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Hormonal and biochemical serum assay in relation to the estrous cycle and follicular growth in Arabian mare

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ABSTRACT

Objective: The current study is an endeavor for profound understanding hormonal and biochemical constituents in Arabian mare serum during the estrous cycle. **Methods:** Ten Arab brood mares of previous foaling records were scanned with ultrasound each other day, and blood samples were collected with each ultrasound examination. The diameter of follicles and corpus luteum was measured. The follicles were classified into small, medium, dominant, 1st and 2nd largest according to their diameter. Hormonal concentrations of thyroxin, progesterone, estradiol and testosterone were assayed. Superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO) and total antioxidants concentrations (TAC) were measured. Besides, the levels of total proteins, albumin, globulin, glucose, total cholesterol, and triglycerides were assayed. **Results:** The data revealed that multiple follicles underwent progressive enlargement (≤30 mm). Only the largest (dominant) follicle reached a maximal diameter of (35.82±1.57) mm during estrous phase. During the luteal phase, the corpus luteum reached a maximum diameter of (31.78±1.4) mm. The serum progesterone levels in mare were significantly (P<0.05) higher in luteal phase than those recorded at time of estrus. The reverse was true for estradiol. However, the levels of testosterone and thyroxin did not significantly change during estrous cycle in mare. No significant difference was observed in the serum levels of total protein, albumin and globulin in both phases of estrous cycle. Similar finding was observed in SOD concentrations. In contrast, the concentrations of glucose, cholesterol and triglyceride tended to be significantly (P<0.05) higher at estrous stage than those recorded at luteal one. The same finding was observed in NO levels. On other hand, TAC significantly increased (P<0.05) in mare serum obtained from luteal phase than those obtained at estrous one. A reverse was true for MDA levels. **Conlcusions:** The steroid hormones and metabolic constituents of mare serum (glucose, cholesterol and triglyceride) as well as NO, MDA and TAC vary according to the stage of the estrous cycle, suggesting their possible role in the process of follicular development in mare.

1. Introduction

Informations on the reproductive parameters of equine like folliculogenesis are needed for clinical and scientific purposes[1,2]. Recently, the mare has been considered a relevant comparative research model for follicle studies because of striking similarities with women in regard to follicle dynamics and hormonal changes during the interovulatory interval[3] and the ultrasonographic changes of the preovulatory follicle before ovulation[4,5]. The biochemical constituents of mare serum have still to be determined. Attention to this aspect of reproductive physiology is needed due to the pivotal role of ovulation in cyclicity and in reproductive management[1]. Data from the horse is scarce and is derived mainly from follicular fluid samples collected from slaughtered animals, with emphasis on retrospective steroid analyses and histological assessment of the stage of the follicular cycle[6]. Changes in biochemical constituents of blood are important indicators of physiological state of an animal[7]. Metabolic changes in the blood serum may be reflected in the biochemical composition of follicular fluid and can indirectly influence oocyte quality[8]. The regular pattern of mares estrous cycle

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relies on the balance among reproductive hormones. On other hand, the data on oxidants and antioxidants in mare estrous cycle are scarce. In equine, oxidant/antioxidant research has focused not only on the stallion because semen viability, motility and semen plasma membrane function involve oxidants and antioxidants[9,10]. The present study was planned for profound understanding hormonal and biochemical events taking place during the estrous cycle of Arabian mare serum in relation to follicular growth.

2. Materials and methods

2.1. Experimental location

This study was conducted at Police Abasia horse stud which were allowed to have the standard measurements of management, Cairo, Egypt (latitude 30′01′′N; longitude 31′21′′E) during summer (May to June 2012). Animals were kept under natural light in an open shelter and outdoor paddock.

2.2. Experimental animals, feeding and design

Ten clinically healthy, non lactating Arab broad mares, aging 5-10 years, weighing 350-400 kg were used in this study. The animals were cyclic and showed signs of estrus regularly. Daily observations for detection of estrus in mare were carried out using stallion of high libido. The animals were fed Egyptian clover (*Trifolium alexandriunm*) and had free access to water and mineralized salt. The animals were provided with concentrated ration and wheat straw a*d libitum*.

