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Antioxidents Markers, Trace Minerals and Steroid Hormones in Preconceptional Arab Mares

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Abstract: Arab brood mares (n=24) underwent weekly gynecological ultrasound examination and blood sampling during their breeding time from detection of mature ovulating follicle (≤ 35 mm) till detection of early pregnancy. Mares were divided according to conception into fertile when pregnancy was detected at 18 -45 days post breeding and sub-fertile when no pregnancy was detected. Sera were separated and subjected to hormonal assaying, copper and zinc measurements in addition to some oxidative stress biomarkers. Independent sample t-test was performed to evaluate data. Both zinc (p=0.02) and copper (p=0.05) levels were high in fertile mares. Superoxide dismutase SOD (p=0.002) and nitric oxide NO (p=0.59) levels were low in fertile mares. Ascorbic acid AA (p= 0.27) and glutathione reduced GSH (p=0.61) were slightly high in fertile mares. Malonaldehyde MDA was nearly similar in both fertile and infertile mares (p=0.99). Estradiol (p=0.02), progesterone (p=0.41), cortisol (p=0.0001) and testosterone (p=0.049) were low in fertile mares. The negative correlation between zinc and cortisol and between SOD with zinc and copper let us conclude that some mares with a sufficient antioxidants reservoir can overcome stress during breeding which are not only increase cortisol levels, but also affects trace minerals and SOD activity and in turn decrease conception either by decline in immunonological status or oxidant/antioxidant imbalance. Supplementing mares with sufficient antioxidants such as zinc, copper and ascorbic acid can overcome such stress and improve conception.

Key words: Mares · Cortisol · Microminerals · Oxidant Biomarkers · Steroid Hormones

INTRODUCTION

Oxidation provides energy for maintenance of cellular integrity and physiological functions. Most of the consumed oxygen forms carbon dioxide and water; however, 1 to 2% of the oxygen is not completely reduced and forms reactive oxygen species [1]. When antioxidant systems are insufficient, oxidative processes may damage DNA, lipids, enzymes and contribute to degenerative changes, including inflammation [2] and aging [3]. Therefore, ROS can play an important role in pathophysiology processes affecting female reproduction such as infertility, preeclampsia, fetal embryopathies, preterm labor and abortions and assisted fertility [4]. ROS and antioxidant enzyme systems are important component of the mammalian reproductive functions [5].

In human reproductive system, ROS and antioxidants perform physiological roles during folliculogenesis, oocyte maturation, luteal regression and fertilization [6]. In equine, oxidant/antioxidant research has focused primarily on male reproduction [7, 8]. However, there are limited reports about the possible effects of ROS in the female reproduction [9].

Micronutrients play a central role in metabolism and in the maintenance of tissue function. The most important category of these micronutrients is the trace elements. Zinc [10] and Copper [11] are essential for the function of growth, developments and the immune system cells and also for the activity of several enzymes. Concentrations of cortisol in horses increased after exercise [12] during isolation stress [13], after restraint via a twitch [14] and after sexual activity [15]. To our knowledge, no data are available relating cortisol to infertility in mares. In horses,

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several studies were conducted on endurance, race and exercise horse to investigate the effect of exercise on the oxidant/antioxidant equilibrium [16] but concerning mare reproduction, data and studies were not available.

This study was conducted to explore levels of some oxidative stress markers during mares' breeding. The values of some antioxidant markers in the blood serum of mares such as superoxide dismutase (SOD), glutathione reductase (GSH), nitric oxide (NO), ascorbic acid (AA) and the content of thiobarbituric acid-reactive substances (malondialdehyde, MDA) were determined. In addition, cortisol, progesterone, estradiol and testosterone levels were also evaluated. The main trace minerals concerning mare reproduction measured in this study were zinc and copper.

MATERIALS AND METHODS

Animals: Arab brood mares (n=24) aged 5-10 years, average body weight (350kg) were selected for regular ultrasonographic examination and blood sampling during their breeding interval. Breeding starts from October till the following May every year in Egypt. Mares belonged to the same horse farm (Police Academy, Abasia), samples were collected during the same interval (January to March 2010) and subjected to the same management. Mares were divided into two groups according to conception after breeding. Fertile mares (n=15) are mares that conceived and became pregnant after natural breeding. Subfertile (n=9) are mares that previously got pregnant but during the study period they were detected non pregnant with ultrasound after repeated breeding (three estrous cycles) without any obvious cause for the infertility.

Ultrasound Examination: A muti-frequency 2.5-7.5 MHz endorectal transducer of NOVEK ultrasound scanner (Germany) belonged to Police Academy horse farm was used for examining mares at weekly intervals for three successive estrous cycles from before breeding for detection of mature ovarian follicle (≥3cm) till detection of early pregnancy (18-21 days post breeding) and confirming it (45 days post breeding).

