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Original article

Endometrial histopathology, bacteriology and cytology outcomes in mares with early embryonic death (EED): a field study

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Abstract

Early embryonic death (EED) is one of the causes of infertility in the mare. We compared endometrial environment in 9 mares with EED and 13 mares in diestrus phase. Cotton swab (CS), cytobrush (CB) and uterine biopsy (B) samples were obtained for the cytological, bacteriological and histopathological examinations. In the first step we compared CS and CB methods to biopsy as a reference method, as B revealed the highest number of positive results in cytological and bacteriological examinations in both groups. In turn, we also compared cytological, bacteriological and histopathological findings between EED and control animals using the B sampling. Although the differences between these groups were not statistically significant ($p \geq 0.05$), there was a tendency to a higher prevalence of subclinical endometritis in the control group, than in the EED group (62% vs 22%). In general, positive bacteriological results were similar in both groups (62% vs 55%), whereas positive cytological results were higher in the control group (62% vs 22%; $p \geq 0.05$). In histopathological examination in EED mares endometrial degeneration was better expressed (all mares were with grades IIB and III on the Kenney-Doig scale); however, the differences between both groups were not statistically significant ($p \geq 0.05$). We could not confirm a clear difference in uterine environment between the two groups. Moreover, the uterine biopsy seemed to be the most reasonable sampling method for diagnosis of endometrial state.

Key words: equine, uterine biopsy, early embryonic loss, pregnancy, sampling methods

Introduction

Early embryonic death (EED) in mares is commonly defined as the death of the embryo between fertilization and day 42 of pregnancy (Newcomb and Cuervo-Arango 2011). The prevalence of EED ranged between 2.6-24% with a mean of 8.6% in different studies (Vanderwall 2008). From a practical point of view this phenomenon can occur at two distinct times: before the first pregnancy check, meaning before 10-12 days of pregnancy, and during the subsequent days of pregnancy (>12 days of pregnancy), that can be diagnosed with ultrasound examination. Early embryonic death in mares is multifactorial. The factors contributing to this occurrence are very heterogeneous and can be as simple as the age of the mare, malnutrition, toxicosis of the maternal

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organism or improper timing of mating or artificial insemination (Van Niekerk 1965, Ball 1988, Darenius 1992, Brendemuehl et al. 1994, Blodgett 2001). This disorder also occurs due to other numerous noninfectious causes, such as: corpus luteum insufficiency (Ball 1988, Sharp 2000), failure of maternal recognition of pregnancy as a consequence of insufficient embryonic vesicle movement (Ginther 1983, Leith and Ginther 1984) or insufficient embryonal production of hormones (Weber et al. 1991, 1991a, Sissener et al. 1995, Herrler et al. 2000). Moreover, abnormalities in the histological structure of the endometrium, e.g. endometrial cysts, fibrosis (Kenney 1978) or embryo development defects in the early stage of pregnancy are considered as important causes of this disorder (Newcomb and Cuervo-Arango 2011). Twin pregnancy (concerning a smaller embryo vesicle) and manual reduction of one embryo during a twin pregnancy, as an extrinsic factor, increases the general incidence of early pregnancy failure (Nout 1996, Wilsher et al. 2012). In the past, insufficient production of progesterone (P4) by the corpus luteum was considered as the most common reason for EED but the latest studies show that the equine embryo can survive long periods of sub-optimal P4 levels (Newcomb 2000); however, mares with correct levels of P4 undergo EED as well (Papa et al. 1998).

