Technological approaches to the problem of double ovulation and twin pregnancies in mares

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Abstract. The aim of the work was to determine and justify the correct technological approach in cases of double ovulation and twin pregnancies in mares. The frequency of single and double ovulations (n=2207), the level of twin pregnancies and the results of manual reduction of excess embryo in mares were studied. The average level of double ovulations was 9.5 % per year, and it had no significant difference in mares of different breeds (Russian Trotter, French Trotter, Standardbred and others). The number of poliovulations in February-March (3.2–4.9 %) and August (5.1 %) was significantly (p<0.05) lower than it was in April-July (9.9–11.3 %). The number of multiple ovulations gradually decreased from young (4–8 yo) to old (>14 yo) mares (11.9 – 5.1 %) (p<0.05). The third part of all mares (35.8 %) with poliovulations repeated it during one season, and after 1–3 successive breeding seasons. The mating of mares with double ovulation resulted in 35.0 % of twin embryos and 28.1 % of single embryos. The success rate of manual reduction of the second embryo on days 12–14 was 85.3 %.

1. Introduction

Twin pregnancy remains a significant problem reducing the efficiency of reproduction in horse breeding in Russia. This takes place on farms with no qualified specialists and ultrasound scanners or in farms with small herd. In farms with ultrasonic devices, abortions of twins are usually rare.

The ultrasound diagnostics of twins and the method of manual reduction of an excess embryo in mares decrease significantly the number of twin abortions (from 20–40 to 3–6 % of all abortions) in the modern horse reproductive practice [1–3].

Various researchers studied the origin, possible ways of the natural development and likely outcome of twin pregnancies, the causes and mechanism of the natural reduction of extra embryos in the uterus [4–9]. The level of twin pregnancy is directly related to the level of poliovulatory cycles in mares, since twins are almost always the result of fertilization of two ova [5, 9], if the interval between ovulations does not exceed the time of sperm survival in the oviducts. Therefore chances of getting twins in the natural mating and insemination with fresh sperm are greater than with chilled or frozen semen. There are a few reports about natural identical twins [10–12]. J.R. Crabtree (2018) suggests that the number does not exceed 1 % of all twins [1]. The cases of identical twins also include those resulted of spontaneous embryo division during/after transfer to the recipient [13–15].

The incidence of poliovulatory cycles in various horse breeds ranges from 8 to 25 % [7]. The increasing percentage of poliovulatory cycles, twin pregnancies and pregnancy loss is reported in Thoroughbred mares and its crosses [3, 17–20]. An individual predisposition, repeatability and heritability of double ovulation in mares were established [21, 22]. The other factors increasing the
level of twin pregnancies are age, reproductive status, season, nutrition and use of drugs to control ovulation in mares [7, 9, 23–25].

There are several methods to avoid the birth of twins in mares: missing of the estrus cycle with double ovulation, manual reduction of one embryo, prostaglandin F2a injection, uterus flushing, ultrasound guided foetal cardiac puncture, aspiration of foetal fluids, cranio-cervical dislocation [1, 26–32]. However, there is a mechanism of natural reduction of twins in mares [6, 9], that occurs in about 60 % of cases [30]. The natural elimination of one or both embryos occurs mainly after fixation of them in the uterus [16]. The loss of twin embryos can occur spontaneously in 75 % of cases with fixation in one horn and in 15 % of cases with fixation in different horns of the uterus [33].

Approximately 70 % of twin pregnancies are unilateral, and only 1 % of them result in the birth of normal full-term offspring [21]. The main reasons of natural reduction of one or both twin embryos are the degree of asynchrony of ovulations in polyovulatory cycles, and therefore difference in size between embryos, unilateral or bilateral fixation of embryos in uterus, orientation of the embryoblasts to each other in embryo vesicles in unilateral pregnancy, poor nutrition [5, 34].

