

Hormonal and Cytogenetic Investigations in Mares with Early Embryonic Death

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Abstract: Early pregnancy loss in the mare is a major cause of infertility and economic loss. The aim of this work was to evaluate the equine embryonic death using hormonal and cytogenetic parameters. Twenty two European non pregnant brood mares aged from 3 to 7 years were used for this study. Animals were periodically examined with ultrasound at days 15, 21, 30, 45 and 60 after ovulation for investigating of follicular development, ovulation and gestation. All mares were diagnosed to have early embryonic death at 45 to 60 days in the second breeding season. Blood samples were collected from mares for detection of hormones, nitric oxide, micronuclei test and chromosome aberrations. Progesterone levels in mares were significantly ($p < 0.0001$) higher after pregnancy diagnosis at 21 days than at 45 to 60 days whereas early embryonic loss was recorded in all mares with ultrasound. Mean leptin levels were significantly ($p < 0.05$) very low during the early pregnancy then increased, but still lower than normal levels during the early pregnancy death. Insulin like growth factor -1 and Nitric oxide did not significantly change in animals during the pregnancy and early embryonic death. A significant ($p < 0.05$) increase in percentage of micronuclei in binucleated lymphocyte and the frequencies of chromosomal abnormalities were observed in mares with early embryonic death than early pregnant mares. In conclusion, cytogenetic analysis is essential in the evaluation of early embryonic death. Further studies are needed to determine dysfunction in hormone production in relation to early embryonic death and their relationship with infectious causing embryonic death.

Key words: Mare • Hormone • Early Embryonic Loss • Micronuclei • Chromosome Abnormalities

INTRODUCTION

Early embryonic loss in the mare is generally defined as pregnancy failure that occurs between fertilization and day 40 to 60 of gestation. Embryos die at various stages of their development, starting from fertilized eggs, through morules, blastocysts to older embryos undergoing organogenesis [1, 2]. The diagnosis of early embryonic loss and recognition of factors contributing to its occurrence have been dramatically improved by the routine use of transrectal ultrasonography for early pregnancy diagnosis. Ultrasonography studies in mare showed a rate of embryonic death ranging from 5 to 24% during 11 and 50 days [3] and a rate of 13.28 % during 19-21 days post-ovulation [4]. Factors that may contribute to the occurrence of embryonic loss in the mare have been classified as intrinsic, extrinsic and embryonic [5]. Intrinsic factors include endometrial disease, progesterone insufficiency, maternal age, lactation, foal-heat breeding,

time of insemination relative to ovulation, site of intrauterine fixation of the embryonic vesicle and maternal chromosomal abnormalities. Extrinsic factors include stress; nutrition; season/climate; transrectal palpation/ultrasonography; sire and/or semen processing/handling; and gamete handling/manipulation for assisted reproductive techniques. Embryonic factors include chromosomal anomalies or other inherent characteristics of the embryo; currently, it appears that embryonic factors are linked to intrinsic (e.g. maternal age) and/or extrinsic (e.g. oocyte handling/manipulation) factors.

Several factors are involved in pregnancy maintenance in brood mares. These factors include adequate progesterone levels [6], adequate supporting uterine environment and absence of non infectious and infectious causes [7]. The conceptus is also an active partner in the successful establishment and maintenance of pregnancy [8]. Progesterone is critical to embryonic survival, the cause-and-effect relationship between

progesterone and spontaneous embryonic loss remains unclear [9]. Reduced progesterone concentrations could be related to endometritis, failure of maternal pregnancy recognition, or luteal insufficiency [6].

