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Oxidative Stress Biomarkers in Mares with Different Reproductive Problems

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ABSTRACT

The present study aimed to investigate some oxidative stress biomarkers and their relations to reproductive problems. Two hundred and ninety two blood samples were collected from 53 pure Arabian mares after reproductive ultrasound examination. Superoxide dismutase (SOD), nitric oxide (NO), catalase (CAT) and the product of lipid peroxidation (malondialdehyde, MDA) were measured in blood serum. In pregnant mares, both MDA ($P<0.0001$) and NO ($P<0.05$) levels were high in mares encountered early embryonic death compared to pregnant ones. SOD concentrations were insignificantly low in mares with early embryonic death while CAT concentrations were significantly high ($P<0.05$) in pregnant mares after anestrum period and those encountered early embryonic death. In non pregnant mares, concentrations of MDA and NO significantly ($P<0.0001$) increased in mares with anovulatory follicle and granulose cell tumor. NO levels significantly increased in repeat breeder mares and those cyclic after abortion. SOD significantly ($P<0.01$) increased after abortion, however, CAT significantly ($p<0.05$) increased at foal heat. In conclusion, mares after early embryonic death, abortion and at foal heat (normal parturition) are exposed to oxidative stress. Supplementation with antioxidant feed additives might overcome this oxidative stress and resume mare reproductive performance.

Key words: Oxidative stress, endometritis, early pregnancy, early embryonic death, mare.

Introduction

Pregnancy failure that occurs between fertilization to day 60 of gestation is called early embryonic loss and embryos die starting from fertilized eggs till after organogenesis (Ball *et al.,* 1986 and Allen, 2000). The diagnosis of early embryonic loss from day 15 to day 60 became possible by the use of transrectal ultrasonography for early pregnancy diagnosis. Using ultrasonography in mare pregnancy diagnosis revealed that the rate of embryonic death ranged from 5 to 24% during 11 and 50 days (Ginther *et al.,* 1985) and 13.28 % during 19-21 days post-ovulation (Papa *et al.,* 1998).

Acute, chronic, or subclinical inflammatory conditions of the uterus are known as endometritis that causes substantial reductions in mare fertility (Hurtgen, 2006). Breeding-induced endometritis (BIE) in the mare is resolved by 36 hr after insemination in resistant mares. However, 10-15% susceptible broodmares fail to do so because of impaired uterine contractility between 7 and 19 hr after exposure to seminal or bacterial challenge, which adversely affects their fertility (Alghamdi *et al.,* 2005). During endometritis, sperm phagocytosis increased, zygotes resulting from fertilization of oocytes with sperm subjected to oxidative stress are less likely to develop to the blastocyst stage, lipopolysaccharides (LPS) and tumour necrosis factor-α (TNFα) impair follicular steroidogenesis growth and ovulation and even embryos exposed to inflammatory mediators during development have fewer trophoectoderm cells (Gilbert, 2011).Most of the consumed oxygen forms are carbon dioxide and water; however, 1 to 2% of not completely reduced oxygen forms are reactive oxygen species (ROS) (Clarkson and Thompson, 2000). ROS can play an important role in pathophysiology processes affecting female reproduction such as infertility, fetal embryopathies and abortions (Agarwal, and Allamaneni, 2004). ROS and antioxidant enzyme systems are important component of the mammalian reproductive functions (Al-Gubory *et al.,* 2010). 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 cells (Gebicki and Bartosz, 2010). When antioxidant systems are insufficient, oxidative processes may damage DNA, lipids, enzymes and contribute to degenerative changes including inflammation (Frohman, 1993).

