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THE HORMONAL COMPOSITION OF FOLLICULAR FLUID IN CYCLING MARES

Gamze Evkuran Dal* and Güven Kasikci
Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine,
Department of Obstetrics and Gynaecology, Avcilar, Istanbul, 34320, Turkey

ABSTRACT

This study was performed to investigate the intrafollicular concentrations of oestradiol, progesterone, anti-Müllerian hormone (AMH), insulin-like growth factor-1 (IGF-1), inhibin-A, inhibin-B, and vascular endothelial growth factor (VEGF) during breeding season in mares. A total of 25 healthy, non-lactating mares with regular oestrous cycles were used. Follicular aspirations were performed from a preovulatory follicle (POF) and a subordinate follicle (SOF) during oestrus. The aspiration procedure was repeated one week later to collect follicular fluid sample during the luteal phase (luteal phase follicle, LPF). Follicular fluid IGF-1, inhibin-A and VEGF concentrations were not statistically different between follicle groups. LPF concentrations of oestradiol was the lowest and significantly different ($P<0.001$) from POF and SOF. The highest progesterone concentrations were measured in POF. SOF concentrations of progesterone were the lowest and significantly different from other follicle types ($P<0.001$). POF AMH concentrations were lower and significantly different ($P<0.001$) from other follicle groups. LPF inhibin-B concentrations were significantly higher ($P<0.001$) than POF and SOF levels. The data suggest that the levels of IGF-1, inhibin-A, and VEGF tend to be higher in preovulatory follicles despite the absence of significancy, however inhibin-B, oestradiol, progesterone and AMH show a variable pattern on the follicular basis throughout the oestrous cycley

Keywords: Aspiration, follicle, follicular fluid, hormone, mare

1. INTRODUCTION

Each follicle has a unique physiological environment that differs from other follicles and from various factors in circulation [1]. Follicular fluid contains numerous biologically active factors and hormones, such as steroid and peptide hormones, prostaglandins, cytokines, insulin-like growth factors and their binding proteins, and many others [2]. Dominant follicle development and differentiation involves complex mechanisms ultimately leading to ovulation. Steroids, growth factors and other peptidergic factors play important roles in folliculogenesis together with pituitary gonadotrophins in local, paracrine and autocrine manners [3].

It was aimed to investigate the concentrations of hormonal contents [oestradiol, progesterone, anti-Müllerian hormone (AMH), insulin-like growth factor-1 (IGF-1), inhibin-A, inhibin-B, and vascular endothelial growth factor (VEGF)] in follicular fluid samples in mares, and to compare their concentrations at different types of follicles during the follicular and luteal phases of oestrus.

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2. MATERIALS AND METHODS

2.1Animals and follicular development tracking

A total of 25 healthy, non-lactating mares with regular oestrous cycles (Thoroughbred, n=21; and Arabian n=4, with a mean age of 13 ± 5) were used during the breeding season in the northern hemisphere (40°59'N 28°43'E). The mares were kept in standard-sized equine boxes during the night and in an outdoor paddock during day with free access to water and mineralized salt. Animals were fed with 1 kg of roughage and 1 kg of concentrate feed per 100 kg bwt.

Follicular activities were monitored by a real-time B mode ultrasound (Medison SA60V, Medison Co. Ltd., Seoul, South Korea) equipped with a 5 MHz linear probe. The ultrasound examinations were carried out three times per week during vernal transition until the first ovulation of the season to determine the onset of ovulatory period. No pharmacological drugs were used to hasten the transition nor to synchronize the oestrus cycles. The ultrasonographic examinations were performed daily during the second oestrous cycle of the season to collect data and to determine the days of follicular fluid aspirations.

2.2Sample collection

Follicular fluid samples collected from mares constituted the study materials. When the preovulatory follicle reached a diameter >35 mm with an accompanying teasing score of 5/5 and ultrasonically detectable uterine edema of UE4 [4], the aspiration of the preovulatory follicle (POF) and a subordinate follicle (SOF) were performed from each mare (Day 0). The aspiration procedure was repeated on Day 7 to collect a luteal phase follicle (LPF) sample. The follicle with the largest diameter was selected from the follicle pool during dioestrus. So, a total of 3 follicles from the same ovary were aspirated per mare.

The procedures were performed when the mares restrained in stocks. Mares were sedated with 0.02 mg/kg i.v. detomidine hydrochloride (Domosedan, Pfizer, Espoo, Finland) prior to follicular fluid aspiration. Additionally, 1.1 mg/kg i.v. flunixin meglumine (Flumeglin, Teknovet, Istanbul, Turkey) was administered for its anti-inflammatory, anti-pyretic and analgesic effects. The mare's tail was wrapped, the feces was removed, and the perineal area was cleaned with skin disinfectant (Zefirolum Fort, Kimpa, Istanbul, Turkey) diluted according to the manufacturer's instructions to prevent possible contamination.