Endometritis can have a significant impact on early embryonic loss (Ball 1988, Darenius 1992, Papa et al. 1998, LeBlanc 2009). It is caused by bacteria or fungus, which are infectious factors involved in the EED pathogenesis (Hughes and Loy 1975, Woods et al. 1987, Ricketts 1999, Dascanio et al. 2001). An experiment conducted by Woods et al. (1985) indicated that embryos recovered from subfertile mares with endometritis were smaller in diameter and a lower number of flushes contained normal embryos in comparison to maiden mares. As a possible pathomechanism, despite an altered uterine environment, it was suggested that endometritis could be an underlying cause of secondary luteal insufficiency (Ginther et al. 1985a). A study of the immunological background of equine EED, which focused on lymphocytes Th1 (subpopulations $CD2^+$, $CD4^+$, $CD8^+$) and NK cell concentration in peripheral blood, shows the vital role of the immunological system in the process (Krakowski et al. 2010). The percentage of the cells mentioned above (as well as their ratio) was significantly higher in the EED group, which indicates the development of an inflammatory reaction in the mares, seen as stimulated lymphocyte Th activity. Further studies indicated acute phase protein serum levels (serum amyloid A, haptoglobin) as reliable markers of ongoing general inflammation, including endometritis (Krakowski et al. 2011).

To diagnose this endometrial dysfunction, the most common sampling methods used are cotton swab, cytobrush or low-volume uterine lavage (Roszel and Freeman 1988, LeBlanc et al. 2007, Nielsen et al. 2010, Overbeck et al 2011, Cocchia et al. 2012, Kozdrowski et al. 2013, Ferris et al. 2015). The efficiency of the different techniques has been widely studied and reported (Wingfield 1978, Aguilar et al. 2006, Kozdrowski et al. 2015). Moreover, the biopsy sampling technique enabled the histopathological classification of endometrial tissue (Ricketts 1975). Unfortunately, these studies were performed in non-mated animals, suffering from endometritis or checked for mating or artificial insemination, whereas such studies in EED mares have been neglected. To the best of our knowledge only one study conducted in the late 1990s focused on cytology, bacteriology and histology of the endometrium in mares that underwent EED (Papa et al. 1998). Another report focused on individual cases of 15 mares with a history of EED; however, no control group was used in that specific experiment (Darenius 1992). The authors of the abovementioned studies indicated endometritis in both acute and chronic form as an important cause of early fetal loss in the mare. Other than that, experiments on equine EED were designed to calculate pregnancy rates in reference to their previous reproductive history (Woods et al. 1987) or in animals inoculated with microorganisms (Hughes and Loy 1975).

To address this research gap, we performed cytology, bacteriology and histology examination of endometrium samples collected from mares with diagnosed EED and physiological P4 concentrations in peripheral blood and compared these results with outcomes obtained in clinically healthy mares in diestrus. Endometrial biopsy was used to detect degenerative changes and to determine their severity. We compared the results of histopathological examination of endometrial tissue collected at the same time as cytological sampling to better understand uterine disturbances in EED mares. The design of our study also allowed the comparison of the bacteriological and cytological results obtained by cotton swab, cytobrush and uterine biopsy sampling in both groups.

Materials and Methods

Animals, experimental groups and study design

Twenty-two multiparous warmblood mares (aged 4-12 years) from six private polish studs were used in this study, which was undertaken during the breeding season of 2016 and 2017 (from April to August). Experimental animals were assigned to one of the two following groups: (1) experimental group (n=9), including mares that suffered from early embryonic death

(up to 40th day of pregnancy); and (2) control group (n=13), consisting of animals in diestrus.

The assignment of mares to the appropriate group was based on full examination of the reproductive tract and collecting breeding history of each mare. Moreover, blood P4 levels were measured to control luteal activity. In our study, only cases of EED with P4 levels similar to the hormone concentration during the luteal phase of the ovarian cycle (5-16 ng/ml) were included. Mares with EED were selected from animals that were examined every 5th day by transrectal palpation and monitored ultrasonographically starting from the 10th day after mating or artificial insemination. Diagnosis of EED included at least one of the following symptoms observed in mares diagnosed earlier as pregnant: growth halt of an embryo vesicle or loss of its spherical shape, lack of an embryonal vesicle or embryo proper, hyperechogenic appearance of the embryo vesicle fluid, high (2 or 3) score of uterine oedema as equivalent to high estrogenisation of the endometrium, fluid in the uterus lumen (Ginther et al. 1985, McCue et al. 2011, Newcomb and Cuervo-Arango 2011).

