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 embryoopathies 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 (NO2) in addition to non-reactive species such as peroxynitrite (ONOO−), and nitrosamines (Rosselli et al., 1998). RNS are mainly derived from
NO, which is formed from O₂ 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).

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.

<table>
<thead>
<tr>
<th>Reproductive condition</th>
<th>Foal heat</th>
<th>Repeat breeder</th>
<th>Cyclic normal</th>
<th>Endometritis</th>
<th>Granulosa cell tumor</th>
<th>Anovulatory follicle</th>
<th>After abortion</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>5</td>
<td>46</td>
<td>40</td>
<td>21</td>
<td>5</td>
<td>20</td>
<td>39</td>
<td>176</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Reproductive condition</th>
<th>Pregnant at foal heat</th>
<th>Early pregnant</th>
<th>Pregnant after anestrus treated</th>
<th>Early embryonic death</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>6</td>
<td>96</td>
<td>7</td>
<td>7</td>
<td>116</td>
</tr>
</tbody>
</table>

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 °C 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 H₂O₂. 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).

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**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<0.01, ** 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.

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 (Dimmel et al., 1997; Mannick et al., 1999, 2001).

In tissues of low O₂ saturation condition, NO reduces ischemic damage by increasing blood flow secondary to vasodilating effect. While, in tissues of high O₂ 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 (Barszczewski et al., 2005). Presence of different isoforms of NO synthase was reported in the equine endometrium of early pregnant mares (Welter et al., 2004) and NO is considered an important mediator implicated in maintenance and termination of pregnancy (Alghamdi et al., 2004). 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 (Yalampalli 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 estrus 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 resorption of atretic follicles was observed and NO sensitive pathways were activated (Roberto da Costa et al., 2005). Near luteal phase, NO levels increased in the endometrium, which 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 embryos. 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 mares.
Arabian mares affected with endometriosis (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 menstrual 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 (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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