fluid, the follicles were classified according to the diameter in three groups: small (less than 15 mm), middle (between 16 to 29 mm), and large (more than 30 mm) follicles. Only follicles that appeared healthy (i.e., well vascularized and having transparent follicular wall and fluid), containing a higher concentration of E₂ than P₄ were considered as dominant follicles.

MDS *in vitro*: The MDS for mare follicles was based on the method developed for porcine follicles *in vivo* [8], with some modifications for an *in vitro* organ culture system [1]. Only E₂ dominant follicles larger than 30 mm were used for perfusion using the MDS. The selected follicles were dissected from the surrounding stromal tissue from the ovaries and cut in 8 pieces. In each piece, a dialysis capillary tube was implanted into the theca layer and fixed to the follicular tissue by Hystoacryl blue (B. Braun-Dexon GmbH, Spangenberg, Germany). Control (perfused with Ringer's solution only) and treatment groups were randomly chosen. For

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the stimulation, equine LH (Sigma, St Luis, U.S.A.), or PGF_{2α} (Sigma, St Louis, U.S.A.) were diluted in Ringer's solution to obtain the required final concentrations of 10 IU/ml and 10⁻⁶ M/ml, respectively. The solution was then infused into the MDS for 4 hr between 4 and 8 hr. During all the incubation period the follicles were perfused with Ringer's solution at a flow rate of 3 ml/hr. After 2 hr of preperfusion fractions of the MDS perfusate were collected up to 12 hr (4 hr/fraction). The order of the perfusate solutions was Ringer's alone, LH and PGF_{2α}. At every 4 hr a fraction of the perfusate was collected from the MDS and stored at -20°C for later analysis. Before each perfusion equine LH and PGF_{2α} were diluted in Ringer's solution. The concentration was determined based on the results of a preliminary experiment where the transfer capacity of the membrane was estimated to be 1%. The following steps of the MDS until the hormonal assay were performed as described by Acosta [1].

Hormonal assay: The perfusates (3 ml) and follicular fluids (300 μl) were adjusted to pH 3.5 using 1 N HCl and 5 N HCl, respectively, and allowed to stand at room temperature for 1 hr. Then 3 volumes of diethyl ether were added and the mixture was shaken for 30 min at a frequency of 250 cycles/min (using Shaker SA 31; Yamamoto Co., Tokyo, Japan). The samples were then allowed to stand for 15 min and placed in a -80°C freezer for 1 hr. The upper diethyl ether fraction was decanted and evaporated. The residue was dissolved in 300 μl assay buffer for steroid and prostaglandin enzyme immunoassays (EIAs) (40 mM PBS, 0.1 BSA, pH 7.2). To estimate the recovery rate, A₄, P₄, E₂, and PGF_{2α} were added to the Ringer's solution (30, 100, 20, and 100 pg/ml, respectively), and the obtained values were 85%, 88%, 75%, and 72%, respectively. The EIA for P₄ and E₂ were performed following protocols already described in other studies [15, 23]. In addition, the recovery rate of P₄, A₄, E₂, and PGF_{2α} was estimated.

The standard curve for P₄, A₄, E₂, and PGF_{2α} ranged between 0.05 to 25 ng/ml, 2 to 1,000 pg/ml, 2 to 2,000 pg/ml, and 10 to 10,000 pg/ml, respectively. The corresponding intra- and inter-assay coefficients of variation of the standard curve were 4.8% and 7.5% for P₄, 6.3% and 8.2% for A₄, 6.3% and 8.5% for E₂, 8.7% and 13.1% for PGF_{2α} respectively. Besides, the ED50 of the assay for P₄, A₄, E₂, and PGF_{2α} was 2.5 ng/ml, 115 pg/ml, 110 pg/ml, and 250 pg/ml, respectively.

Statistical analyses: The effects of the infused substances on the release of steroids (P₄, A₄, E₂) and PGF_{2α} were compared with the control values using ANOVA and followed by Student's *t*-test. Differences are considered significant at a probability less than 5% (*P*<0.05). The absolute concentrations of the hormones in the MDS fractions (mean ± SEM) are given in the figure legends.

RESULTS

Hormonal concentration in the follicular fluid: The concentration of P₄, A₄, and E₂ and PGF_{2α} in the small, middle

and large follicular fluids are shown in Fig. 1. The P₄ concentration in the follicular fluid was similar among the different sizes of follicles. Concentration of A₄ in middle and large follicles was higher (*P*<0.05) than small follicles. The highest level of E₂ (665 ± 74 ng/ml) was found in large follicles and the lowest level (64 ± 20 ng/ml) was found in small follicles. PGF_{2α} concentration was not significantly different observed among the follicle of different sizes.