The diestrus phase was determined by transrectal palpation and ultrasound examination of the uterus and ovaries, progesterone level measurement (5-16ng/ml) as well as behaviour observations. In all animals of both groups, 3 endometrial samples were simultaneously obtained using a cotton swab (CS), cytobrush (CB) and uterine biopsy (B). In the EED group, uterine samples were taken as soon as the embryonic loss was confirmed. The diestrus group of animals was sampled between the 6th and 11th day of the cycle (day 0=ovulation day). Diagnosis of endometritis in animals was based on either positive cytology or both positive cytology and bacteriology results. CS, CB and B samples were used for bacteriology and cytology, and B for histopathology as well.

Uterine sampling techniques

In all animals of both groups, uterine samples were obtained using a cotton swab catheter (Minitube, cat. No. 17214/2950), followed by a cytological brush (Minitube, cat. No. 17214/2960) and uterus biopsy forceps (Kruuse, cat. No.141965). Preparations of each mare as well as uterine sampling were done according to generally accepted procedures (Love 2011). All samples were transported to the laboratory in provided containers and stored at room temperature until further analysis. All collected uterine samples were first used for bacteriological examinations to ensure the most reliable results possible, followed by a cytological. Tissue samples collected by uterine biopsy additionally underwent histopathological examination.

Laboratory examinations

Bacteriological examination

Culture mediums used in bacteriology analysis were: Columbian agar with 5% defibrinated sheep blood and Edwards agar with 5% defibrinated sheep blood (Oxoid, UK), and for the antibiogram Mueller-Hinton Agar and Mueller-Hinton Agar with 5% horse blood addition were used. Plates were first incubated for 48 h at 37°C. Colonies were then classified based on their appearance, hemolyse type and ability to produce catalase, oxidase and coagulase. Gram staining was also performed. Final classification was obtained using latex tests (Staphytect Plus, PathoDxtra Strep Grouping Kit; Oxoid, UK) and biochemical API tests (API 20E, 20 NE, API Staph, API Strep, bioMerieux, France). Samples were considered as positive if growth of monoculture was observed, and negative in the case of mixed growth of more than 1 colony or no bacterial growth (Nielsen 2005).

Cytological examination

Cotton swabs, brushes and biopsy samples were smeared on a basic microscope glass slide and impregnated with Cytofix (Samco, Poland) and left to dry at room temperature. Two methods of staining were performed: Shorr's in Kubicek's modification and Diff-Quik (HEMAVET, Kolchem), in parallel duplicate glasses. Slides were evaluated under a light microscope with 400x magnification. In every sample, 200 cells were counted in total. The presence of polymorphonuclear leukocytes (PMNs) was evaluated and calculated as a percentage of epithelial cells seen in the smear. Samples considered as endometritis positive contained PMNs over 0.5% of all cells counted (Nielsen 2005).

Histopathological examination

Biopsy samples were fixed in 10% formaldehyde for 2 days before staining. Routine staining with hematoxylin-eosin was performed. Evaluation of samples began with light microscopy under 400x magnification. Presence and infiltration of PMNs in the epithelium and stratum compactum was assessed to confirm or exclude any inflammation. Moreover, the category, according to the generally used scale of histopathological evaluation of the equine endometrium by Kenney-Doig, was determined for each sample (Snider et al. 2011). Grade IIA was considered as a cut-off for differentiation of pathological and physiological endometrium.

Table 1. Results of cytological and bacteriological examinations of all animals used in the study, in regard to the method used. CS-cotton swab, CB- cytobrush, B-biopsy. Positive result in cytological examination refers to samples containing PMNs>0.5%, negative: PMNs<0.5%. Positive result in bacteriological examination refers to samples with substantial growth of monocolony, while negative result includes samples with mixed growth of bacterial colonies or no growth.