Blood Sampling: Blood samples were collected via jugular vein puncture with each ultrasound examination at 9 to 11 am. Sera were separated and stored at -20°C for biochemical analysis.

Biochemical Analysis

Determination of Oxidative Stress Markers: Activity of SOD (Cat number SD2521) in blood serum (inhibition rate percent) [17], GSH [18] (Cat number GR2511), NO (Cat number NO2533) [19], AA (Cat number MD2515) [20], MDA [21] was assayed by the measurement of MDA (Cat number MD2120) levels on the base of MDA reacted with thiobarbituric acid at 532 nm, using commercially (Bio diagnostic, Egypt) supplied kits (Kit number MD2529) and Zinc [22] was assayed by spectrophotometer using commercially supplied kits (Biodiagnostics, Egypt). Serum Cu concentrations were determined by flame atomic absorption spectrophotometer in the presence of lan-thanum chloride on a Perkin-Elmer Model 5000 with an AS-50 autosampler (Perkin-Elmer, Nonvalk, CT).

Hormone Assaying: Serum cortisol [23], progesterone [24], estradiol [25] and total testosterone [26] concentrations were analyzed using a commercially available Enzyme immunoassay kit supplied by Medical Biological Service S.R.L. (Milano, Italy). Sensitivity, intra- and inter-assay CV were 0.4 µg/dl, 2.9% and 3.8%, for cortisol, 10pg/ml, 9.1% and 9.8% for estradiol, 0.1ng/ml, 10.6% and 12.6% for progesterone and 0.022ng/ml, 6.6% and 7.3% for testosterone, respectively.

Statistical Analysis: All data are presented as means± standard error (S.E.) of the means. Data were analyzed using SPSS [27]. The obtained data were analyzed by the effect of reproductive condition using independent sample t-test, Pearson correlation coefficients were also performed between the assayed hormones and antioxidants.

RESULTS

Levels of NO (Table 1) were not significantly different between fertile (24.87±1.68) and subfertile mares, but its levels are slightly high in subfertile mares (26.59±2.55). AA levels are high in fertile (26.85±9.87) compared to subfertile (14.96±3.52). MDA levels are similar in fertile (4.93±0.79) and subfertile (4.94±1.58) mares. GSH concentrations are low in subfertile (10.07±1.39) mares. Zinc and copper (p=0.052) are significantly high in fertile mares. SOD levels are significantly (p=0.027) low in fertile mares (Table 1).

Table 1: Mean± SE values of blood serum levels of NO (µmol/L), ascorbic acid (AA, mg/L), GSH (mg/dL), MDA (nmol/ml), SOD(U/ml), Zinc (mg/l) and copper (mg/l)

Condition	Fertile (Conceived)	Subfertile (Not conceived)	
N	15	9	P-value
NO	24.87±1.68	26.59±2.55	0.59
AA	26.85±9.87	14.96±3.52	0.27
MDA	4.93±0.79	4.94±1.58	0.99
GSH	11.49±2.43	10.07±1.39	0.61
SOD	1942.6±38	3250.7±338	0.02
Zinc	0.129±0.01	0.108±0.01	0.02
Copper	0.57±0.04	0.42±.006	0.05

Table 2: Mean± SE values of E2 (pg/ml), P4 (ng/ml), testosterone (ng/ml) and cortisol (ng/ml) in cyclic and repeat breeder mares

Condition	CONCEIVED	NOT CONCEIVED	P -Value
Cortisol	15.67±1.61	27.18±1.90	0.0001
E_2	316.62±27.28	422.14±31.93	0.028
P_4	5.5692±1.22	7.3044±1.65	0.41
T	2.09±0.41	5.27±1.60	0.049
E2:T	241.7±79.58	98.09±48.8	0.215

Table 3: Pearson correlation coefficients between levels of cortisol, Progesterone (P4), esreadiol (E2), Testosterone (T), Zinc, copper,Reduced glutathione (GSH), Ascorbic acid (AA),Lipid peroxidase product(Malonaldehyde, MDA), Superoxide dismutase (SOD), E2/Tin blood serum of brood mars

