Contents lists available at ScienceDirect

Asian Pacific Journal of Reproduction

Journal homepage: www.elsevier.com/locate/apjr

Document heading 10.1016/S2305-0500(13)60077-9

Oxidant/antioxidant status during foal heat in Arab mares and their relation to ovarian hormones

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ARTICLE INFO

Article history:
Received 5 August 2012
Received in revised form 8 August 2012
Accepted 10 September 2012
Available online 20 September 2012

Keywords:
Oxidative stress
lysozyme
Nitric oxide
Foal heat
Mare

ABSTRACT

Objective: To study the role of some oxidative stress biomarkers in conception during foal heat. **Methods:** Ultrasound scanning was used to confirm the presence of mature ovulating follicle (>30mm) in mares exhibited foal heat on days 7 to 9 postpartum. Daily blood samples were collected from parturition till foal heat to measure equine lysozymes, nitric oxide (NO), catalase (CAT), total proteins and globulins. Lipid peroxide (MDA), reduced glutathione (GSH), ascorbic acid (AA) and zinc were measured during foal heat expression form day 7 to 10 post foaling. **Results:** Lysozymes were high on days 1 and 9 postpartum but low level was recorded on day 6. NO, total proteins and globulin peaked on days 5 and 6 but low level was recorded on day 9. High MDA ($P<0.03$), zinc ($P<0.05$) levels were observed on days 8 and 9. High GSH was observed on day 9. AA ($P<0.03$) and CAT levels were low on days 8 and 9. Non conceived mares had high MDA, low zinc, AA, CAT and GSH. **Conclusions:** Mares during the foal heat are subjected to oxidative stress on days 8 and 9 as expressed by low AA, zinc, and GSH and high MDA and mares with sufficient antioxidant capacity can overcome this stress and conceive. Some mares need to be supplied during foal heat with antioxidants to improve their immunity and overcome oxidant/antioxidant imbalance to conceive. This study recommends the use of ascorbic acid and zinc as supplements during foal heat.

1. Introduction

Parturition has recently been considered an acute inflammatory process during which the uterus and cervix undergo a series of deep structural and functional reorganizations[1]. Involvement of the oxidant system in several reproductive processes is investigated during follicular development, ovarian steroidogenesis, ovulation, corpus luteum formation and function, luteolysis, germ cell function, maintenance of pregnancy and beginning of parturition[2]. Gebicki and Bartosz presented evidences supporting the theses that proteins are the primary targets of reactive oxygen species (ROS) in cells and that the protein radicals and other reactive protein derivatives generated act as intermediates, propagating the oxidative damage to other cell components[3].

Nitric oxide (NO) plays several roles in reproduction. It stimulates gonadotropin-releasing hormone (GnRH) secretion by activating ovary and is hypothesized to play a role in steroidogenesis[4]. It is implicated in the control of gonadotrophin secretion at both hypothalamic and hypophyseal levels, luteinizing hormone (LH) surge mechanism, sexual behaviour, estradiol synthesis, follicle survival and ovulation[5]. Estrogen may mediate its vascular effects through stimulation of endothelial NO synthesis[6]. It regulates ovarian blood flow before ovulation[7]. In the genital tract of mares, NO appears to be involved both in follicular growth and ovulation[8] and is also a potential mediator of luteal development and maintenance, angiogenesis, and blood flow[9]. It also regulates uterine blood flow during the estrous cycle in mares[10].

Lysozymes are hydrolytic enzymes, characterized by their ability to cleave the beta-[1,4]-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer. In the animal kingdom, three major distinct lysozyme types have been identified, including the c-type (chicken

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or conventional type), g-type (goose-type) and i-type (invertebrate type) lysozyme[11]. Lysozymes protect the host against pathogenic infection[12]. Equine lysozymes are also antibacterial proteins and have been implicated in innate immunity which act as a bacteriolytic enzyme identified in the many body fluids and tissues[13].

