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Title: The relationship between the positive identification of the embryo proper in equine pregnancies aged 18 to 28 days and its future viability: a field study

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Abstract: Early embryonic loss (EEL) negatively affects the reproductive efficiency of equine reproduction. A sign of future EEL is when the embryo proper (EP) fails to develop within the embryonic vesicle after 30 days of gestation. The earlier the identification of impending EEL the earlier the mare can be re-bred to allow a second chance of pregnancy. The objectives of this study were to determine the percentage of embryonic vesicles with a visible EP at 18 to 28 days of gestation and to study the association between the presence/absence of the EP at different days of gestation and the future viability of the pregnancy. A total of 1256 pregnancies were identified and followed by transrectal B-Mode ultrasonography 12 to 45 days post-ovulation in mares of the same Thoroughbred farm. The identification of the EP was attempted once during Days 18 to 28. Pregnancy re-confirmation was performed on Days 35 to 45. The percentage of vesicles with an EP increased gradually from Day 18 (2.8%) to Day 21 (86.9%) P < .05. From Day 20 onwards, the EEL rate of mares with vesicles without an EP was significantly higher (P < .05) than that of vesicles with a positive identification of an EP. In conclusion, the EP of the equine vesicle can be identified reliably with B-mode ultrasonography in the majority of mares (> 71%) on Day 20 of gestation. The lack of a positive identification of an EP from Day 20 onwards is associated with a higher EEL rate.

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The relationship between the positive identification of the embryo proper in equine pregnancies aged 18 to 28 days and its future viability: a field study

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ABSTRACT

Early embryonic loss (EEL) negatively affects the reproductive efficiency of equine reproduction. A sign of future EEL is when the embryo proper (EP) fails to develop within the embryonic vesicle after 30 days of gestation. The earlier the identification of impending EEL the earlier the mare can be re-bred to allow a second chance of pregnancy. The objectives of this study were to determine the percentage of embryonic vesicles with a visible EP at 18 to 28 days of gestation and to study the association between the presence/absence of the EP at different days of gestation and the future viability of the pregnancy. A total of 1256 pregnancies were identified and followed by transrectal B-Mode ultrasonography 12 to 45 days post-ovulation in mares of the same Thoroughbred farm. The identification of the EP was attempted once
The percentage of vesicles with an EP increased gradually from Day 18 (2.8%) to Day 21 (86.9%) \( P < .05 \). From Day 20 onwards, the EEL rate of mares with vesicles without an EP was significantly higher \( (P < .05) \) than that of vesicles with a positive identification of an EP. In conclusion, the EP of the equine vesicle can be identified reliably with B-mode ultrasonography in the majority of mares \( (> 71\%) \) on Day 20 of gestation. The lack of a positive identification of an EP from Day 20 onwards is associated with a higher EEL rate.

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1. **Introduction**

Early embryonic loss (EEL) negatively affects the reproductive efficiency of equine reproduction. It can be defined as pregnancy failure that occurs between fertilization and day 42 of gestation [1]. Although an important number of losses occur between fertilization and day 12 of gestation [1], under field conditions embryonic failure in the mare can only be detected with ultrasound after day 11 to 12 of gestation by repeated transrectal examinations. Under these circumstances, the detected incidence of EEL between days 12 and 40 is on the order of 10 to 15% in young mares and 20 to 30% in aged mares [2]. A review on EEL from several controlled and field studies provided a weighted mean of embryo losses between day 10 and 60 of gestation of 8.6% (1519 embryo failures of 17581 pregnancies) with a range from 2.6 to 24% [1].

Early pregnancy diagnosis is essential for mare management, especially to reduce the need for continued estrus detection. Repeated diagnoses are needed to identify early embryonic failure. The earlier a failure can be discovered, the better the chance
of establishing a new pregnancy, and the least time lost. Prediction that an apparently viable pregnancy could fail would further aid the detection of any failure and reduce the time lost in establishing a new pregnancy.

Examination of mares either daily or every two days for ovulation diagnosis allows embryo age to be calculated to ± 12 h or ± 24 h respectively. Examination of the corpus luteum at first detection may further define the time of ovulation and hence embryo age. Embryo development is closely related to embryo age and vesicle diameter at 11 to 15 days post-ovulation, date at which is a good guide to future viability [3]. At this time, the embryonic vesicle grows between 2.9 and 4.5 mm per day [4]. The expected mean diameter of the embryonic vesicle at 13 and 15 days post-ovulation is 12.4 ± 1.8 mm and 20.1 ± 3 mm [4]. At this stage of development, a reduction in diameter equivalent to ≥ 2 days of growth is highly indicative of future embryo failure [3].