Reactive nitrogen species (RNS) include NO and nitrogen dioxide (NO_2) in addition to non-reactive species such as peroxynitrite ($ONOO^-$), and nitrosamines (Rosselli *et al.,* 1998). RNS are mainly derived from

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NO, which is formed from O_2 and L-arginine, and its reaction with the SO anion, which forms peroxynitrite (Burton and Jauniaux, 2010). Peroxynitrite is capable of inducing lipid peroxidation and nitrosation of many tyrosine molecules that normally act as mediators of enzyme function and signal transduction (Rosselli *et al.,* 1998). NO plays several roles in reproduction. In both ovarian cells-derived and vascular endothelial cell-derived, NO has an essential role in the physiology and biology of the ovary with respect to regulation of folliculogenesis and ovulation (Rosselli *et al.,* 1998). NO appears to be involved in both follicular growth and ovulation (Pinto *et al.,* 2002) and also is a potential mediator of luteal development and maintenance, angiogenesis, and blood flow (Ferreira-Dias *et al.,* 2011). It also regulates uterine blood flow during the estrous cycle in mares (Honnens *et al.,* 2011).

Thiobarbituric acid-reacting substances (TBARS), which measure primarily malondialdehyde derived from lipid peroxidation, as well as other breakdown products from oxidatively modified proteins, carbohydrates and nucleic acids (Guichardant *et al.*, 2004).

This study was conducted to explore the changes in oxidative stress biomarkers in mares with different reproductive problems by measuring superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO) and the content of thiobarbituric acid-reactive substances (malondialdehyde, MDA).

Material and Methods

Animals

Brood Arab mares (3-15 years old) belonged to El-Basaten Horse Club, Police Academy Horse Stud (Abbasia, Cairo, Ministry of Interior), Al-Zahraa Pure Arabian Horse Stud and Private Arabian Horse Studs (Cairo) were located under investigation. Mares (n=53) were kept in an indoor paddocks. Mares were kept under natural day-light and temperature. They were maintained on a commercial pelleted ration and hay or barley and hay during summer with free access to water. Barley and good quality Egyptian clover (*Trifolium alexandrinum*) is available from October to May every year where breeding commences.

Ultrasound scanning

A multi-frequency 2.6-7.5 MHz endo-rectal transducer of NOVEK ultrasound scanner (Germany) belonged to Police Academy Horse farm and a scanner 200 (Pie medical, Netherlands) equipped with 6-8 linear-array real time B-mode transducer was used for examining mares at weekly intervals before breeding for detection of mature graffian follicles (≥30 mm). When a large follicle >30mm and uterine edema was scanned, mare was referred to a stallion for confirming estrus signs and excluding persistent anovulatory follicles.Early pregnancy, endometrial and ovarian abnormality were assessed by ultrasound with reference to previous breeding history of the mare. Both ovarian and uterine reproductive events were recorded.

Blood sampling

One hundred and seventy six blood samples (Table 1) were collected from non pregnant mares and 116 samples were collected from pregnant mares (Table 2) via Jugular vein punctures in plain vacuum tubes. Harvested sera were stored at -20°C till assaying.

Table 1: Number of blood samples collected from non pregnant mares.

Reproductive condition	Foal heat	Repeat breeder	Cyclic normal	Endometritis	Granulosa cell tumor	Anovulatory follicle	After abortion	Total
No. of samples	5	46	40	21	5	20	39	176

Table 2: Number of blood samples collected from pregnant mares.

Reproductive condition	Pregnant at foal heat	Early pregnant	Pregnant after anestrum treated	Early Embryonic death	Total
No. of samples	6	96	7	7	116

Oxidative stress biomarker measurements

For measuring MDA, the assay utilized developed thiobarbituric acid-reactive substances (TBARS) which were formed as result of reaction of thiobarbituric acid with malondialdehyde (MDA) in acidic medium at 95 ºC for 30 minutes (Ohkawa *et al.,* 1979). Concerning NO, in acid medium and in the presence of nitrite, the formed nitrous acid diazotise sulphanilamide and the product are coupled with N-(1–naphthyl) ethylenediamine (Montgomery and Dymock, 1961). Concerning CAT, it reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor (Aebi, 1984). SOD assay relied on the ability of the SOD to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetra zolium dye (Nishikimi *et al.,* 1972).

Commercial diagnostic kits were supplied by Biodiagnostic (29 Tahreer St., Dokki, Giza, Egypt) for assay of values of serum MDA, NO, CAT and SOD.