Limited reports about the possible effects of ROS in the female reproductive system are available[14]. Data on oxidants and antioxidants in mare reproduction are scarce. Postpartum period in the mares is characterized by rapid uterine involution and in some mares, an estrous period (foal heat) which is often fertile. Early postpartum endometrial cells undergo apoptosis but proliferation of cells is predominant during the 2nd week. Increased expression of estrogen receptors allow the endometrium to respond to estrogen during foal heat and endometrium respond to progesterone in diestrus[15]. Because of the mare's ability to come in foal heat and ovulate very soon, a thoroughly performed control of the puerperal period is of particular importance. It is desirable to breed mares at the first postpartum estrus (foal heat) in order to maintain a 12-month foaling interval and to produce earlier foals for racing and sales[16]. Conception rates are lower and embryonic losses are higher for approximately 2.5 month postpartum of thoroughbred mares, despite management practices that employ early postpartum breeding[17]. Our objectives were to characterize some of the oxidative stress biomarkers in Arab mares around foal heat, to relate them to conception at foal heat and to use these values as reference values to improve conception in mares subjected to oxidative stress during postpartum heat by using antioxidants supplementation for either dietary or treatment.

2. Material and methods

2.1. Animal sampling and examination

Sixteen multiparous Arabian mares (5-12 years old) belonged to Al-Zahraa Arab Horse stud were included in the present study. Animals were maintained under veterinary observation and fed Egyptian clover and concentrated ration formulated to fulfill energy requirement for lactating mares. From parturition (January to March 2007) till after foal heat expression using the selected stallion or day 11 postpartum, each animal was daily subjected to blood sampling via jugular vein puncture. Sera were preserved at -20 °C till antioxidant analysis. Mares were scanned with ultrasound (scanner 240, Pie Medical, Netherlands) equipped with endorectal linear array 6-8 MHz transducer on the days of natural breeding (day 7, 9 and 11 postpartum) to ascertain the presence, location, size(>30 mm in diameter) and morphology of the mature and ovulating follicle.

2.2. Blood antioxidant and biochemical analysis

Blood serum glutathione (GSH) reduced[18], nitric oxide (NO[19], lipid peroxide methane dicarboxylic aldehyde (MDA)[20], ascorbic acid[21], zinc[22] and catalase (CAT)[23] were measured by spectrophotometer using commercial kit (Bio-diagnostic, Egypt).

Lysozyme activity (μg/mL) was measured by using agarose gel cell lysis assay[24]. Lysozyme activity was measured by the lysoplate assay method. Briefly, heat-killed *Micrococcus lysodeikticus* (Sigma, 500 mg/L) were suspended in 1% agarose gel. Melt agarose was poured in Petri dishes to a depth of 4 mm. Twelve wells (1.5 mm in diameter) were cut in the agarose. Wells were filled with 2 μL of standard dilutions of chicken egg white lysozyme (0-500 μg/mL) or with serum samples. Petri dishes were incubated at RT for at least 18 h. The diameter of the lysis zones around each well was measured and the area (μm^2) was calculated and used as a result. The lytic zones were proportional to the concentration of lysozyme.

Serum levels of total proteins (g/dL) and albumin (g/dL) were determined spectrophotometerically[25,26] using diagnostic kit (StanBio, USA). Serum level of globulins was determined by subtracting the values of albumin from those of total proteins.

2.3. Statistical analysis

Data are presented as mean±SEM. Data were subjected to statistical analysis using SPSS software[27]. Simple one way ANOVA was done to identify the effect of days from foaling (day 1) till after foal heat expression (day 10). The Duncan's Multiple Range test was used to separate between significant means. Independent sample *t*-test was used to test differences in means of variables between conceived or non-conceived mares at foal heat. Pearson bivariate correlation coefficients were also calculated between studied variables.

3. Results

Reduced GSH levels around the foal heat are insignificantly high on day 9 than on day 7, day 8 and day 10. Moreover, its levels are insignificantly low in non-conceived mares at foal heat (Table 1). MDA levels are significantly ($P<0.05$) high on day 8 and day 9 compared to that on day 7 and day 10. A slight increase in MDA level was observed in non-conceived mares. Zinc levels are significantly ($P=0.05$) low on day 7 and day 10 compared to that on day 8 and day 9, and insignificantly low levels are recoded in non-conceived mares. Ascorbic acid level is significantly ($P<0.05$) high on day 7 and day 10 compared to that on day 9, and it is also high in conceived mares than non-conceived ones.