Several hormones play important roles in fertilization, implantation and maintenance of pregnancy in several animals' species. The role of insulin-like growth factor I (IGF-I) as a paracrine-autocrine modulator of steroidogenesis in the conceptus was investigated by Hofig *et al.* [10]. It has been hypothesized that there is a positive 'feed forward system' by which embryonic oestrogen enhances uterine secretion of IGF-I. Both IGF-I and IGF-II displayed different spatial and temporal patterns of expression during early pregnancy and had a role in the process of attachment and implantation [11]. The synthesis and secretion of IGF-I by both the embryo and endometrium underscore the importance of this growth factor in early pregnancy. Uterine expression of IGF-I is widely documented [12]. Insulin like growth factor-I is also produced from the medium and large follicles during early pregnancy in the mare [13].

In the horse, leptin is a hormone produced by adipose tissue and its peripheral concentration reflects fat mass [14]. The possible role of leptin has been investigated in the mare on maintenance of ovarian activity during seasonal anestrus [15] and during foaling and lactation [16]. Following leptin discovery in adipose tissue, it has been also detected in the placenta, amniotic fluid and in fetal plasma as early as week 18 of gestation [17]. It has been demonstrated that leptin concentration in the cord serum is elevated and independent of maternal serum concentration [18]. Leptin produced by the placenta during pregnancy makes a substantial contribution to maternal circulating levels during pregnancy [19].

Nitric oxide (NO) is generated from L-arginine by the action of the enzyme NO synthase in vascular and neural tissues of many organs [20]. NO is important for implantation [21, 22], embryonic survival and development, as well as trophoblast outgrowth and cell migration [23]. After implantation, NO plays an important role in regulating uteroplacental-fetal blood flows and transfer of nutrients and oxygen from mother to fetus [20]. NO and polyamines are essential to angiogenesis and embryogenesis, as well as fetal-placental growth [24, 25].

Chromosomal analyses are an important and necessary part of the etiological research couples with recurrent fetal wastage [26] and reproductive failure [27]. Micronuclei represent whole chromosomes or chromosome fragments that have been lost from the cell nucleus during mitosis or meiosis [28, 29]. Heddle *et al.*

[30] suggested that micronuclei may form by one of four basic mechanisms: 1) mitotic or meiotic loss of an acentric fragment; 2) a variety of mechanical consequences of chromosomal breakage and exchange; 3) mitotic or meiotic loss of whole chromosomes; 4) as a result of apoptosis. Analysis of chromosomal number and morphology has been considered important to diagnose the cause of infertility in mares when other possible causes have been excluded [31, 32]. It has been estimated that 2% of stallions have chromosomal defects [33]; however, the relationship between chromosomal aberrations and stallion fertility is largely unstudied [34, 35]. In a referred population of subfertile stallions, Kenney *et al.* [35] found 18 of 36 stallions had abnormal karyotypes. Cytogenetic studies are also considered important in understanding the pathogenesis of some types of embryonic and fetal death [36]. There have been relatively few cytogenetic studies relating chromosomal abnormalities to early embryonic loss in horses [37]. Therefore, this study aimed to analyze blood of mare with early embryonic loss for hormonal picture, micronuclei and chromosomal abnormalities.

MATERIALS AND METHODS

Animals: Twenty two European non pregnant brood mares aged from 3 to 7 years were used for this study. Mares were imported to a horse farm during summer season of 2008 and breeding trials were started from the next summer of 2009 after adaptation to Egyptian environment for one season. These animals were always kept under regular veterinary observation and fed Egyptian clover barseem (*Trifolium alexandrinum*) or barseem hay beside concentrated ration that was formulated to meet energy requirement. Water was provided *ad libitum*.

Ultrasonographic Examination (US): Animals were periodically examined with ultrasound at days 15, 21, 30, 45 and 60 after ovulation for control of follicular development, ovulation and gestation. NOVEK (Germany) ultrasound scanner equipped with endorectal 2.5-7.5 multi-frequencies B-mode linear array transducer was used to evaluate ovaries and uterus.