Statistical analysis

Data are presented as Mean±SEM (Standard error) using SPSS (2007) software version 16.0. Analysis of variance one way ANOVA of pregnant and non pregnant mares was used. Results are presented in plots with error bars.

Results

In pregnant mares, significant high MDA ($P<0.0001$) levels were recorded in mares encountered early embryonic death compared to those bred at foal heat, early pregnant and those bred after receiving treatment for induction of estrus (Figure 1).

Significant high NO ($P<0.05$) were observed in mares encountered early embryonic death compared to those get pregnant at foal heat and after receiving treatment for anestrum. Early pregnant mares had insignificantly high NO concentrations compared to the two other pregnant groups (Figure 1).

CAT concentrations were significantly high ($P<0.01$) in pregnant mares after anestrum treatment and those encountered early embryonic death compared to those get pregnant at foal heat while, the CAT of early pregnant mares were insignificant compared to other three groups. SOD concentrations were insignificantly varied among the four groups (Figure 2).

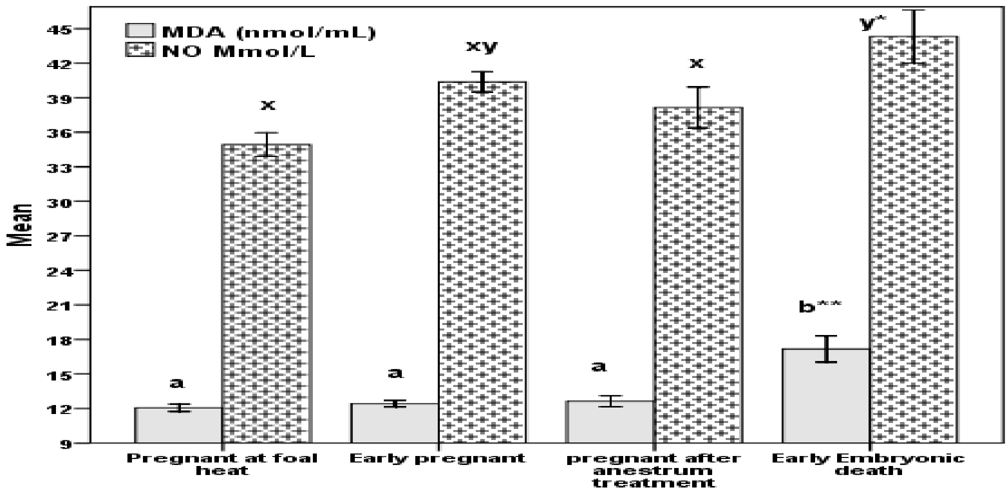

Fig. 1: Mean MDA and NO concentrations with error bars. Means with different superscripts (a, b)are significant at $P<0.05$. Means with different superscripts (x, y) are significant at $P<0.05$. * means significant at $P<0.05$, ** means significant at $P<0.0001$.