CAT levels are insignificantly low in non-conceived mares compared to those in conceived mares (Table 1), and its levels were low on day 8, day 9 and day 10 compared to that on day 7. Levels of NO, total proteins, globulins (Figure 1), CAT and lysozymes (Figure 2) were not significantly affected by day from foaling till day 10, but levels of lysozymes increased linearly from day 8 and show a peak on day 10. Also, NO, total proteins and globulins showed a peak on day 5 and day 6. There were three peaks in levels of CAT activity on day 4, day 6 and day 10 post foaling (Figure 2). The increase in CAT activity was accompanied by a decrease in lysozymes activity except on day 5 and day 7.

Ascorbic acid has a high negative correlation with both GSH ($r=-0.51$; $P=0.07$) and MDA ($r=-0.60$; $P=0.03$). A negative non-significant correlation is also found between ascorbic acid and zinc ($r=-0.49$; $P=0.1$). A significant negative correlation is found between ascorbic acid and progesterone (P_4; $r=-0.59$; $P=0.04$) but a high negative non-significant one is found with estradiol (E_2; $r=-0.81$; $P=0.4$). GSH is significantly correlated with E_2 ($r=0.66$; $P=0.03$) and has a negative significant correlation with MDA ($r=-0.33$; $P=0.04$). MDA is significantly correlated with NO ($r=0.36$; $P=0.03$). NO is significantly correlated with both total proteins ($r=0.52$; $P=0.001$) and globulins ($r=0.47$; $P=0.003$). P_4 is significantly correlated with zinc ($r=0.46$; $P=0.004$) and lyzozymes ($r=0.28$; $P=0.04$). A non-significant correlation is found between zinc and both lyzozymes ($r=0.30$) and E_2 ($r=0.33$) (Table 2)

Table 1

Antioxidant levels during foal heat and in either conceived or non-conceived mares.

Days from foaling	GSH (mg/dL)	MDA* (nmol/mL)	Zinc* (mg/L)	Ascorbic acid* (mg/L)	CAT (IU/L)
7	11.60±3.13	0.79±0.66^{a}	0.108±0.008^{a}	20.09±2.18^{c}	416.7±27.6
8	11.67±1.86	8.09±1.09^{c}	0.135±0.001^{b}	11.58±1.30^{b}	186.7±18.4
9	16.65±2.83	6.68±0.76^{bc}	0.125±0.004^{ab}	4.78±2.20^{a}	243.3±11.2
10	10.78±0.87	1.32±1.86^{ab}	0.114±0.002^{ab}	19.50±2.10^{c}	409.7±56.3
Conceived mares	18.49±2.30	4.13±0.62	0.136±0.007	12.71±1.23	443.8±111.9
Non-conceived mares	16.70±2.40	4.40±0.39	0.132±0.006	6.42±5.07	382.6±55.1

*Means significant at $P<0.05$; means with different superscripts in the same column are significantly different at $P<0.05$.

Table 2

Pearson correlation coefficients between antioxidant and biochemical indexes.

Indexes	GSH	MDA	Zinc	Lysozymes	Proteins	Globulin	NO	P_4	E_2
Ascorbic acid	−0.51	−0.60*	−0.49	−0.11	−1.00**	−1.00**	−0.17	−0.59*	−0.81
GSH	1.00	−0.33*	0.18	0.05	0.26	0.38	−0.17	0.28	0.66*
MDA		1.00	0.05	0.09	0.25	−0.03	0.36*	0.07	0.02
Zinc			1.00	0.30	−0.22	−0.20	−0.01	0.46**	0.33
Lysozymes				1.00	0.15	0.10	−0.11	0.28*	0.02
Protein					1.00	0.89**	0.52**	−0.49*	−1.00**
Globulin						1.00	0.47**	−0.25	−1.00**
NO							1.00	−0.07	−0.16
P_4								1.00	0.06
E_2									1.00

*Means correlation is significant at $P<0.05$, and ** means correlation is significant at $P<0.01$.