A real-time B-mode ultrasound equipped with a 5 MHz curvilinear vaginal probe (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China), a 12-G double lumen aspiration needle (12-Gauge Double Lumen Equine Ovum Pick-Up Needle and Collection System, COOK Veterinary Products, Brisbane, Australia) which was attached to the probe, and an aspirator (Labotect Aspirator 3, Labotect, Rosdorf, Germany) were used for transvaginal ultrasound-guided follicular aspiration (Figure 1). The process was performed as described by Carnevale [5].

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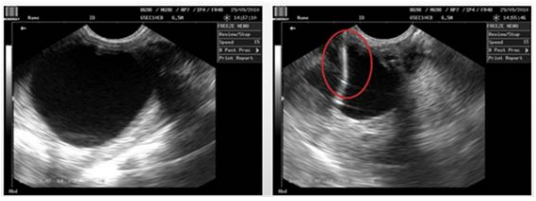

Figure 1: Transvaginal ultrasound-guided follicular aspiration from a preovulatory follicle (red circle: aspiration needle)

Follicular fluid samples were centrifuged (NF 800R, Nüve, Ankara, Turkey) at 3000 xg for 15 min and supernatants were stored at –20 ℃ until analysis.

2.3Analysis of samples

Analysis were performed by three different methods as described below according to the manufacturers' instructions.

Follicular fluid concentrations of oestradiol and progesterone were analysed by radioimmunoassay (RIA) method using commercial kits (Ultra-sensitive RIA, DSL4800, Beckman Coulter, Inc., and RIA for progesterone, IM1188, Immunotech, respectively). The sensitivity of oestradiol and progesterone RIA kits were 2.2 ng/mL and 0.05 ng/mL. The intra- and inter-assay precisions were ≤8.9% and ≤12.2%; ≤5.8% and ≤9.0%, respectively. IGF-1 concentrations were analysed by immunoradiometric assay (IRMA) using a kit with sensitivity of 2.0 ng/mL (IRMA IGF-1, A15729, Immunotech). The intra- and inter-assay precisions of the kit were ≤6.3% and ≤ 6.8%. Enzyme-linked immunosorbent assays (ELISAs) were used for analysing AMH (AMH Gen II ELISA, A79765, Beckman Coulter, Inc.), inhibin-A (Inhibin A ELISA, DSL-10-28100t, Beckman Coulter, Inc.), inhibin-B (Inhibin B Gen II ELISA, A81303, Beckman Coulter, Inc.), and VEGF (Horse VEGF ELISA kits, CSB-E13799Hs, Cusabio Biotech Co., Ltd.) concentrations. The sensitivities of ELISA kits were 0.08 ng/mL, 5.0 pg/mL, 2.6 pg/mL, and 8.0 pg/mL, respectively. The intra- and inter-assay precisions of the kits were ≤5.4% and ≤5.6%; ≤6.0% and ≤7.8%; ≤3.8% and ≤5.6%; ≤8.0% and ≤10.0%, respectively.

2.4 Statistical analyses

IBM SPSS Statistics (version 10.00; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Comparison of hormone concentrations measured in POFs, SOFs, and LPFs were performed with one-way analysis of variance. Tukey's test was used for determining which follicle groups were significantly different from each other. $P<0.05$ was considered statistically significant.

3. RESULTS

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The mean diameters of follicles before aspiration were recorded as 41.4 ± 3.9 mm for POF, 21.1 ± 5.1 mm for SOF and 19.5 ± 5.3 mm for LPF. Any case of double preovulatory follicle formation which leads to double ovulation, as well as dioestrus ovulation was not encountered. The presence of ultrasonically well-identified and developed corpus luteum (CL) was verified in all mares on Day 7.

Follicular fluid IGF-1, inhibin-A and VEGF concentrations were not statistically different between POF, SOF and LPF, however, all three parameters had their highest concentrations in POF. LPF concentrations of oestradiol was the lowest and significantly different (P<0.001) from POF and SOF. The highest progesterone concentrations were measured in POF. SOF concentrations of progesterone were the lowest and significantly different from other follicle types (P<0.001). POF AMH concentrations were lower and significantly different (P<0.001) from other follicle groups. LPF inhibin-B concentrations were significantly higher (P<0.001) than POF and SOF levels (Table 1).

Table 1: Preovulatory, subordinate and luteal phase follicle concentrations of progesterone, oestradiol, AMH, IGF-1, inhibin-A, inhibin-B and VEGF

Hormones	POF		SOF		LPF		P value
	Mean	SE	Mean	SE	Mean	SE	
Progesterone (ng/mL)	56.46[a]	2.33	31.68[b]	2.97	48.27[a]	3.79	<0.001
Oestradiol (pg/mL)	3.18[a]	0.11	3.22[a]	0.30	0.94[b]	0.13	<0.001
AMH (ng/mL)	31.05[b]	3.47	92.49[a]	9.31	93.71[a]	9.83	<0.001
IGF-1 (ng/mL)	81.23	7.00	67.64	7.31	70.39	8.61	0.420
Inhibin-A (ng/mL)	25.02	1.12	22.72	1.35	22.92	1.53	0.411
Inhibin-B (ng/mL)	1.07[a]	0.04	1.12[a]	0.05	1.33[b]	0.03	<0.001
VEGF (pg/mL)	54.53	5.31	44.72	3.16	40.64	3.68	0.059

a,b Means within rows bearing different superscript letters are significantly different. SE, Standard Error