	P4	E2	T	zinc	copper	GSH	AA	MDA	NO	SOD	E2/T
Cortisol	0.09	0.67**	0.55*	-0.64**	-0.14	0.12	-0.20	-0.05	-0.14	0.35	0.06
P4	1	0.31	0.56*	0.31	0.22	0.07	0.29	-0.15	0.00	-0.01	-0.48
E2		1	0.44	-0.42	0.02	-0.25	-0.04	-0.14	-0.41	0.34	0.02
T			1	-0.27	-0.28	-0.01	-0.01	-0.12	0.24	-0.10	-0.65*
zinc				1	0.46*	-0.01	-0.02	0.09	0.32	-0.45*	-0.11
copper					1	0.03	0.12	0.07	-0.01	-0.48*	0.16
GSH						1	-0.11	-0.42	-0.07	0.00	0.45
AA							1	0.06	0.02	-0.19	-0.14
MDA								1	0.34	-0.06	-0.14
NO									1	-0.37	-0.50
SOD										1	0.33

*significant at P<0.05 level, ** significant at p<0.01 level

Cortisol levels (Table 2) were significantly (p= 0.0001) low in fertile mares (15.67±1.61) compared to subfertiles (27.18±1.90). Estradiol levels are significantly (p= 0.028) low in fertile mares (316.62±27.28) compared to subfertile mares (422.14±31.93). Progesterone levels are not significantly low in fertile (5.5692±1.22) in comparison to subfertile mares (7.3044±1.65).Testosterone levels are significantly (p=0.049) low in fertile mares (2.09±0.41) compared to subfertile mares (5.27±1.60).

Cortisol had strong positive correlation with estradiol (E2, r= 0.67) and testosterone (r= 0.55; Table3) but negative significant correlation was found with zinc (r= - 0.64). Progesterone had a strong correlation with only testosterone (r=0.56). Estradiol has negative but non significant correlations with both zinc and NO and positive non significant correlations with both testosterone and SOD. Zinc has a negative significant correlation with SOD (r= -0.45) and a significant positive one with copper (r=0.46). Copper has also a negative correlation with SOD (r= -0.48). GSH has a non significant negative correlation with MDA. MDA has a non significant positive correlation with NO. NO has a negative non significant correlation with SOD.

DISCUSSION

In this study, GSH, AA, zinc and copper levels in subfertile mares were lower than those subsequently conceived. The same was observed in trained thoroughbred horses before supplementing them with antioxidants whatever their age or gender [28]. The slight insignificant decline of GSH in this study may reflect increased stress on infertile mares. GSH antioxidant system is foremost among the cellular protective mechanisms. Depletion of this small molecule is a common consequence of increased formation of ROS during increased cellular activities. Antioxidant imbalance was also observed after three months in the control horses, reflected by a significant decrease in GSH, SOD [28]. Exhaustive exercise depletes glutathione and simultaneously generates free radicals. This is evidenced by increases in lipid peroxidation, glutathione oxidation and oxidative protein damage [29]. Ishida *et al*. [30] confirmed the expression of Cu/ZnSOD and Mn-SOD genes in the equine tissues by RT-PCR and in situ hybridization. In brood mares of this study, the sensitivity of the mares to slight increase in stress may decrease the immunological status and in turn starts to deplete GSH.

The increase in trace minerals in fertile mares which in turn got conceived compared to subfertile that failed to conceive agrees with Stanton *et al*. [31] who reported that cows receiving organic trace minerals exhibited higher pregnancy rates to AI than those receiving inorganic trace minerals. Improved reproductive performance has been also reported in dairy cows receiving organic mineral supplements [32], which were attributed to improve repair of damaged uterine tissue following calving. As well as, organic trace minerals supplemented cows tended to have a higher pregnancy rate to AI than did inorganic supplemented cows [33]. Although, Wichert *et al*. [34] recorded no clinical signs for zinc and copper deficiency in horses in Bavaria and referred the absence of clinical signs to vitamin E supplementation in adult non-reproducing and nonperforming horses fed diet deficient in trace minerals. Trace-elements, such as zinc and copper play an important catalytic role for the enzymatic activity of SOD (Zn, Mn, Cu) [35, 36], so their decline in infertile mares is related to increased activity of SOD and this also confirmed by the negative correlation between them and SOD. This was observed in horses from a region where feedstuffs were known to be Cu- and Zn deficient [37]. Since zinc decreases ROS production [38], this study proved similar results in fertile mares where high zinc is accompanied by high GSH, NO, AA concentrations and low SOD. Training Standardbreds for 3 months increased both AA and SOD [28]. The increase in the levels of SOD in the serum of subfertile mares was accompanied by a decline in both zinc and copper and the negative correlation between SOD with both zinc and copper in this work is in agreement with Fang [39] who concluded that insufficient protein intake results in deficiency of zinc [a cofactor of Cu,Zn-SOD], indirectly affecting superoxide removal system. In contrast, SOD activity was unchanged by feeding a Cu- and Zn supplemented diet for 24 days to horses fed to be in a mineral-deficient state but a longer diet adaptation period, preceded by a more dramatic state of deficiency, would better serve as a model to examine the impact of Cu and Zn status on SOD activity [40].