Method of sampling	Cytology (n=23)		Bacteriology (n=23)	
	Negative (-)	Positive (+)	Negative (-)	Positive (+)
CS	21 (95%)	1 (5%)	14 (64%)	8 (36%)
CB	19 (86%)	3 (14%)	12 (55%)	10 (45%)
B	13 (59%)	9 (41%)	7 (32%)	15(68%)

Table 2. Results of cytological, bacteriological and histopathololgical examination of biopsy samples of endometrium from mares with EED (EED group) and mares in diestrus (control group) and results of Fisher exact test Grades in histopathology section correspond to Kenney-Doig scale. Positive cytology was based on the number of PMNs found in the smear (PMNs>0.5%). Positive bacteriology was based on the substantial bacterial growth of maximum monoculture. Statistical significance was set to $p \leq 0.05$.

Method	Result	EED group	Control group	Fisher exact test p value
Cytology	Negative	7 (78%)	5 (38%)	p=0.25
	Positive	2 (22%)	8 (62%)	
Bacteriology	Negative	4 (45%)	5 (38%)	p=0.23
	Positive	5 (55%)	8 (62%)	
Histopathology	Grade ≤ IIB	6 (67%)	12 (92%)	p=0.18
	Grade III	3 (33%)	1 (8%)	

Data analysis

In both groups of mares, the percentage of animals with positive bacteriological and cytological results was calculated. Regarding histopathology, the percentage of animals with Kenney-Doig scale grade was also calculated. The Fisher Exact test was used to determine differences between the experimental and control group in bacteriological, cytological and histopathological results. Because of the small number of animals graded with I or IIA on the Kenney-Doig scale within experimental groups, two statistical groups were defined: 1 (consisting of mares graded I, IIA and IIB), and 2 (consisting of mares graded III), to asses and compare the distribution of histopathology grades. Statistical significance was set at $p \leq 0.05$. Moreover, to compare cytology and bacteriology results and to determine the agreement between the methods used for acquiring samples (CS, CB, B), Cohen's kappa coefficient (κ) was calculated (software: *IBM SPSS Statistics 24*). The agreement was assessed regarding biopsy as a reference method, as this technique revealed the highest number of positive results in both cytology and bacteriology (Table 1). Therefore, all the above outcomes and comparisons between the two experimental groups of animals reflect the results of this particular technique.

Results

The highest number of positive outcomes for bacteriological and cytological examination in both groups were obtained using the biopsy sampling technique (Table 1). Positive cytology results were observed in both groups: in 2/9 (22%) of the EED mares and in 8/13 (62%) of the diestrus animals. A substantial growth of bacterial monocolonies was also found in both groups: in 5/9 mares (55%) of the EED group and 8/13 mares (62%) of the control group (Table 2). According to generally approved criteria for endometritis, 2 (22%) EED and 8 (62%) diestrus animals were diagnosed as having endometritis. We found no statistically significant differences between analyzed groups in cytological examination (Table 2).

Bacterial growth was found in 8/13 (62%) control mares and in 5/9 (55%) EED mares. Bacteria species found in the EED group included β-hemolytic *Streptococcus* group C (*Streptococcus equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus*) and *Escherichia coli*. Bacteria found in the control group included: β-hemolytic *Streptococcus* group B (*Streptococcus agalctiae*), group C, group D (*Streptococcus equinus*), *Staphylococcus sp.* and *Bacillus*. *Escherichia coli* growth was considered a contamination and was counted as negative. Only in some mares of both groups was more than one monoculture growth observed (in the EED group n=1, in diestrus group n=3),

Table 3. Results of cytological and bacteriological examinations (samples obtained by biopsy), according to Kenney-Doig scale (number of mares and %) in all studied animals (n=22). In cytology "-" is negative and "+" is positive (PMNs>0.5%); in bacteriology "-" is no growth and "+" is substantial bacterial growth of monoculture.