The method of manual reduction of twins at early stages of pregnancy became routine practice in horse reproduction. The operation is performed by transrectal squeezing the uterine horn and crushing the smallest embryo under ultrasound control. The success of the procedure depends on the age and size of the embryos, their location in the uterus, the qualification of the performer and may achieve 90 % [4]. The most common approach for twin embryo reduction to single embryo in mares is transrectal crushing of one embryo in the mobile phase of pregnancy (day 11-16 after ovulation). It is possible to separate twin embryos in this period, if they are close to each other. After fixation twins (day 17–18) in one horn of the uterus manual reduction of one embryo become more dangerous for surviving of the second embryo. As reported, the efficiency of twin embryos crushing between days 21 and 31 was 70 % and between days 31 and 45 – 23 % [32].

The aim of the work was studying the frequency of double ovulations in different horse breeds in Russia, the ratio of synchronous and asynchronous ovulations in one or both ovaries, repeatability of double ovulation in mares’ estrus cycles, pregnancy rate in double ovulation cycles and the success level of the manual reduction of twins on day 12–15.

2. Methods and materials

Data of mares’ fertility during 2013–2016 years at the “Lokotskoy” stud (Bryansk region, Russian Federation) were collected. There were from 139 to 167 mares of three main breeds (Russian Trotter, Standardbred and French Trotter) and other breeds per year at the stud at that period. Careful ultrasound control of follicle growth and ovulation was conducted. The mares were covered or inseminated with fresh/cooled semen every 24–36 hours close to ovulation. The result was checked on day 11–12 after ovulation. The embryo crushing procedure was carried out between days 12 and 15 with ultrasound control.

We calculated the number of cycles with single and double ovulations, synchronous and asynchronous ovulations in one or both ovaries, pregnancy rate and results of manual reduction in mares with twin pregnancy. The data were statistically analyzed by Student-Fischer t-test.

3. Results and discussion

The average number of double ovulations in mares was 9.5 % per year (Table 1). The distribution of poliovulations does not have significant differences by month during the main breeding season: April (10.8 %), May (10.5 %), June (9.9 %) and July (11.3 %). Some studies (H.Merct, W. Jochle, 1999) have shown an increase in the percentage of twin pregnancies from February-March (0.9 %) to April-May (1.34 %) and June-July (1.76 %) [34]. The same observations are reported by J. R. Newcombe (2000): the incidence of multiple ovulation was the highest in May and June, 28.4 % and 22.3 %, respectively, compared with 20.8 %, 21.8 % and 18.2 % in March, April and July [11]. According to our calculations the number of poliovulations in February-March (3.2–4.9 %) and August (5.1 %) was significantly (two to three times) lower (p <0.05) than it was from April to July.
The analysis of the polyovulations level in mares of different horse breeds (Table 2) showed that in Standardbred mares it was 12.7 %. The average frequency of double ovulations in mares of other breeds was lower (8.18–11.88 %) but the difference is not significant. The highest double ovulations level was reported in thoroughbred mares (19 %) [21].

Table 3. Frequency of multiple ovulations in mares of different age

| The age of mares, years | Number of estrus cycles | Poliovulatory cycles |
|------------------------|-------------------------|----------------------|
|                        | total, n                | n                    | %        |
|                        |                         |                      |          |
| 4-8                    | 556                     | 66                   | 11.9±1.37  |
| 9-13                   | 352                     | 29                   | 8.2±1.46   |
| 14-18                  | 125                     | 8                    | 6.4±2.18  |
| ≥19                    | 39                      | 2                    | 5.1±3.52  |

*p<0.05, *ap <0.05, *bp <0.05, *cp <0.05, *dp <0.05

Table 4 demonstrates that synchronous ovulations were twice more often (68.34 %) than asynchronous ovulations (31.66 %) in mares (p<0.001). These data do not agree with data from other sources [8, 36]. Among all synchronous ovulations 45 % occurred in one ovary and 23.3 % in both ovaries (p<0.001). Asynchronous ovulations in one or two ovaries were distributed equally. Our results suggest that the probability of detecting twin embryonic vesicles of the same size in mare uterus is greater than the vesicles of different sizes.