Hormone Assay: Blood samples were obtained via venipuncture at 15 to 21 (early pregnancy) and 45 to 60 days (pregnancy loss) after ovulations. Serum was harvested, pooled and stored at -20°C until hormonal assay. Progesterone was assayed using ELISA commercial kit for quantitative determination of

progesterone in serum with inter and intra-run precision coefficients of variation of 2.9 and 4.8 %, respectively and a sensitivity of 0.05ng/ml [38]. Insulin like growth factor-1 (IGF-1), (BioSource Europe S. A. Belgium) [39] and MutliSpecies Leptin RIA Kit (Linco Research) [40] were estimated by radioimmunoassay (RIA). The limit of sensitivity, intra- and inter-assay coefficients of variation were 3.4 ng/ml, 1.9% and 4.1% and 1.0 ng/ml, 2.8% and 8%, respectively.

Nitric Oxide (NO): NO was assayed using ELISA [41]. For measuring serum nitrite according to Rajaraman *et al.* [42], 100 µl of serum samples were mixed with an equal volume of freshly prepared Greiss reagent, incubated for 10 min at room temperature and absorbency measured at 570 nm using a micro titer plate reader. The nitrite level in serum samples was calculated by comparing the optical density against the nitrite standard curve of sodium nitrite in distilled water.

Cytogenetic Analysis: Blood samples were collected in vials containing heparin as anticoagulant from mares at 15 to 21 days (early pregnancy) and at 45 to 60 days (early embryonic loss) for micronuclei test and chromosome aberrations.

The *In vitro* Micronuclei (MN) Test: The test was carried out according to Fenech and Morley [43]. Whole blood cultures from the mares were set up by adding 0.4 ml whole blood to 5 ml culture medium consisting of RPMI 1640 supplemented with 15 % fetal bovine serum, 2mM L-glutamine, antibiotics (100 units/ml penicillin and 100 µg streptomycin/ml) and 1.0 % phytohemagglutinin. Cytochalasin B was added to the cultures at 44 h post initiation at final concentration 5 µg/ml. Twenty four hours later the cells were centrifugated, resuspended in hypotonic saline (75 mM KCL), centrifuged again and fixed twice in fixative (acetic acid and methanol; 1:3) for 20 min. The cell suspension was dropped on wet slides and the air dried preparations were stained with 4 % Giemsa in Sorensen's buffer, pH 7.4. Scoring was done at 100 X magnification. 1000 binucleated cells/ animal were counted for the presence of micronuclei. The data were statistically analyzed using Fisher exact test. Replicative index (RI), a measure of cell division kinetics was calculated by scoring 500 cell/sample, by counting the percent of cells containing 1,2,3 or more nuclei / individual.

$$RI = [(1x \% \text{ mononuclear cells}) + (2x \% \text{ bi}) + (3x \% \text{ tri}) + (4x \% \text{ tetra})]/n.$$

Chromosomal Aberrations: Lymphocyte cultures were prepared according to Halnan [44]. Blood cells were cultured for 72 h at 38°C in 5 ml TCM-199, 1ml fetal calf serum and 0.1 ml phytohaemagglutinin (PHA). After incubation, cells were treated with colchicines (0.05%) for 2 h, then with a hypotonic (0.075M KCL) for 30 min. After fixation in acetic acid: ethanol (1: 3) solution, the cells suspension was dropped on wet slides then flamed to dry. The slides were stained with Giemsa stain and covered with DPX mounting media for chromosomal analysis. Chromosomal abnormalities were recorded in at least 50 metaphase spreads for each animal.

Statistical Analysis: Data was presented as means ± Standard error of mean (SEM) and subjected to statistical analysis according to Snedecor and Cochran [45].

RESULTS

Ultrasonographic Examination: Large nonechogenic (black) follicles were recorded during behavioural estrus. The echogenic (homogenous grey) corpus luteum was recorded after ovulation and persists during luteal stage of the estrous cycle. Estrus was detected by visual observation of animal behaviour and US. Large ovulating follicles of >3.5 cm in diameter were recorded during estrous behavior. The presence of embryonic vesicle of 1.5 to 2 cm in diameter after successful breeding associated with corpus luteum indicates pregnancy. Absence of the embryonic vesicle, abnormal shape and absence of corpus luteum associated with estrous behavior indicated early embryonic death. All mares were diagnosed as early embryonic death at 45 to 60 days in the second breeding season after their breeding with Egyptian native breed stallions.