Kenney Doig scale	Cytology (–) Bacteriology (–)	Cytology (–) Bacteriology (+)	Cytology (+) Bacteriology (–)	Cytology (+) Bacteriology (+)
I	0 (0)	0 (0)	0 (0)	0 (0)
IIA	2 (9.00)	1 (4.54)	0 (0)	0 (0)
IIB	4 (18.18)	2 (9.00)	0 (0)	9 (40.90)
III	0 (0)	3 (13.63)	0 (0)	1 (4.54)

Table 4. Agreement between biopsy (B) and the cotton swab (CS), and between cytobrush (CB) and biopsy (B), according to Cohen's kappa coefficient. In "Results in methods being compared" section, the upper character in the box refers to biopsy, the bottom character concerns the result of compared technique (in agreement with the corresponding row).

Method of examination	Comparison between a pair of methods	Results in methods being compared				Cohen's kappa coefficient (κ)
		- and -	- and +	+ and	+ and +	
Cytology	B vs CS	14	0	7	1	0.154
	B vs CB	13	1	5	3	0.340
Bacteriology	B vs CS	7	0	7	8	0.421
	B vs CB	6	1	6	9	0.384

no bacterial growth was also observed in both groups (in the EED group n=3, in diestrus group n=2). We found no statistically significant differences between analyzed groups (Table 2).

Histopathologically, we found endometrium within the physiological range (Kenney-Doig scale I) in none of the animals. In the EED group 6 mares graded IIB and 3 graded III. In the control group the distribution of grades was different and featured grades IIA, IIB and III (n=1, n=11, n=1, respectively). We found no statistically significant differences between analyzed groups (Table 2).

Collation between the results of bacteriology and cytology with the histopathological outcome of every sample obtained by biopsy is presented in Table 3. It shows a tendency to increased numbers of mares affected with bacteria and/or enhanced PMNs number with a higher grade of Kenney- Doig scale.

Comparison of different sampling methods showed that Cohen's kappa coefficient, in the cytological examination, indicated poor (κ=0.154) and fair (κ=0.340) agreement between cotton swab-biopsy, and cytobrush-biopsy comparison, respectively. Similarly, in the bacteriological examination: there was only moderate (κ=0.421) and fair (κ=0.384) agreement between the following pair of sampling methods: cotton swab-biopsy, and cytobrush-biopsy comparison, respectively (Table 4).

Discussion

Early embryonic loss in the mare is a complex and multifactorial disease (Vanderwall 2008). In our study, progesterone concentration in EED mares was similar to its level in control mares and was within the range typical for early pregnancy. That proves that insufficiency of corpus luteum was not the factor contributing to EED in our experimental mares.

In our study we focused on a comprehensive diagnostics of uterine disturbances in EED cases using a cotton swab, cytobrush and biopsy simultaneously, to investigate the endometrial environment as a potential cause of EED. Endometritis has been cited as an important factor in embryonic and early fetal loss in mares; however, only very limited, controlled studies have been performed on the relationship of endometritis and embryonic loss (Ball 1988). In the studies of Papa et al (1998) and Darenius (1992), most of the affected mares showed compromised endometrium and had endometritis of different severity, sometimes combined with periglandular fibrosis. According to our criteria, we confirmed subclinical endometritis in 2 out of 9 (22%) EED mares, whereas this disorder occurred in 8 out of 13 (62%) control mares. This shows that subclinical endometritis is a very common disease in mares; however, is not always the sole cause of EED and may not be as important factor contributing to EED etiopathogenesis as previously suspected. However, our control group, composed of non-inseminated mares in the luteal phase, only partially fulfills requirements for control animals. The cytological and bacteriological results in EED mares were not fully indicative for endometritis, and surprisingly these findings in the control mares were even inferior to the results in the EED group (54% vs 22% positive cytology, respectively). Similarly,

substantial bacterial growth was found in 62% diestrus mares, while only 55% of EED mares were affected. However, in most cases of EED, the only bacteria species found was *Streptococcus* group C, while bacteriological findings obtained in control mares were more diverse and consisted of 3 bacteria species (*Streptococcus* group B, C, D, *Staphylococcus,* and *Bacillus*). Nevertheless, results acquired from uterine culture can lead to false positive or negative results and have been regarded as a poor predictor of fertility (Digby 1978). However, when bacterial growth is present it is always connected with lower pregnancy rates (Riddle et al. 2007). Since, in our EED cases, the only bacterial growth was type C *Streptococcus*, these bacteria can be considered as a potential cause of pregnancy failure. Nevertheless, we found no statistically significant differences between the two experimental groups in either cytological or bacteriological examinations performed. In view of this, in our opinion, each case of EED should be analyzed carefully and considered individually, because some differences seem to be impossible to unravel by group comparison.