Table 4. Frequency of synchronous ovulations in mares of different horse breeds

| Breed                  | total  | single | double |
|------------------------|--------|--------|--------|
| Russian Trotter        | 672    | 617    | 55     | 8.18±1.06 |
| Standardbred           | 181    | 158    | 23     | 12.71±3.21 |
| French Trotter         | 244    | 215    | 29     | 11.88±2.07 |
| Other *                | 152    | 139    | 13     | 8.55±1.77  |
| In all                 | 1249   | 1129   | 120    | 9.61±2.69  |

*a*druft, Orlov Trotter, halfbred, sport breeds

In our research the number of multiple ovulations in mares of different ages (Table 3) gradually decreased from young to old mares (11.9 – 5.1 %). Our data is contrary to the records of Deskur S. et al. (1985), according to the twin pregnancy rate in thoroughbred mares it is raised with age – 2.8, 3.4, 3.6 , 6.8 % for mares of 4–7, 8–11, 12–15 and 16-20 years old respectively [35].
Table 4. The ratio of synchronous and asynchronous double ovulations in one or both ovaries in mares estrus cycles

| Ovulations | Synchronous (0–12 hours) | Asynchronous (12–36 hours) |
|------------|--------------------------|-----------------------------|
|            | n | %                      | n | %                      |
| in one ovary | 54 | 45.00±4.54\(^c\) | 19 | 15.83±3.33            |
| in both ovaries | 28 | 23.33±3.86\(^d\) | 19 | 15.83±3.33            |
| Total       | 82 | 68.34±4.25\(^a\) | 38 | 31.66±4.25\(^b\)        |

\(^{a,b,p}<0.001, \(^{c,d,p}<0.001\)

Analysis of the repeatability of poliovulations in mares over four seasons (Table 5) showed that ovulations of two follicles were repeated in more than third part of mares (36.4 %). Half of these mares (50 %) had one double ovulation per season, which was repeated in a season or two. In a quarter of mares (28.6 %) estrus cycles with double ovulation were repeated both during one season and in 1–2 successive seasons. However most of the mares became pregnant after mating/insenmination, so it was impossible to notice their further cyclic activity in one season. Nonetheless it is reasonable to combine the second and the third groups, and they will make up almost 80 % of mares with predisposition to repeat poliovulations. The results show that the mares are individually susceptible to double ovulation and, consequently, twin pregnancy.

Table 5. Repeatability of double ovulations in mares during four seasons

| Mares with double ovulations | n | % |
|-----------------------------|---|---|
| Total                       | 77| 100|
| Recurrence of double ovulations | 28| 36.4±9.08\(^a\) |
| Including:                 |   |    |
| more than one double ovulation in one season | 6 | 21.4±16.7 |
| one double ovulation in one season with repeating in 1–2 seasons | 14 | 50.0±13.4 |
| more than one double ovulation in one season with repeating in 1-2 successive seasons | 8 | 28.6±15.98 |

\(^{a,p}<0.05\)

The data of Table 6 indicate that insemination/mating of mares in estrus cycles with double ovulation does not necessarily lead to fertilization of both eggs, and therefore, to produce twins. G.J. Ginther (1983) reported that pregnancy rates were higher (P<0.01) for double ovulations 0–2 days apart (83–87 %) than for single ovulations (54 %) [25]. In our studies twin embryos were detected by ultrasond scanning in 35 % (28.6–44.4 %) of cases, and single embryos in 28.1 % (11.1–38.1 %) of cases. So among covered mares with double ovulation only a third part of them had 2 embryos on day 12–15. The highest predisposition for fertilization of both eggs in cycles with double ovulation was found in Standardbred mares (44.4 %) and the lowest one was in French Trotter mares (28.6 %). It is the evidence of the natural barrier of multiple pregnancy at the stage of fertilization and/or first stages of embryogenesis in oviducts.

Table 6. The results of ultrasound examination of covered mares on day 12-14 post double ovulation

| Breed                  | Mares covered in double ovulation cycles, n | 0 embryos | 1 embryo | 2 embryos |
|------------------------|-------------------------------------------|-----------|----------|-----------|
|                        | 0 embryos | % | 1 embryo | % | 2 embryos | % |
| Russian Trotter        | 43        | 16 | 37.2 | 11 | 25.6 | 16 | 37.2 |
| Standardbred           | 18        | 8 | 44.4 | 2 | 11.1 | 8 | 44.4 |
| French Trotter         | 21        | 7 | 33.3 | 8 | 38.1 | 6 | 28.6 |
| Other *                | 20        | 7 | 35.0 | 7 | 35.0 | 6 | 30.0 |
| Total                  | 102       | 38 | 36.9 | 28 | 28.1 | 36 | 35.0 |