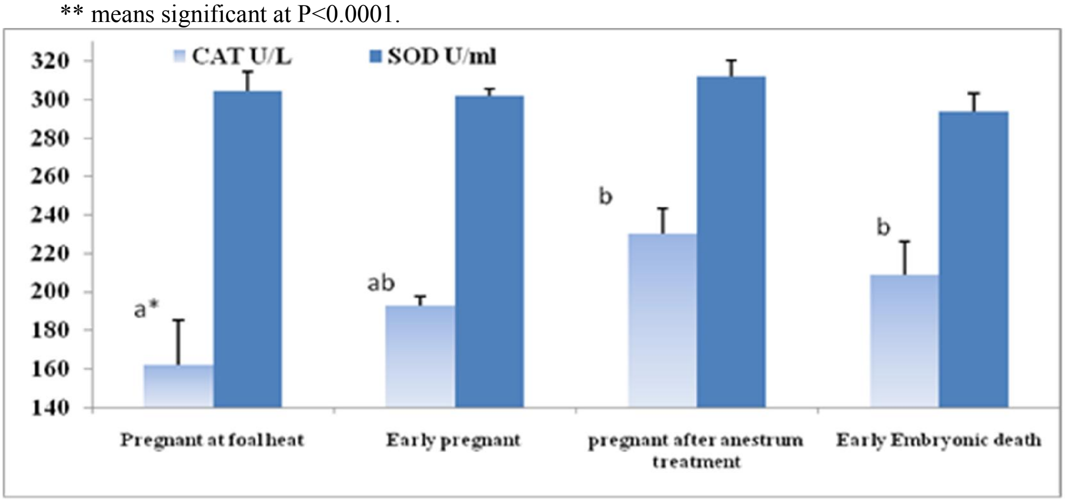

Fig. 2: Mean SOD and CAT concentrations with error bars. Means with different superscripts (a, b) are significant at $P<0.05$. * Means are significant at $P<0.01$.

In non pregnant mares, concentrations of MDA significantly (P<0.0001) increased in mares with anovulatory follicle and granulose cell tumor compared to those at foal heat and those cycling normally (Figure 3).

NO levels significantly (P<0.0001) increased in granulose cell tumor, anovulatory follicle and after abortion compared to those at foal heat, endometritis or those cycling normal (Figure 3).

Significant (P<0.05) high CAT levels were recorded in mares at foal heat compared to all non pregnant mares of all groups.

Significant (P<0.01) high SOD concentrations were observed in mares after abortion compared to mares at foal heat, with granulose cell tumor and those with endometritis.

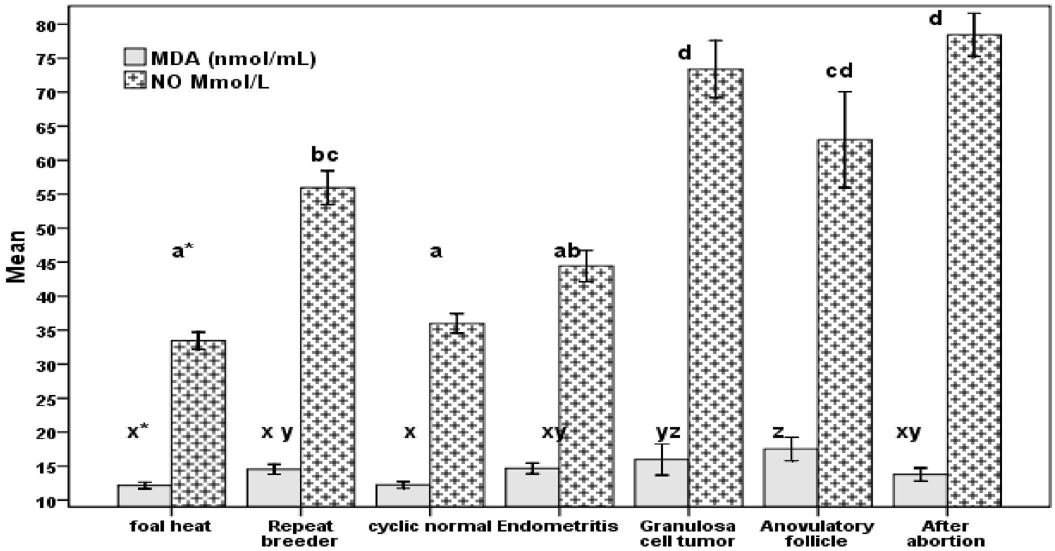

Fig. 3: Mean MDA and NO concentrations with error bars. Means with different superscripts (a, b) are significant at P<0.05. Means with different superscripts (x, y) are significant at P<0.05.* means are significant at P<0.0001