Effect of LH on the local release of P₄, A₄, E₂, and PGF_{2α} by MDS: Infusion of LH increased the release of P₄ and A₄ (150–180%) compared to the control group (*P*<0.05). However, no changes were observed in E₂ release as the result of LH stimulation. The PGF_{2α} release acutely increased (3 fold) after LH infusion compared with control group (*P*<0.001) from 4 to 8 hr of experimental period (Fig. 2.1).

Effect of PGF_{2α} on the release of P₄, A₄ and E₂: PGF_{2α} infusion stimulated the release of P₄ and A₄ (165 ± 25 % and 212 ± 26 %, respectively) compared with control. In other hand, no effect E₂ release was observed during the same time period (Fig. 2.2).

DISCUSSION

In the present study, follicular fluid from different sizes of follicles were analyzed. There were no differences in P₄ concentrations among follicles sizes. A₄ concentrations were higher in middle and large follicles compared to small follicles. Furthermore, E₂ concentration was very high in large follicles. The PGF_{2α} concentration was not different among follicle size during the experimental period. These results disagree with Watson and Hirrchs [22] who found high concentration of steroids in the preovulatory mare follicular fluid.

The E₂ has been considered as one of the important steroid hormone responsible for follicular maturation [11]. Similar as Kenny's [14] results, the concentration of E₂ was observed low in small follicle, increased in middle follicle, and was high in large follicles. The large amount of E₂ release when the follicle reaches more than 30 mm in diameter is due to the ability of the granulosa cells in synthesizing oestrogen. Gastal *et al.* [10] observed that the

Fig. 1. P₄, A₄, E₂ and PGF_{2α} concentration in follicular fluid analyzed by EIA in the small (vertical hatched bars), middle (shaded bars) and large follicles (hatched bars). a, b, c indicate significant differences among different groups.

Fig. 2.1

Fig. 2.2

Fig. 2.1. Effects of 10 IU/ml LH infusion (shade bars) on P₄, A₄, E₂, and PGF_{2α} release from microdialyzed dominant follicular tissue (n=4) *in vitro* expressed as percentage of basal release. * *P*<0.05, * *P*<0.001 compared with respective controls (open bars) at the same time-point (Student's *t*-test).

Fig. 2.2. Effects of 10⁻⁶ M/ml PGF_{2α} infusion (shaded bars) on P₄, A₄, and E₂ release from microdialyzed dominant follicular tissue (n=6) *in vitro* expressed as percentage of basal release. * *P*<0.05 compared with respective controls (open bars) at the same time-point.

differences in E₂ concentrations between larger and smaller follicles increased progressively. Those authors observed that the first significant increase in systemic E₂ occurred the day before or at the beginning of the pre-ovulatory follicle selection. Alternatively, follicular wall treated with LH using MDS system has not stimulated the E₂ synthesis. This point was also observed by Watson and Hinrichs [22], they found that the E₂ does not increase in the period immediately before ovulation.

The LH infusion in the preovulatory follicular tissue resulted in moderate secretion of P₄, and A₄ as was observed by Sirois *et al.* [18]. In addition, the secretion of PGF_{2α} in the large follicle was also increased. No changes in E₂ secretion were observed. Based on these data it could be possible to say that LH blocks estradiol secretion and that PGF_{2α} plays a role in the ovulation process by stimulating

the granulosa cell in order to release P₄, preparing to the corpus luteum formation.

The infusion of PGF_{2α} stimulated the secretion of P₄ and A₄. The HCG administration in early pregnant mares can cause embryonic resorption [3] and Troedson *et al.* [20] reported that treatment with PGF_{2α} could interfere the corpus luteum formation. Others [12, 16] reported that equine corpus luteum might be refractory to PGF_{2α} during the first four days after ovulation. Also, there is a report performed *in vivo* indicating that the PG administration in presence of large ovulatory follicle can shorten the time to estrus [17, 22]. The increase in P₄ in large follicles is presumably due to luteinization, in consequence the PG production from follicular cells were stimulated by LH [5, 6].

In conclusion, the LH stimulates steroids and PGF_{2α} production in theca interna cells and luteinization of follicular cells in large follicles. Also, PGF_{2α} infusion has stimulated P₄ and A₄ release, which mean that PGF_{2α} seemed to have a mediator role to stimulate those hormones. The nature of the mechanisms involved in the biologic selection of dominant follicles and the role of LH is still a perplexing research problem in mare reproduction.

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