2.3. Ultrasonic scanning (US)

Estrous cycle was divided into two distinct phases (estrous and luteal phase) according to US and estrus behaviour. Ultrasound examination was performed each other day to study the follicular dynamics in mares during one regular estrous cycle (21 successive days). Ovarian scanning was used as previously described[11] using a NOVEK ultrasound scanner (Germany) equipped with multi-frequency linear array real time B-mode transducer of 2.6-7.5 MHz was used for scanning mares. A non-echogenic (black follicles) were counted and grouped according to their diameter into small (5-10 mm), medium (10-25 mm) and large follicles (≥25 mm). The diameter of the 1st and 2nd largest follicle was also recorded. Echogenic (homogenous grey) corpus luteum was recorded on both ovaries and its diameter was recorded during luteal stage of the estrous cycle.

2.4. Blood samples

Blood samples were collected via jugular vein with each ultrasound examination. Sera were separated and stored at −20 °C for biochemical analysis, antioxidants and hormone assaying.

2.5. Hormonal assay

Progesterone was measured using solid-phase ^{125}I-progesterone RIA (Coat-A-Count Progesterone; Diagnostic Product Corporation; Los Angeles, USA) according to the reference [12]. The assay sensitivity was 0.05 ng/mL. The inter- and intra-run precision coefficients of variation were 2.9% and 4.8%, respectively. Estradiol was assayed using RIA DSL-43100 commercial kit of diagnostic kit laboratories according to the reference [13]. Intra- and inter-assay coefficients of variation were 5.9% and 10.1%, repectively. Sensitivity of the assays was 2.0 pg/mL. Testosterone was assayed according to the reference [14]. The sensitivity of the assay was 0.038 ng/mL. The intra- and inter-assay precision was 4.5% and 6.3%, respectively. Total thyroxine was assayed according to the reference [15]. Sensitivity of the assay was 0.4 μg/dL. The intra- and inter-assay precision was 3.0% and 3.7%, respectively.

2.6. Metabolites assay

Serum total protein, albumin, glucose, cholesterol and triglycerides as well as malondialdehyde (MDA) and total antioxidant capacity (TAC) concentrations were determined spectrophotometrically in the collected samples. Total proteins were determined using bicinchinonic acid assay[16]. The albumin was assayed using ELISA[17]. Globulin concentrations in the samples were determined by subtracting albumin concentrations from total proteins concentrations. Serum glucose was assayed using glucose oxidase[18]. Serum concentration of total cholesterol was determined by an enzymatic colorimetric method[19] using cholesterol kit of Biodiagnostic. Triglycerides levels were measured using the competitive inhibition enzyme immunoassay technique[20].

2.7. Antioxidant and MDA assay

Superoxide dismutase (SOD) was estimated using an SOD assay kit (Fisher Scientific Company LLC; San Diego, USA) based on nitroblue tetrazolium reduction test[21]. Nitric Oxide (NO) was assayed using ELISA as previously described[22] using commercial available kits (Bio-diagnostic). Lipid perxidation was assayed by measuring malondialdehyde (MDA) levels[23] using commercially supplied kits (Bio-diagnostic). TAC concentrations were assayed using chemical colorimetry[24].

2.8. Statistical analysis

Data were subjected to statistical analysis using SPSS/PC[25]. Estrous and luteal data were also analyzed for period effects using repeated measure analysis of variance (ANOVA), and the Duncan′s multiple range test was used in separating between significant means. Data were expressed in mean±SEM, and the significance was set at $P<0.05$. Non-parametric correlation coefficients were performed using the Pearson′s method.

3. Results

3.1. Variation of follicles and corpus luteum in the estrous cycle of Arabian mare

The US examination revealed that during the estrous phase of the cycle (Table 1), multiple follicles underwent progressive enlargement (≤30 mm). Only the 1st largest (dominant) follicle reached a maximal diameter of (35.82±1.57) mm during estrous phase. On other hand, the mean diameters of the subordinate follicles (the 2nd largest) at the beginning of deviation are (20.38±1.37) mm. During the luteal phase, the corpus luteum became dominant and reached a maximum diameter of (31.78±1.40) mm compared to that recorded at estrous one [(24.30±2.79) mm)]. The diameter of small and medium follicle did not significantly change during estrous cycle.

3.2. Hormonal concentrations of mare serum in relation to estrous cycle

The serum progesterone levels in mare were significantly higher ($P<0.05$) in luteal phase of estrus cycle than those recorded at time of estrus. Conversely, the serum concentration of estradiol-17 beta were significantly ($P<0.05$) elevated at estrous phase than that recorded at luteal one, whereas the serum testosterone and thyroxine levels did not reveal any significant differences between estrous and luteal stage (Table 2).

3.3. Metabolite concentrations of mare serum in relation to estrous cycle

Table 3 shows no significant difference was observed in the serum levels of total protein, albumin and globulin in both phases of estrous cycle. In contrast, the concentrations of glucose, cholesterol and triglyceride tended to be significantly higher ($P<0.05$) in estrous stage than those recorded at luteal one.