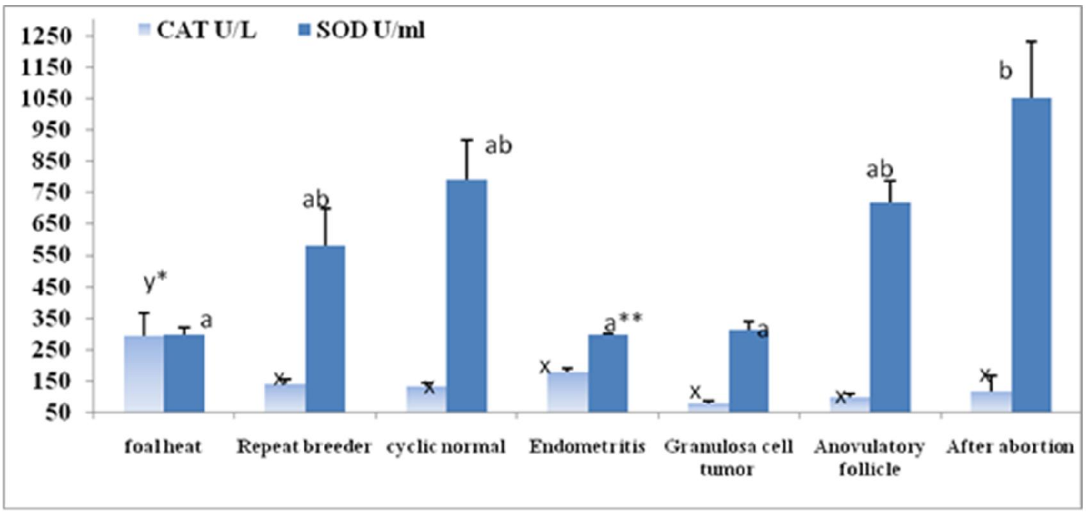

Fig.4: Mean SOD and CAT concentrations with error bars. Means with different superscripts (a, b) are significant at P<0.01(**). Means with different superscripts (x, y) are significant at P<0.05(*).

Discussion

Nitric oxide is a reactive free radical, synthesized from L-arginine by nitric oxide synthase (NOS) which catalyzes the mixed functional oxidation of a guanidino nitrogen atom of L-arginine to yield L-citrulline and NO (Kwon *et al.,* 1990 , Moncada *et al.,* 1991). NO may occasionally be harmful to tissue by producing secondary toxic products. It exerts a wide range of biologic effects through activation of soluble guanylyl cyclase (Alderton *et al.,* 2001; Keynes and Garthwaite, 2004), but it can also interact directly with thiol group (SH) on certain cysteine residues, resulting in protein nitrosylation (Stamler, 1994). Those cysteine residues have regulatory roles in enzymes and other proteins. Cysteine nitrosylation modulates enzyme activity and alters protein functions. Caspases are among the proteins whose functions are modulated by nitrosylation, and S nitrosylation of the active cysteine residues of caspase-3 and -9 resulting in inactivation of the enzymes (Dimmeler *et al.,* 1997; Mannick *et al.,* 1999, 2001).

In tissues of low O_2 saturation condition, NO reduces ischemic damage by increasing blood flow secondary to vasodilating effect. While, in tissues of high O_2 saturation condition, it leads to directly toxic metabolites by reacting superoxide and forming some toxic substances such as peroxynitrite in the cells (Wright *et al.,* 1992). NO is involved in the regulation of blood flow through the reproductive tract (Barszczewska *et al.,* 2005). Presence of different isoforms of NO synthase was reported in the equine endometrium of early pregnant mares (Welter *et al.,* 2004). NO is considered an important mediator implicated in maintenance and termination of pregnancy (Yalampalli *et al*., 1993,1994). The significant increase of NO levels in mares after losing their embryos compared to pregnant mares is in agreement with previous studies on rabbits (Sladek *et al.,* 1993), and human (Buhimschi *et al.,* 1995) as NO is also generated by the uterus to inhibit uterine contractility during pregnancy but after early embryonic death uterine contractility may increase to clear the uterus from remnants of the absorbed embryo and to prepare the uterus to the next estrous and ovulation. In contrast, a previous study reported non significant low NO in mares encountered early embryonic death compared to early pregnant mares (Ezzo *et al.,* 2011). Also, circulating levels of nitrite/nitrate were similar in successful and unsuccessful implantation and unrelated to the outcome of pregnancy (Fábregues *et al.,* 2000).