Endometrial biopsy is a quick and reasonable cytological and bacteriological diagnostic tool and provides the most reliable results. It also provides material for histopathological analysis (Nielsen 2005). The results of the histopathological examination in the EED group showed 100% of mares graded IIB and III, and therefore degenerative processes of endometrium in the EED group might be involved in this pathology. However, there were no statistically significant differences between both groups. The histopathological status of the endometrium can strongly influence embryonal mortality in the mare because the crucial processes of maternal recognition of pregnancy take place in this uterine structure (Allen 2001).

Noteworthy are the valuable results obtained using biopsy tissue samples for cytological and bacteriological examination, as we were able to confirm the presence of bacteria or PMNs in numerous mares with negative results based on other sampling methods. There are many studies identifying clinical and subclinical endometritis using different diagnostic methods (Blanchard et al. 1981, Watson et al. 1987, Snider et al. 2011, Cocchia et al 2012, Kozdrowski et al. 2013, Katila 2016). The most reasonable approach to diagnose inflammation in the equine endometrium, even in its subclinical form, was proposed to be the cytobrush method (Cocchia et al. 2012). It has been suggested that cytobrush should be used as a routine method allowing the improvement of the diagnostics, because cytobrush was in many aspects superior to the cotton swab outcome (Cocchia et al. 2012). This opinion is also confirmed by our data (45% positive results in bacteriology using CB vs 36% using CS and 14% positive cytology results vs 5% using CS). However, standardized interpretation of cytological findings in the mare is not available and should still be refined (Cocchia et al. 2012, Walter et al 2012). We compared the accuracy of three different techniques of sampling for bacteriologic and cytologic examination as an additional aim of our study and as mentioned above, the uterine biopsy seemed to be the most reasonable sampling method. This finding is in line with earlier suggestions of Nielsen (2005). It should also be stressed that there was a relationship between a high grade of histopathological degeneration and increased bacterial load and number of PMNs.

Equine endometritis as a potential cause of EED is very difficult to study because of the wide array of variables associated with each affected mare (Darenius 1992, Papa et al. 1998). Although, for the diagnosis of the uterine status, we used all currently available methods, we were not able to expose a clear EED cause in all mares. Due to this, there is a need to perform more detailed analyses with the use of molecular biology methods, to confirm the expression of other factors possibly involved with EED. These kinds of studies have not been available in EED mares until now. In dairy cattle affected by repeat breeding disorder similar to EED in mares, enhanced expression of some inflammatory mediators such as TNF α, iNOS, PGFS and others have been confirmed in subclinical endometritis (Janowski et al. 2013, Kasimanickam et al. 2014). Research on another genus showed that low concentrations of nitric oxide are essential for the development of an early buffalo embryo, whilst its high concentrations can be toxic to the blastocyst (Saugandhika et al. 2010). Moreover, a genetic analysis indicated that chromosomal abnormalities have a significant influence on embryonal survival during pregnancy, suggesting karyotypisation of mares that underwent EED repeatedly (Lear et al. 2008). Further studies are needed to clarify the importance of these molecular processes in EED mares with negative clinical, bacteriological and cytological examination. It seems that in many clinical cases factors and mechanism causing EED are beyond a conventional diagnostic spectrum. Surprisingly, there is very limited data available on this topic.

In conclusion, diagnostic failure in some EED cases regarding the uterine environment is related to complications in the categorization of the endometrium as healthy or affected by bacterial, cytological or degenerative processes using standard methods of sampling. The uterine biopsy seems to be the most reasonable method of sampling for cytological and bacteriological diagnostics when combined with a deeper evaluation of the histopathological degeneration of the endometrium.

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