This study proved that AA related to fertility as its levels are high in fertile mare and is also correlated with progesterone. Ascorbic acid and zinc are regarded as the scavengers of excessive superoxide anions production, although simultaneously zinc was found to be a dose dependent inhibitor of SOD-like activity [41]. AA activity increased also during early pregnancy in mares' uterine flushing. Moreover, total recoverable ascorbic acid was also affected [$P<0.01$] by day of the estrous cycle and pregnancy [42].

Malondialdehyde [MDA] is an indicator of the lipid peroxidation and, the amount of produced MDA was used as an index of the lipid peroxidation. Although in brood mares of this study MDA was not different in both groups but it has a negative correlation with GSH and a positive one with NO. In goats, MDA level began to increase sharply within 1–4 days following the insertion of sponges. These high levels were maintained in both groups until the sponge withdrawal and it was arrived to highest level at the day of sponge withdrawal. Following the sponge removal, MDA level declined rapidly to below basal level in vitamin E treatment group but remained high in the control group [43].

This study found a negative correlation between SOD and NO. NO differently regulates SOD according to exposure time of lutein cells to ROS during luteolysis and NO reduces SOD levels to facilitate excess intraluteal ROS during luteolysis [44]. NO regulates also ovarian function [45]. Moreover, locally produced NO is important for the maintenance and increase ovarian blood flow during the preovulatory period [46]. In agreement with Rosselli *et al*. [47], NO synthesis increases with follicular development. In contrast to Rosselli *et al*. [47] a negative correlation was recorded between NO and estrogen which may led us suggest a feedback control mechanism between them. NO also behaves as a potential antioxidant agent by virtue of its ability to reduce other molecules [6]. NO could act with a dual action (protective or pro-oxidant) in CL development during the estrous cycle [48]. The correlation between NO and MDA found in this study agree with other reports [49]. Modulation of this balance may have important clinical implications.

The increased levels of NO and their association with increased estradiol levels and in turn increased blood flow to the genital organs either physiologically during follicular and luteal growth or due to slight uterine inflammatory response during breeding. The negative correlation between NO and E2 indicates the synergistic roles between them. As NO is the main mediator for estrogen-induced stimulation of uterine blood flow in addition to other estrogen–independent actions [50]. The high NO and E2 levels found in subfertile mares confirmed that uterine nitric oxide synthestase (NOS) system plays a major role in regulation of uterine perfusion during the estrous cycle in mares by increasing uterine blood flow during estrus and early luteal phase in mares [50]. Although this study found no correlation between NO and P4 but NO seem to act as luteotrophic factors in mares by stimulating progesterone production.

The present study found increased concentrations of cortisol, testosterone [T], estradiol [E2] and progesterone [P4] in mares that did not conceive and this increase was significant for all of them except P4 and the similar correlation of T with both E2 and P4 is confirmed since the equine species is characterized by its ability to convert strongly androgen-to-estrogen, principally by aromatase localized in follicular theca interna and granulosa cells [51] and in the corpus luteum [53]. Testosterone [T], the main endogenous anabolic androgen, is produced by the ovary of the cycling mare [53] and by the corpus luteum in early pregnant mares [54]. E2 can stimulate antioxidant enzyme expression [55] and reducing superoxide [O2-] and/or peroxynitrite [ONOO-, 56]. In this study, E2 stimulated SOD production and inactivated or depleted GSH, NO and zinc. In overectomized estrogen deficient female hamster, estrogen replacement showed significant protective effects for antioxidant reservation against exercise training oxidative stress [57]. Testosterone, not estrogen, acts in the corpus luteum to stimulate progesterone production and Mn-SOD expression in rats [58]. In dairy cows, Rapoport *et al.* [59] recorded a positive significant ($P<0.001$) correlation($r=0.66$) between SOD and progesterone during the luteal stage of the estrous cycle. In this study P4 is not only high in subfertile mares but is also correlated with AA, copper and zinc. Progestogens act as glucocorticoid agonists and have long-lasting suppressive effects on the hypothalamic–pituitary axis [60]. As increased progesterone concentrations delayed the onset of estrus and inhibited the pre-ovulatory LH surge by negative feedback in cows Duchens *et al*. [61], in this study it may declined conception through disturbing oestrous cycle length and delaying ovulation.

CONCLUSION

Stress subjected on mares during breeding season could hinder conception. Mares that overcame this stress are those having a reservoir of oxidative stress combating elements such as trace minerals, SOD, GSH and AA. This study recommended supplying mares during breeding season by trace minerals and ascorbic acid to achieve high conception rate.

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