In the current study, the increased NO in repeat breeder mares and after abortion compared to mares cyclic normally or at first foal heat may refer to the elevated nitric oxide generated in non pregnant uterus during proestrus (Yallampalli *et al.,* 1998). Endometritis is associated with impaired reproductive performance which directly affected by bacterial products, such as LPS (endotoxin), or indirectly by inflammatory mediators, such as NO and oxidative stress affecting sperm, ovarian, uterine and embryonic function (Gilbert, 2011). Different isoforms of NO synthase was reported in the equine endometrium of cyclic mares (Welter *et al.,* 2004) and those susceptible to post-breeding endometritis (Alghamdi *et al.,* 2005). Beside, NO played an important role in the endometrium, where changes in vascular function occur throughout the estrous cycle (Reynolds *et al.,* 1992). Moreover, endothelial NOS was observed in endometrial glands, endothelial cells, fibroblasts, blood and lymphatic vessels. Endometrial eNOS expression was the highest in the follicular and mid-luteal phases while it was found to be the lowest in the early luteal phase. In the follicular phase, hyperplasia of endometrial tissue with respect to myometrium was detected. NO may play some roles in both proliferative and secretory phases of endometrial development in the mare (Roberto da Costa *et al.,* 2007). Nearly, a similar NO levels in mares with endometritis were recorded to those cyclic normally or at foal heat. This is similar to our previous study which reported that mares with reproductive tract infections had similar NO concentrations to those cyclic normally but insignificantly low compared to early pregnant mares and those with upper-respiratory and reproductive tract infection (Abo El-Maaty *et al.,* 2014). The decreased circulating NO in mares with infectious clinical endometritis may refer to the consumption of circulating NO as a free radical to decrease the effects of bacterial toxins on endometrial cells. On the contrary, susceptible mares with breeding-induced endometritis had a higher NO in their uterine secretions and greater uterine inducible NOS (iNOS) expression compared with resistant mares (Alghamdi *et al.,* 2005). Moreover, during the estrous preceding successful or unsuccessful conception, concentrations of NO did not significantly change in repeat breeder mares encountered several unsuccessful bleedings (Abo El-Maaty *et al.,* 2012b), suggesting a possible role of NO, either directly or in a NO-associated pathway, in delayed uterine clearance (Alghamdi *et al.,* 2005).

NO-generating system is also present in the equine ovary (Pinto *et al.,* 2003). NO is involved in the ovulatory process along with the fact that the granulosa and theca interna cells start to luteinize before ovulation in response to an ovulatory stimulus (Collins *et al.,* 1997, Kerban *et al.,* 1999). The significant higher NO concentrations in mares with anovulatory follicles or those with granulosa cell tumor of this study were also higher than that previously recorded during normal estrous cycle of mares where circulating NO reached a maximum level 3 days before ovulation and at day 5 after ovulation (Abdelnaby et al., 2015). These results confirms that NO has been implicated in ovulation (Bonello *et al*., 1996), steroidogenesis (Van Voorhis *et al*., 1994) and follicle survival (Chun *et al*., 1995). NO synthesized by the rat ovary participate in ovulation and atresia (Shukovski and Tsafriri, 1995), by controlling of ovarian vessel relaxation to accommodate the necessary changes in blood flow, blood volume, and plasma exudation that accompany follicle rupture (Bonello *et al.,* 1996 and Yamauchi *et al.,* 2006). It is likely to be the most important role of NO.