Hormone Assay: Plasma hormonal concentrations are shown in Table 1. Progesterone levels in mares were significantly ($p < 0.001$) higher at early pregnancy than at early embryonic loss. Mean levels of Insulin like growth factor -1 did not significantly change in animals during the two periods of pregnancy and early loss. Mean leptin levels were significantly ($p < 0.05$) very low during early pregnancy then increased but still lower than normal levels during the pregnancy loss.

Table 1: Hormonal and nitric oxide values in mare with early embryonic death comparing with the same mare at pregnancy (Means \pm SE)

Animal	Progesterone** (ng/ml)	IGF1 (ng/ml)	Leptin* (ng/ml)	Nitric oxide (NO, μ mol)
Pregnant	7.33 \pm 1.13 ^b	331.8 \pm 30.1	0.61 \pm 0.13 ^a	41.65 \pm 6.98
Early embryonic death	2.18 \pm 0.37 ^a	439.34 \pm 35.8	1.13 \pm 0.22 ^b	39.19 \pm 5.73

Insulin like growth factor -1 (IGF-1), *P=0.05, **P<0.0001

Table 2: Micronucleus level in binucleated lymphocytes in mare with early embryonic death comparing with the same mare at pregnancy

Animal	Number of animals	No of binucleated cells	No of MN in binucleated cells	% of MN in binucleated cells \pm S.E	Replicative index
Pregnant	22	22000	87	0.39 \pm 0.44	2.0
Early embryonic death	22	22000	281	1.27 \pm 0.40*	1.8

No of binucleated 1000/animal. * Significant at P< 0.05 compared to cyclic level

Table 3: Mean percentage of chromosome aberrations in mare with early embryonic death comparing with the same mare at pregnancy

Animal	Number of animals	Number of metaphases	Number of abnormal metaphases	Chromosome aberrations (Mean % \pm S.E) without gaps	Types of abnormal metaphases			
					Gaps	Fragment	Break	Deletion
Pregnant	22	1100	11	1.0 \pm 0.35	5	5	4	2
Early embryonic death	22	1100	34	3.09 \pm 0.41*	8	14	16	4

No of metaphases 50/animal. * Significant at P< 0.05 compared to cyclic level.

Nitric Oxide: Nitric oxide did not significantly change in animals during the two periods.

Cytogenetic Analysis

Micronucleus Test: Table 2 reflect a significant ($p<0.05$) increase in the percentage of micronuclei in binucleated lymphocyte in mares with early embryonic death than during pregnancy. No differences between the replicative indices in mares with early embryonic death as comparing to the normal pregnancy was observed in these study.

Chromosomal Aberrations: Chromosomal aberrations in mare lymphocytes are presented in Table 3. The percentage of abnormalities significantly ($p<0.05$) increased in mares with early embryonic death than normal pregnant mares. The percentage reached 3.09 ± 0.41 in mares during early embryonic death as compared with 1.0 ± 0.35 during pregnancy.

DISCUSSION

The decrease in the levels of progesterone in mares suffered from early embryonic death in the present study agrees with those of Volkmann *et al.* [46]. In many species, progesterone has an important role in maintaining uterine quiescence during pregnancy. It reduces uterine contractility by hyperpolarizing the myometrium and by reducing the number of gap junctions and receptors for contractile agents in the myometrium [47, 48]. During the early pregnancy the only source of progesterone for