There is little change in the diameter of the embryonic vesicle from about Day 16 to 17, after fixation of the embryonic vesicle at the flexure of the uterine horn [5], and even in viable pregnancies its diameter can vary from under 20 mm to over 30 mm (Newcombe, unpublished data). From about Day 17 onwards therefore, vesicle diameter becomes a less reliable indicator of future viability.

Identification of the embryonic disc or ‘embryo proper’ (EP) with discernible heart beat is often the ‘gold standard’ for accurate pregnancy diagnosis and embryo viability, especially where endometrial cysts may be confused with an embryonic vesicle or ovulation dates are uncertain. The EP is first visualized with B-Mode (Real-time) ultrasonography on Day 19 to 22 (Day 0 = day of ovulation) [6]. The heart beat can be visualized as soon as the EP is also detected. This can be greatly aided by color-Doppler ultrasonography. In one study, in which color-Doppler was used, the
EP and heart beat were first detected at 19.1 ± 0.1 days of gestation [7]. In the latter study, an early indicator of the future EP development could be identified at the future position of the EP even 3 days earlier (16.4 ± 0.2 days). This was represented by a colored spot, indicating blood flow, in the endometrium at the ventral aspect of the vesicle opposite to the mesometrial attachment and adjacent to the wall of the vesicle [7]. However, under field conditions, the use of simple B-Mode ultrasonography is more common practice.

The presence of an embryonic vesicle without the development of an EP (anembryonic versicle) beyond day 30 of gestation has been considered abnormal and linked to pregnancy failure [8]. The objective of this study was to determine the ability to detect the EP and heart beat under field conditions in a large population of mares between Days 18 and 28 of pregnancy and to study the association between failure to detect the EP at different days of gestation with the future viability of that pregnancy.

2. Materials and methods

2.1. Detection of ovulation and post-mating treatment

Over four breeding seasons, 1256 embryonic vesicles from 1109 different mares were detected at routine ultrasonographic pregnancy diagnosis by a single operator at a Thoroughbred (TB) farm in the UK (northern hemisphere). Mares were examined with an ultrasound scanner (DP-6600Vet, Mindray Co., Ltd, USA) equipped with a linear array 5-10 MHz transducer every two days for the detection of ovulation and again if a second ovulation was suspected. The day of ovulation was defined as Day 1 and depending on palpation and the appearance of the ovulatory area or early CL on
ultrasound [9], the day of first detection of ovulation was defined as either Day 1 (when the ovary with a previously recorded preovulatory follicle presented an ill-defined ovulatory area of low echogenicity with or without a central lacuna containing echo mobile particles) or Day 2 (when the ovary with the previously preovulatory follicle presented a well defined hyperechoic corpus luteum with or without a central lacuna containing solid strands of fibrin). All mares were mated by natural cover before ovulation by one of five TB stallions of proven fertility (≥ 65% per cycle) within the same farm. All mares received the same post-mating treatment consisting of an intrauterine infusion of 12 mL of a mixture of injectable procaine penicillin (1800 mg) suspension (6 mL Depocillin®, Intervet, Cambridge, UK) and 900 mg of framycetin (6 mL of Framomycin® 15% injection, Novartis Animal Health, Camberley, UK) one to two days post-mating followed by a bolus of 25 IU of oxytocin (Oxytocin Leo; Leo Pharma Laboratories Ltd, Princes Risborough, UK) administered intravenously one day later.

2.2. Pregnancy diagnosis

Initial pregnancy diagnosis was performed on Days 12 to 14. The next examination was performed six to 10 days later (Days 18 to 24) and briefly searched for evidence of a hyperechoic body of 1.5 to 2.5 mm diameter with a discernible heart beat (EP) around the perimeter of the vesicle (Fig. 1). Suspect bodies in which the heart beat was not discernible were not included as positive identifications. No more than one minute was spent looking for the EP. All data from pregnancy diagnoses at Days 18 to 28 were obtained from single mares, therefore each mare was examined only once between Days 18 and 28 of gestation.
Twin management was performed by manual reduction between Day 13 and Day 16. These mares were re-examined either two or four days later. Mares in which on Days 18 or 19, the remaining vesicle appeared normal were not re-examined until 6 to 8 days later and were not therefore included in the Day 20 to Day 23 data. Further examinations were made on Days 30 to 32 and 42 to 45 to detect embryo failure. Embryo failure was assumed when the embryonic vesicle was no longer visible ultrasonographically or when the fluid-filled vesicle had failed to develop an EP after Day 30. The fetal stage was defined as a pregnancy of > 42 days that showed a positive heart beat.