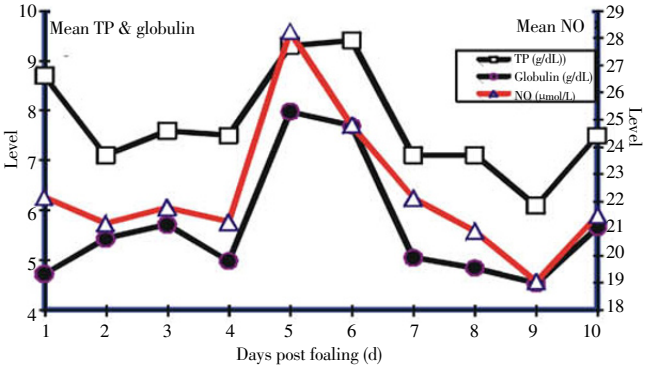

Figure 1. Levels of NO, total proteins (TP) and globulins from foaling till day 10 after foaling.

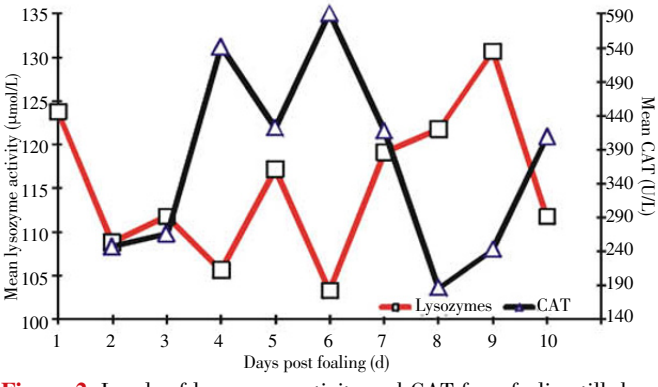

Figure 2. Levels of lysozymes activity and CAT from foaling till day 10 after foaling.

4. Discussion

The evaluation of pre- and peri-conception oxidative stress (increase in oxidative markers) and antioxidant status (reduce or insufficient antioxidant defense mechanisms) by appropriate and reliable techniques appears of utmost importance for preconception health care. Antioxidant/prooxidant status varied between individuals due essentially to differences in diet and environmental conditions. In addition, enzymatic and dietary antioxidants are both components of interrelated and complex systems that interact with each other to control ROS production and thereby they ensure defenses against oxidative stress and prevent cellular damage[28]. Antioxidants protect plasma membrane against peroxidative damage. The generation of lipid peroxides could alter plasma membrane fluidity which is necessary for sperm oocyte fusion[29]. From our point of view, zinc and ascorbic acid which are considered as important antioxidants can be supplemented to animals, and the levels of ascorbic acid and zinc were low on day 9 and in non-conceived mares because ascorbic acid has been proposed as electron donor for some transplasma membrane redox systems, since ascorbic acid may act as an oxidant at low concentrations and an antioxidant at high concentrations[30,31]. Similar to the low ascorbic acid levels in Arab mares observed here on day 9 (foal heat), Rapoport *et al*. recorded high ascorbic acid levels in dairy cows mature corpus luteum, and its levels increased from day 4 to day 12 (Luteal stage) and then decreased on day 20 (day of the next estrus) of the estrous cycle[32].

Interestingly enough, the current study found a positive correlation between estrogen and both GSH and zinc but a negative one was observed with ascorbic acid. Given this background, it could be inferred that oestrogen could have been stimulated and primed the immune response. Indeed, it has been amply stimulated[33]. Zinc can also reduce the free radicals and protect the cells from its harmful effect by its antioxidant action[34].

The increase in GSH levels on day 9 post foaling and in mares conceived when bred at foal heat compared to non-conceived let us suggest its role during ovulation and subsequently conception since GSH is a low molecular

weight scavenger, protects gametes and embryos against oxiradical damage by ROS[35]. Addition of GSH synthesis compounds such as P-mercaptoethanol[36] and cysteamine[37] to the maturation medium improved bovine embryo development up to the 3rd cleavage stage and also enhanced blastocyst formation following fertilization during *in vitro* maturation, and the effect of GSH during IVF on the proportion of blastocysts is dependent on GSH concentration[38].