*druff, Orlov Trotter, halfbred, sport breeds

When two embryonic vesicles are detected in the uterus during ultrasound examination, it is recommended to remove one of them as quickly as possible. If they are of different size the smaller
embryo is usually removed [7, 29]. The procedure of crushing of the excess embryo is carried out more easily in the mobile phase and in the case of bilateral fixation. Small day 10-11 embryos (less than 10 mm in diameter) are suggested more difficult to destroy then larger prefixation embryos [29]. With unilateral fixation the success of manual reduction is 50 %, which is less than the level of the natural twin embryo survival [6]. Therefore, some practitioners prefer not to interfere in the natural process up to day 35 of pregnancy, before the cycle of development and regression of the endometrial cups in the mare’s uterus will start. In our work (Table 7) the success of manual reduction of one of twin embryos ranged from 80 to 100 %.

Table 7. The result of manual reduction of the second embryo on day 12-15 of twin pregnancy

| Year | Twin pregnancy detected | Successful reduction | Unsuccessful reduction* |
|------|-------------------------|----------------------|------------------------|
|      | n | % | n | % |
| 2013 | 7 | 6 | 100.0 | – | – |
| 2014 | 9 | 8 | 88.9 | 1 | 11.1 |
| 2015 | 10 | 8 | 80.0 | 2 | 20.0 |
| 2016 | 8 | 7 | 83.3 | 1 | 16.7 |
| Total | 34 | 29 | 85.3 | 4 | 14.7 |

*both embryos were crushed (n=2) or the second embryo died later (n=2)

Our observations showed that after asynchronous ovulation the vesicles of different sizes tend to move together through the uterus and are usually close to each other. They are difficult to be separated and easily become damaged during crushing procedure. It was evident that after asynchronous ovulation frequency of unilateral fixation of twin embryos was greater (p<0.01) than the frequency of bilateral fixation (10/11 and 1/11, respectively), and the embryo reduction was greater (p<0.01) for unilateral fixation (14/19) after asynchronous ovulation (9/11) than for bilateral fixation (0/9) after synchronous ovulation (5/17) [6]. Therefore, it would be reasonable not to remove the excess embryo, since it is a high probability of fixation of both embryos in one horn and the natural reduction of the smaller vesicle. With synchronous ovulation, the embryos are usually of the same size and, according to repeated ultrasound examinations (2–3 hour period), are located at different distances from each other. It is most convenient to crush one of them, when the distance between vesicles is more than 2 cm. We also noticed that it is more difficult to destroy a small day 11 embryo than larger embryos of day 12–15. However, crushed day 14–15 vesicles leave more fluid in the uterus, which may be a potential problem for the survival of the second embryo [29]. Therefore, we consider that the optimal time for extra embryo removal is day 12–13 of pregnancy. But if both embryos are not in the uterus at this time (> 2 days asynchrony between two ovulations), it is necessary to repeat ultrasound exams and to perform the crushing procedure later.

4. Conclusion
The level of double ovulation in mares of different breeds at the “Lokotskoy” stud (Russia, Bryansk region) has no significant difference and fluctuates between 8.2 and 12.8 %. The distribution of poliovulations in mares from April to July was relatively equally (9.9–11.3 %) but it was two to three times higher than in February-March and August (p >0.95).

The number of multiple ovulations in mares of different ages gradually decreased from young to old mares (11.9 – 5.1 %) (p <0.05). Synchronous ovulations were twice more often than asynchronous ovulations in mares (p<0.001). The third part of all mares with poliovulations tends to repeat it. From this part, 80 % of the mares had double ovulations repeated during one season and in 1–2 successive breeding seasons. The mating of double ovulated mares leads to the finding of 35 % twin and 28.1 % single embryos on days 12–15 after ovulation. Insemination of mares in poliovulatory cycles provides the finding of twins in 28.4 – 44.6 % of cases. The success of manual reduction of the excess embryo in the mobile phase is 80–100 %. Therefore, it is advisable not to miss the estrus cycle with double ovulation in mares.
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