2.3. Statistical analysis

Binary logistic regression was used to compare the difference in embryo detection rate amongst days of gestation (Days 18 to 28) and to compare the early embryonic loss rates between vesicles with a visible EP and those with no EP at a given day of gestation. The predictive value of detecting an EP within the vesicle at a given day of gestation on the future viability of the pregnancy was performed by estimating the sensitivity and the specificity of the test at each day of pregnancy diagnosis relative to ovulation.

The specificity was expressed as the number of vesicles with an EP detected that survived to the fetal stage (true negatives) divided by the number of vesicles with an EP detected that survived to the fetal stage (true negatives) plus the number of vesicles with no EP detected that survived to the fetal stage (false positives).

The sensitivity was expressed as the number of vesicles with no EP detected that failed before the fetal stage (true positives) divided by the number of vesicles with no EP detected that failed before the fetal stage (true positives) plus the number of vesicles with an EP detected that failed before the fetal stage (false negatives).
vesicles with an EP detected that failed before the fetal stage (false negatives). The specificity and sensitivity of the identification of an EP diagnostic test at each day of pregnancy (Days 18 to 28) are given in percentages (%).

3. Results

The overall EEL of the study between Day 18 and 45 was 5.6% (70/1256). The EP detection rate increased gradually ($P < .05$) from Day 18 to Day 21 when it reached the maximum. After that day, the number of vesicles with an EP detected became constant (Fig. 2).

Embryonic vesicles with an EP detected on Days 20 to 24, 26 and 28 were less likely ($P < .05$) to fail than those with a negative identification of the EP at similar gestational ages (Fig. 3). The EEL incidence in vesicles with an EP was not influenced ($P > .05$) by the day of gestation in which the EP was detected (Fig. 3). In contrast, the longer in gestation that the vesicle remained without a visible EP, the more likely ($P < .05$) it was to fail subsequently (Fig. 3).

The sensitivity of the diagnostic test on the future viability of the pregnancy was relatively high (> 92%) throughout the gestation period studied (Table 1), in part due to a low number of pregnancy failures resultant from vesicles with a positive identification of an EP at earlier stages of gestation (Days 18 to 28; false negatives). In contrast, the specificity of the test was low on Days 18 to 20 (Table 1), because many vesicles with no EP detected on Days 18 to 20 still developed normally to the fetal stage (false positives). The specificity of the test increased substantially from Day 21 onwards.
4. Discussion

Causes of embryo loss are multiple, maternal and embryonic, intrinsic and extrinsic and have been reviewed [10-12]. Previous investigations have shown that early vesicle diameter is well correlated with future pregnancy survival [8,13,14]. Small for age (SFA) pregnancies are found in mares of all ages [8,15] and occur more frequently following multiple ovulation [3,16]. Those vesicles most retarded in development may be unable to block luteolysis so that the vesicle is eliminated as the mare returns to estrus [11]. In addition, SFA vesicles are more likely to fail to develop an EP [8].

If the time of ovulation is known accurately, then a SFA vesicle can be diagnosed at early stages. Then the failing pregnancy can be terminated to allow return to estrus with the subsequent possibility to establish a new pregnancy as early as possible. A delay in embryonic vesicle diameter equivalent to two or more days’ growth has been proposed to be highly indicative of subsequent pregnancy failure [11]. However, most practitioners tend to leave these pregnancies until later stages (25 to 30 days of gestation) to give an “extra chance” of survival. If by then, the vesicle still remains without an EP, the pregnancy can be safely terminated [8]. On some occasions, however, embryo vesicles whose diameters fall within the normal range for their age still fail to develop an EP [8].