The higher MDA observed in mares with early embryonic death compared to pregnant mares and the higher levels in mares repeat breeding, with endometritis and after abortion indicate the oxidative stress that reduced conception and prevents maintenance of embryoes. In agreement with our results, plasma concentrations of MDA were compared in healthy mares and in mares with endometritis (Abo El-Maaty *et al.,* 2014). Moreover, the level of MDA was significantly increased in the mares with endometritis due to increased lipid peroxidation in purebred

Arabian mares affected with endometritis (Yaralioglu-Gurgoze *et al.,* 2005). The increase in oxidative stress (OS) corresponds to compensation by the antioxidant systems so that the MDA concentration (index of lipid peroxidation) remains constant; in other words, the oxidation/antioxidant protection system tends to be equilibrated for the entire menstrual cycle. In agreement with our results, levels of MDA were significantly higher in the liver, spleen, kidney and brain of pregnant-infected mice compared with apparent healthy pregnant mice. Although MDA levels were significantly higher in the placenta of pregnant-infected mice compared with pregnant mice (Sharma *et al.,* 2012). In addition, lipid peroxide level was highest among infertile women with endometriosis (Szczepańska *et al.,* 2003). MDA concentrations are significantly low in mares with reproductive tract infection compared to early pregnant and normal cyclic mares (Abo El-Maaty *et al.,* 2014). This increase in oxidative stress during the menstural cycle in women corresponds to compensation by the antioxidant systems so that the MDA concentration remains constant; in other words, the oxidation/antioxidant protection system tends to be equilibrated for the entire menstrual cycle (Cornelli *et al.,* 2013). In agreement with the increased MDA concentrations in mares encountered abortion or early embryonic death, women with idiopathic recurrent pregnancy loss (RPL) showed increased MDA levels (El-Far *et al.,* 2007). The higher MDA concentrations observed in mares encountered early embryonic death indicated the great damage to the endometrium due to the death of the embryo. In agreement with our previous results, after normal foaling and during the foal heat, mares are subjected to oxidative stress on days 8 and 9 as expressed by high MDA and mares with sufficient antioxidant capacity can overcome this stress in addition to foaling, lactation and conception stressors (Abo El-Maaty *et al.,* 2012a). The increased MDA concentrations in repeat breeder mares indicated subclinical endometritis which affect fertility due to exposing mares to oxidative stress in addition to managemental stress during handling and securing mares at natural breeding. In agreement with previous results higher MDA were reported during follicular phase compared to luteal phase of equine estrous cycle (Abo El- Maaty and El-Shahat, 2012). During the estrous preceding successful or unsuccessful conception, concentrations of MDA did not significantly change in subfertile mares encountered several unsuccessful breedings but significant high SOD were only reported. This study proved a significant negative relation between SOD, zinc and copper but a non significant direct one with estradiol and cortisol (Abo El-Maaty *et al.,* 2012b). Moreover, placental and foetal growth restrictions are attributed to increased placental malondialdehyde (MDA) (Al-Gubory *et al.,* 2014).

SOD levels were lowest among infertile women with endometriosis. Low activity of antioxidant enzymes in the peritoneal fluid of infertile women with endometriosis probably do not influence fertility in these women, but these factors may play a role in the development of the disease (Szczepańska *et al.,* 2003).

In agreement with our results SOD levels were significantly higher in the liver, spleen, kidney and brain of pregnant-infected mice compared with pregnant mice and they remained unaltered in the placenta of pregnant-infected mice compared with pregnant mice (Sharma *et al.,* 2012). Another study showed significantly low levels of SOD, in women with idiopathic recurrent pregnancy loss (El-Far *et al.,* 2007). In women, SOD levels remain essentially constant with no significant cycle dependent changes (Massafra *et al.,* 2000).

Furthermore, CAT activity was significantly lower in all the organs of pregnant-infected mice compared with pregnant mice (Sharma *et al.,* 2012). Another study showed significantly low levels of CAT in patients with idiopathic RPL (El-Far *et al.,* 2007). In women, CAT levels remain essentially constant with no significant cycle dependent changes (Massafra *et al.,* 2000).

Placental oxidative stress is linked with poor prenatal development and pregnancy losses. The end products of lipid peroxidation, with concomitants alterations in placental antioxidants, namely copper-zinc containing superoxide dismutase (SOD1), manganese containing (SOD2), glutathione peroxidases (GPX), glutathione reductase (GR) and catalase (CAT) activities may be involved in placental and foetal growth restriction (Al-Gubory *et al.,* 2014).

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