3.4. Antioxidant capacity of mare serum in relation to estrous cycle

Serum SOD concentrations did not significantly differ between estrous and luteal phase of mare estrous cycle (Table 4). However, NO levels tended to be significantly higher during estrous than those recorded in luteal stage ($P<0.05$). On other hand, TAC activity was significantly higher ($P<0.05$) in mare serum obtained from luteal phase than those obtained at estrous one. A reverse was true for MDA levels.

Table 1

Follicular activity (mean±SEM) in relation to stages of estrous cycle in Arabian mare. (mm)

Stages of estrous cycle	Diameter of follicle				Diameter of corpus luteum
	Small	Medium	1st largest (dominant)	2nd largest	
Estrous phase	9.84±0.16	15.86±1.65	35.86±1.57[a]	26.38±1.13[a]	24.30±2.7[a]
Luteal phase	9.44±0.19	15.37±0.75	20.37±2.79[b]	16.82±1.31[b]	31.78±1.4[b]

Data with different superscripts within the same column are significantly different ($P<0.05$).

Table 2

Serum hormonal concentration (mean±SEM) in relation to stages of estrous cycle in Arabian mare.

Stages of estrous cycle	Progesterone (P_4) (ng/mL)	Estradiol-17 beta (E_2) (pg/mL)	Testosterone (ng/mL)	Thyroxine (T_4) (μg/mL)
Estrous phase	0.80±0.09[a]	83.21±10.7[a]	0.69±0.12[a]	5.89±0.26[a]
Luteal phase	3.23±0.05[b]	79.76±7.7[b]	0.80±0.08[a]	5.19±0.17[a]

Data with different superscripts within the same column are significantly different ($P<0.05$).

Table 3

Serum metabolites concentration (mean±SEM) in relation to stages of estrous cycle in Arabian mare.

Stages of estrous cycle	Total proteins (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)
Estrous phase	5.62±0.1[a]	4.70±0.1[a]	0.92±0.1[a]	50.53±2.7[a]	148.45±1.4[a]	59.15±1.4[a]
Luteal phase	5.50±0.1[a]	4.60±0.1[a]	0.90±0.0[a]	40.36±2.0[b]	144.22±1.1[b]	52.79±0.9[b]

Data with different superscripts within the same column are significantly different ($P<0.05$).

Table 4

Serum concentrations of SOD, NO, MDA and TAC (mean±SEM) in relation to stages of estrous cycle in Arabian mare.

Stages of estrous cycle	Super oxide dismutase (SOD) (U/mL)	Nitric oxide (NO) (μmol/L)	Malonaldhyde (MDA) (nmol/mL)	Total antioxidant capacity (TAC) (mmol/L)
Estrous phase	1 023.3±189.0[a]	13.17±0.14[a]	13.43±0.58[a]	2.1±2.1[a]
Luteal phase	915.3±131.4[a]	10.82±0.08[b]	11.64±.039[b]	5.9±4.6[b]

Data with different superscripts within the same column are significantly different ($P<0.05$).

3.5. Correlation between biochemical parameters and follicular diameter

There was a positive correlation between diameter of corpus luteum and progesterone level (r=0.56, P=0.001). However, the diameter of dominant follicles was significantly correlated with hormonal level of estrogen (r=0.55, P=0.01). Thyroxine level was correlated with diameter of the 1st and 2nd largest follicle (r=0.49, P=0.046; r=0.45, P=0.084 respectively). Glucose, NO and TAC levels were correlated with diameter of the largest follicle (r=0.48, P=0.007; r=0.42; P=0.056; and r= 0.49, P=0.041; respectively). Beside, TAC was correlated with diameter of the small (r=0.69; P=0.000 1). Nevertheless, the diameter of the medium follicle is correlated with cholesterol (r=0.37; P=0.041).