pregnancy maintenance is the corpus luteum and any decrease in its levels indicates insufficiency in its production from the corpus luteum due to luteolysis. Several factors could lead to luteolysis and a decrease in peripheral levels of progesterone during early pregnancy which ends pregnancy in mares. Slight inflammatory reagents in the uterus initiate the production of prostaglandins which induces luteolysis and in turn ends pregnancy [49]. The embryo must reach an adequate size by day 16 of pregnancy in order to produce sufficient interferon-tau to prevent luteolysis and achieve successful maternal recognition of pregnancy [50, 51]. Interferon-tau production by the embryo depends greatly on an appropriate pattern of maternal progesterone secretion, especially during the first week after ovulation, when the early embryo is developing [52]. Moreover, restricted conceptus mobility also results in luteolysis in the mare and subsequent decline in progesterone leading to embryonic death. This supports the notion that unrestricted mobility of the equine conceptus, allowing it to interact with most of the uterine endometrium is necessary for luteal maintenance and conceptus survival [53].

The non significant decrease in the levels of insulin like growth factor-I (IGF-I) in this work indicated that its role during early pregnancy is not as important as progesterone. IGF-I has an autocrine and/or paracrine role in attachment and implantation [54, 55]. IGF-I stimulates progesterone production [56], but circulating IGF-I concentration does not seem to be directly related to CL function [57].

Leptin levels in the present study significantly increased during the two periods of pregnancy and pregnancy loss and this may be attributed to the increase in the body fat and weight of the animals after adaptation to different environments and the regulaty of the cyclicity of the animals since they were long day breeders in their home environment and became adapted to the breeding season in Egypt which starts from October and ends by May. Leptin has been detected in the placenta, amniotic fluid and in fetal plasma [58].

Fábregues *et al.* [59] agreed with our results that circulating levels of nitrite/nitrate are similar in successful and unsuccessful implantation and unrelated to the outcome of pregnancy. This precludes the use of serum NO measurement as a marker of implantation and successful pregnancy. In spite of that leptin [60], nitric oxide [25] and insulin like growth factor -I [11] are expressed in the placenta and the embryo but till now their role has not yet been cleared.

With respect to cytogenetic analysis, our study showed a significant increase in percentage of micronuclei in binucleated lymphocyte in mares during early embryonic death. Similarly, micronucleated cell rates were found to be increased in lymphocytes of patients with infertility and two or more spontaneous abortions [61]. An increased level of micronuclei has been shown to be a marker of chromosome damage. The detection of micronuclei has been used as a rapid and sensitive screening system for evaluating genotoxic risk in populations [62].

Our data demonstrated that, there is a significant increased in structural and numerical chromosomal aberrations in mares during early embryonic loss than pregnancy. Lear *et al.* [63] reported three mares with repeated pregnancy loss before day 65 of gestation each had a chromosomal translocation. Wood *et al.* [64] found that more abnormal embryos were recovered from mares with early embryonic death. In this respect, Mahmoud *et al.* [65] and Mahmoud [66] recorded chromosomal abnormalities in oocytes and *in vitro* produced embryos in buffaloes that lead to early embryonic death. Increasing maternal age is clearly associated with decreased oocyte quality, which may reflect chromosomal and/or other inherent changes within the oocyte that apparently do not affect fertilization rates, but dramatically increase the incidence of early embryonic loss in aged mares.

In this study, only single structural aberrations were observed which explained the less adverse effect on the cell survival. In the same time, the rate of chromosomal aberrations was relatively low which is insufficient alone

to cause embryonic loss but may interact with other factors to increase the probability of pregnancy loss. Ahmed *et al.* [67] cited that environmental factors may play a role in the incidence of chromosomal abnormalities since the studied animals were imported and was under the influence of environmental changes. Maternal infections factor during pregnancy may or may not impact fetal development. Givens and Marley [68] in their review and Abdel-Hafeiz *et al.* [69] reported that equine herpesvirus 1 as a cause of late fetal death. Moreover, several studies showed that viruses [70] and after vaccination against viruses [71] lead to chromosomal damage.

CONCLUSION

Cytogenetic analysis is essential tool in the evaluation of early embryonic death. Further studies are needed to determine dysfunction in hormone production in relation to early embryonic death and their relationship with infectious causing embryonic loss.

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