The results of the current study showed that by Day 20, the majority of embryonic vesicles had an ultrasonographically visible EP detected by B-Mode under field conditions. This is in agreement with a previous controlled study in which the EP was first detected on Days 19 to 22 (Day 0 = day of ovulation) [6]. In the current study, after Day 21 of gestation the percentage of new vesicles with an EP did not increase significantly along with gestational age.
On Day 20 of gestation there already exists a significant difference between the future EEL rate of mares with vesicles without an EP and those that have. This difference becomes increasingly greater as the age of gestation increases. However, at 20 to 24 days of pregnancy, even though there is a positive association between the absence of the EP and future pregnancy loss, it seems not advisable to terminate these pregnancies owing to the low specificity of the diagnostic test. For example, on Day 24, the specificity of the test was 50% which means that half of the vesicles without EP detected at that date will still develop one and survive beyond the fetal stage.

The low specificity of the test on Days 21 to 24 may be due to two reasons: failure to identify the EP on the ultrasound by the operator; and individual “physiological” variation of mares in the interval between fertilization and the development of the EP to a sufficient size at which it can be reliably detected on ultrasound.

From Day 26 onwards, both the specificity and sensitivity are high (> 92%). In fact, all pregnancies on Days 26 and 28 without an EP were lost (specificity of 100%). However these results are based on a relative small number of vesicles (3 pregnancies for each day) and therefore the clinical implications must be interpreted with caution.

It seems that the optimum time to perform a second pregnancy diagnosis in mares would be on Day 21 to 22. At that stage of gestation, if an EP is identified within the vesicle, the pregnancy is highly likely to progress to the fetal stage. This is indicated by the high sensitivity of a positive diagnosis of the EP within the vesicle on these days (> 95%). On the other hand, when the EP is not visible, it indicates that the vesicle is more likely to be abnormal and therefore more frequent examinations should be performed to monitor the development of the embryonic vesicle. Probably by around Day 26 to 28, if the vesicle remains without an EP, it can be considered safe to terminate the pregnancy provided that the ovulation date is known accurately.
In conclusion, the EP of the equine vesicle can be identified reliably with B-mode ultrasonography in the majority of mares (> 71%) on Day 20 of gestation. The percentage of vesicles with an EP increases significantly from ay 20 to 21 and then it reaches a plateau. The lack of a positive identification of an EP from Day 20 onwards is associated with a higher EEL rate.

References


Table 1
Embryo proper detection rate and predictive value of viability of pregnancy by detecting the embryo proper at different stages of early gestation.

<table>
<thead>
<tr>
<th>Day of gestation</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
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<tr>
<td>Pregnancies (n)</td>
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<td>90</td>
<td>389</td>
<td>245</td>
<td>171</td>
<td>105</td>
<td>48</td>
<td>50</td>
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<td>31</td>
</tr>
<tr>
<td>Vesicles with EP (%)</td>
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<td>31.1</td>
<td>71.4</td>
<td>86.9</td>
<td>87.1</td>
<td>90.4</td>
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<td>85.7</td>
<td>100.0</td>
<td>90.3</td>
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<td>Pregnancy losses (n)</td>
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<td>18</td>
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<td>98.2</td>
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<td>94.0</td>
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<td>40.0</td>
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</table>
Fig. 1
Fig. 2
Figure 3
Fig. 1. B-Mode sonograms of four pregnancies from four mares at different stages of gestation. 20 d E +: pregnancy Day 20 of gestation (Day 1 = day of ovulation); note the embryo proper (EP) at 5 o’clock position (white arrow). 24 d E −: pregnancy Day 24 of gestation; the EP could not be found, this pregnancy failed before day 42. 27 d E +: pregnancy Day 27 of gestation; note the large EP (white arrow) and the developing allantoids (black arrow). 42 d F +: pregnancy Day 42 of gestation; note the developing amnios (white arrow) around the fetus (black arrow).
Fig. 2. Percentage of vesicles with an embryo proper (EP) detected by B-Mode ultrasonography at different stages of early gestation. Significant difference ($P < .05$) in EP detection rate amongst days of gestations is indicated by different letters.
Fig. 3. The association between the ability to detect the embryo proper (EP) within the embryonic vesicle on Days 18 to 28 of gestation (Day 1 = day of ovulation) and the future pregnancy failure before day 42. Within mares with vesicles with no EP, different letters amongst days of pregnancy indicate significant difference ($P < 0.05$) in embryo loss rate. The embryo loss rates in mares with vesicles with an EP were not different ($P > .05$) amongst days of gestation. Within day of gestation, an asterisk (*) indicates a difference ($P < .05$) in the future early embryonic loss rates between mares with vesicles with an EP and those without one.