4. Discussion

In the present study, the minimum level of P_4 and the maximum level of E_2 were detected on day of estrus in Arabian mares. This is accompanied by an increase in follicular size until reaching its larger size of (35.82± 1.57) mm during estrus and ovulation. Similar finding was obtained by Amer *et al*.[26] and Ginther *et al*[27]. The growth of the dominant follicle was associated with certain intra-follicular E_2 and P_4 in mares[26]. In addition, US examination revealed that follicular development occurred throughout the estrous cycle and large follicles (≤30 mm) can occur even at luteal stage.These findings coincide with other reports[28,29]. The present study reveals that both sex hormones undergo fluctuations for maintenance of estrous cycle among Arabian mares[29,30]. A high relation between ultrasonography findings and hormonal concentration showed the increase of E_2 and the decrease of P_4 concentration, corresponding to the phase of the estrous cycle at which the experiments were performed[26,29,31]. With the increasing follicular size from <30 mm to >30 mm in diameter, there were a significant increase in the hormonal concentration of estrogen and a decrease in the concentration of serum progesterone[26]. Similar finding was observed in the present study. Thyroid hormones are imperative for normal ovarian functions and follicular growth as well as in general metabolic functions in the body. Herein, the concentration of thyroxin hormone was not significant changed at day of estrus and luteal stage. Since, Arab mares cycle continued all the year round and they ovulated continuously throughout the year[32]. Similarly, there was no significant fluctuations in T_3 or T_4 at any stage of estrous cycle[33]. On other hand, the testosterone levels were not significant at day of estrus or luteal stage in our finding. The low level of testosterone reported herein during estrous cycle of mare could be attributed to an increase in granulosa cell numbers and/or aromatase activity[34]. However, Silberzahn *et al*. reported that the testosterone levels were high at estrus in mares peripheral plasma and 11-13 d before the next estrous either before or after the fall in progesterone levels[35]. The levels of serum total proteins, albumin, and globulin were not affected by stage of cycle in the present study. This result indicates that the serum contents of these metabolites do not change during follicular growth. Similar findings were reported by Collins *et al*[36] in mare. Glucose plays an important role in ovarian metabolism because it is considered as the major energy source for the ovary. The serum level of glucose tended to be significantly higher at day of estrus than those recorded in luteal stage in the present study. Similar finding was observed in Arabian mare[37]. The maintenance of physiological levels of glucose is a perquisite for optimum fertility and reproductive success in mare[38]. Herein, the serum concentrations of cholesterol and triglycerides significantly (P<0.05) increased at estrus as compared to those collected from luteal stage. Cholesterol is considered the precursor of all steroid hormones including estrogen and progesterone[39]. Brood mares are subjected to different oxidative stressors during estrus, pregnancy, parturition and lactation. In human reproductive system, ROS and antioxidants perform physiological roles during folliculogenesis, oocyte maturation, luteal regression and fertilization[40].

The values of MDA significantly (P<0.05) increased at estrous phase than those obtained from luteal one in the present finding. However, TAC was significantly (P<0.05) higher in luteal stage than their counterparts at estrus. The high MDA concentrations recorded here in mare serum at estrus may be attributed to an increase in the production of ROS. These ROS were suggested to originate mainly from steroidogenesis occurring in granulosa cells[41]. The higher levels of TAC obtained from mare serum at luteal stage play an important role in the defense systems against oxidants[42]. However, data are not available to indicate how TAC and MDA concentrations are affected by the stage of estrous cycle. The results of the present study represent, to the best of our knowledge, the first documentation of presence of NO in mare serum and its variation with stages of estrous cycle. NO, an important intra ovarian factor, regulates the process of follicular development due to its multifaceted role in angiogenesis, vasodilation, and regulation of follicular basement membrane permeability, steroidogenesis and ovulation[43]. The presence of NO in mare serum and its variation with the follicular size is indicative of its possible involvement in the process of follicular development in the species. The higher concentration of NO in estrous phase of

this study corresponding to the follicle size during common growth phase in mare suggests its active role in follicular growth during this phase, possibly due to its regulatory action on angiogenesis and vasodilatation. Follicles are dependent upon angiogenesis and regulation of blood vessel function for their normal growth and function. This is evident from the growth of the inner network of capillaries in the theca interna concurrent with a period of rapid follicular growth and differentiation[44,45]. Increase in vascularity leads to the selection of a dominant follicle, whereas reduced vascularity leads to follicular atresia. Therefore, the lower NO concentration in luteal stage of mare observed in the present study could be one of the factors that probably hamper their progressive development due to decreased vascularity leading to atresia. An important feature of follicular selection and dominance is the associated increase in estradiol production[46]. Further studies are needed to elucidate the role of NO in follicular development during estrous cycle in mare. In conclusion, the data indicated that the steroid hormones (estrogen and progesterone) and metabolic constituents of mare serum (glucose, cholesterol and triglyceride) as well as NO, MDA and TAC vary according to the stage of the estrous cycle, suggesting their possible role in the process of follicular development in mare.

Conflict of interest statement

We declare that we have no conflict of interest.

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