CAT plays an important role in protecting organisms against oxidative damage caused by ROS by degrading surplus hydrogen peroxide[39]. Similar to the low CAT levels found in mares on days 8 and 9 (foal heat expression) compared to days from foaling to day 7 but in dairy cows, CAT increased from day 4 to 16[32] and declined to low levels on day 20 (next ovulation) of the bovine estrous cycle. Zinc, GSH, CAT and ascorbic acid were low in non-conceived mares during the foal heat. In agreement with our results, El-Deeb and El-Bahr recorded significantly ($P \leqslant 0.05$) lower values of CAT, NO and GSH in equine rhabdomyolysis horses than in the control[40]. In cows, a significant high correlation ($r=0.50$, $P<0.001$) was found between progesterone and CAT levels[32], but a non-significant low one ($r=0.25$) is found in the present study, maybe because a foal heat is an exceptional cycle, progesterone levels are low from foaling till shortly before ovulation in mares[41], and the cows experimental period was during luteal phase of a regular estrous cycle. Moreover, the luteal phase of bovine estrous cycle is longer than the standing heat but that of equine is characterized by longer estrous period that lasts from 5 to 9 d and this work was performed during only the estrous period of the equine estrus.

Similar to stress, vigorous exercise can induce an increase of plasma lipid peroxide concentration but did not change CAT activities[42]. However, we found high concentrations of lipid peroxide during days 8 and 9 just before ovulation. In sheep, a negative correlation was recorded between MDA and ascorbic acid ($r=-0.28$)[43], but our result is higher and more significant.

Although values of lysozymes in saliva did not differ between immunodeficient and healthy subjects and both age and sex have no significant effect on its values[44], this study performed on the same group of mare demonstrated a significant increase in lysozyme activity on days 9 and 10 post-foaling and near ovulation, which may be correlated with an antimicrobial polypeptides that play a role in innate immunity[45]. Equine lysozyme is also an evolutionary link between structurally homologous proteins-lysozymes and lactalbumins[46]. It has an active site of lysozymes, acts as a bacteriolytic enzyme and has calcium ion binding site, characteristic for alpha-lactalbumins[47]. The presence of lysozymes as antimicrobial components is found not only in airway secretions[48] but also widely distributed in tissues and external secretions such as saliva[49], cervical mucus[50], tear[51], milk and blood serum[44]. Their increase around ovulation led us assume either a role in ovulation process or to combat intrauterine bacteria during natural breeding and to investigate this role in mares suffering from subclinical post breeding endometritis.

NO plays a local role in the control of uterine function[52]. In agreement with Rosselli *et al.*[53], NO synthesis increases with follicular development where the ovulating follicle increases in growth rate from foaling, reaches its maximum diameter on day 5 and then has an evident change in its shape after day 5 post foaling. Moreover, the role of hormones in regulating NO synthesis during follicular development and ovulation was obvious. In contrast to the report of Rosselli *et al.*[53], a negative correlation was recorded between NO and estrogen. NO regulates ovarian function[54], but it remained unclear whether these effects were due to NO generated in the vasculature and neurons within the ovary or were due directly to NO generated by various cells within the ovary. Moreover, locally produced NO is important for the maintenance and increase ovarian blood flow during the preovulatory period[7]. As an oxidant, NO showed a correlation with MDA. It has a correlation with both total proteins and globulins because NO is generated from L-arginine by the action of NO synthase[28].

It could be concluded that during early postpartum period, some mares need to supplemention with antioxidants such as ascorbic acid and zinc in addition to some dietary antioxidants in order to decrease the oxidative stress caused by parturition and lactation and to improve their health, immunity and conception at foal heat.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors wishes to thank all the staff members of Al-Zahraa Arab Horse Stud for their cooperation during blood sampling and ultrasound examination. Animal sampling and ultrasound are allowed from Al Zahraa Arab Horse Stud. Chemicals and hormones are supplied and analyzed by authors in both National Research Centre and Animal Reproduction Research Institute.

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