4. DISCUSSION

Although there has been scientific interest in equine follicles since the 1920s, detailed studies for identifying follicular mechanisms in mares gained momentum after the introduction of rectal ultrasonography in addition to systemic hormone measurements. Finally, the development of the ultrasound-guided transvaginal ovarian puncture technique provided easy access to equine ovaries in vivo and provided opportunities for the collection of follicular fluid samples, injection

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of test substances, and experimental manipulations of follicles [6]-[7]. It is a non-surgical procedure which repetitive applications do not have a negative effect on donor mare fertility [8]-[9], and can be easily applied even in pregnant mares [8]. In the present study, ultrasound imaging of the ovary was obligatory for sample collection. The ultrasound-guided transvaginal ovarian puncture technique was successfully performed without any complication. The CL develops without any alteration in its formation or function following transvaginal follicular fluid aspiration, as after spontaneous ovulation [10]. The presence of an ultrasonically well-identified CL during luteal phase follicle aspiration in this study supports the formation of a functional CL following preovulatory follicle aspiration.

Numerous studies have been conducted on intrafollicular hormones in mares. It is reported that ovarian activity in mares is managed not only by the hypothalamus and pituitary but also by the ovary itself. In addition, local ovarian mechanisms are believed to play important roles in various stages of follicular development [7]-[11]. Androgens and progestins are substrates for oestradiol synthesis [12]. Androgen concentrations are higher in subordinate follicles whereas progesterone concentrations start to increase by the onset of deviation [13]-[14]. Although a role has not been defined for progesterone at deviation, this increase is suggested to be a result of being an intermediate steroid for androgen synthesis. Inhibin-A is also known to be responsible for LH-induced androgen production, which is necessary for oestradiol production [13]. Watson et al. [15] compared follicular fluid hormone levels in mares during the vernal transition and breeding season. Inhibin-A, oestradiol and progesterone concentrations were found higher in dominant follicles than in subordinate and transitional follicles. They stated that high inhibin-A concentrations exhibited an anti-atretogenic effect while oestradiol and progesterone concentrations were high because of elevated steroidogenic activity in dominant follicles. The results of the present study supports progesterone and inhibin-A level elevation seen in preovulatory follicles. Although follicular fluid inhibin-A concentration were not statistically different (P=0.420) between follicle groups, the levels tended to be higher in preovulatory follicles. Additionally, progesterone concentrations in POF were found higher than other follicles. It may be conceived as a sign of interaction between these two hormones in oestradiol production.

Progesterone level increase in POF plays a supporting role in follicle rupture in cattles, which is another monovulatory species like mares [16]. Moreover, leakage of follicular fluid containing progesterone into the oviduct during ovulation facilitates the sperm acrosome reaction needed for fertilization [17]. IGF-1 and progesterone levels can be used as biochemical markers for determining oocyte maturation, according to the results of a study on the potential relevance of follicular fluid hormone levels on in vitro oocyte maturation in mares [18]. The high progesterone concentrations measured in POF may have a similar manner with those previous reports.

In a study evaluating intrafollicular hormone levels during follicle selection in mares, inhibin-A concentrations were increased in future dominant follicles (16.0–19.9 mm), as with IGF-1, whereas inhibin-B concentrations remained unchanged, even though the follicles increased in size and maturity [13]. The inhibin-A and IGF-1 concentrations showed a similar patten in the

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present study; and the similarity between inhibin-B concentrations in POF and SOF is consistent with the findings of the study above.

Preovulatory and small antral follicular fluid hormone levels have been measured in women and AMH concentrations in small antral follicles were found higher than in POF. AMH is suggested to act as an aromatase inhibitor and to induce FSH-stimulated granulosa cell differentiation [1]. It a useful parameter for measuring ovarian reserves by its strong relationship with antral follicle count in mares, as well as in women [19]. In the present study subordinate and luteal phase follicles, which are small in diameter, had higher AMH concentrations. AMH showed a dramatic decrease in preovulatory follicles ($P<0.001$). Follicular fluid inhibin-B and AMH concentrations of the present study suggest an interaction between these two hormones which may lead to an accompanying role in small follicle development.

VEGF plays important roles during follicle selection by increasing blood flow velocity and blood flow area of the dominant follicle [20]. VEGF concentrations in follicles with mature oocytes were found higher than in follicles with immature oocytes and can be used as a potential marker for oocyte maturation [21]. Although oocyte quality was not assessed in the present study, the higher VEGF concentrations detected in POF, which are expected to contain mature oocytes according to their developmental stage rather than other follicle groups, exhibits compatible results with previous studies and suggests the important role of VEGF in supplying the requirements for increasing vascularity of the growing follicle.

In conclusion, each follicle individually has its own hormonal content, and the concentrations of hormones vary by developmental stage. Further investigations on transitional periods and in deep anoestrus, considering age factors, will provide new information for evaluating follicular development in mares.

Authors' Declaration of Interest

The authors have declared no competing interests.

5. Acknowledgements

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