Increase of pregnancy rate after multiple periovulatory inseminations in mares

Steigerung der Trächtigkeitsrate von Stuten mittels mehrfachen periovulatorischen Besamungen

Abstract

Objective: There exist differences in the reproductive behavior of stallions and mares under free-running and domestic in-hand breeding conditions. Contrary to artificial insemination programs, a stallion mates a mare multiple times per estrus under natural conditions. The objective of this study was to determine if multiple periovulatory artificial inseminations (MI), four times in two different time intervals instead of two, would result in increased pregnancy outcome or higher incidences of breeding induced endometritis.

Material and methods: Eighty-two estrous mares were allocated randomly to one of three experimental groups. They were administered intramuscular deslorelin (1.25 mg) to induce ovulation, and 24 h later, they were inseminated either twice (group DI), four times in relatively short intervals (group MISI) or four times in relatively long intervals (MILI), after division of one commercial insemination dose into two or four portions, respectively. Uterine sampling for bacteriological and cytological analysis was conducted directly before the first insemination and 24 h after the last insemination.

Results: Mares of the MI groups showed a higher pregnancy rate than mares of the DI group. There was no difference in bacteriological and cytological results between the three groups. In addition, mares of the MISI group showed less intrauterine fluid accumulation 24 h after the last insemination than mares of the DI and MILI group.

Conclusions and clinical relevance: We conclude that multiple periovulatory inseminations in a close time frame to ovulation lead to an increase of pregnancy results per cycle, and suggest, that they do not lead to impaired inflammatory reactions of the uterus in healthy fertile mares.

Zusammenfassung

Gegenstand und Ziel: Zwischen freilebenden und unter domestizierten Bedingungen gehaltenen Hengsten und Stuten bestehen deutliche Unterschiede im Reproduktionsverhalten.

Im Gegensatz zum Prozedere bei der künstlichen Besamung paart sich der Hengst in der Natur während eines Zyklus mehrmals mit der Stute. Ziel dieser Studie war es zu bestimmen, ob vierfache Besamungen in verschiedenen Zeitabständen zur Ovulation im Gegensatz zu zweifachen in einer höheren Trächtigkeitsrate resultieren und ob solche Mehrfachbesamungen eine erhöhte Inzidenz von belegungsinduzierter Endometritis nach sich ziehen.

Material und Methoden: 82 zyklische Stuten verschiedener Rassen wurden nach dem Zufallsprinzip in eine von 3 Behandlungsgruppen eingeteilt. Danach wurden sie im Rahmen einer standardisierten Untersuchung und 24 Stunden nach einer Ovulationsinduktion mittels 1.25 mg Deslorelin i.m. entweder zweimal (Gruppe DI) oder viermal (Gruppe MI) besamt, wobei letzteres in Kurzzeit- (Gruppe MISI) oder Langzeit-Intervallen (Gruppe MILI). Hierzu wurde jeweils eine Besamungsdosis in 2 bzw. 4 Portionen aufgeteilt. Direkt vor der ersten Besamung und 24 Stunden nach der letzten Besamung wurde sowohl eine bakteriologische als auch eine zytologische Untersuchung durchgeführt.

Ergebnisse: Stuten der MI-Gruppe wiesen eine signifikant höhere Trächtigkeitsrate pro Zyklus auf als Stuten der DI-Gruppe. Die Ergebnisse der Bakteriologie und Zytologie ergaben keine signifikanten Unterschiede zwischen den 3 Gruppen. In der MISI-Gruppe wiesen signifikant weniger Stuten Flüssigkeit im Uterus auf als in der MILI- und DI-Gruppe.

Schlussfolgerungen und klinische Relevanz: Die Ergebnisse dieser Studie zeigen, dass multiple periovulatorische Besamungen zu einer Erhöhung der Trächtigkeitsrate führen, und es dabei bei gesunden, fertilen Stuten zu keiner Zunahme von Entzündungsreaktionen und mikrobieller Kontamination des Uterus nach der Besamung kommt.

Keywords: artificial insemination, bacteriology, cytology, fertility, horse
Schlüsselwörter: künstliche Besamung, Bakteriologie, Zytologie, Fruchtbarkeit, Pferd

1. Introduction

Artificial insemination (AI) represents a key procedure in the modern equine breeding industry, aiming to ensure adequate pregnancy rates using defined portions of ejaculates. From an economic point of view, it is common to limit the number of inseminations per estrus

cycle (17) in order to reduce costs and minimize the risk of repeated endometrial inflammation (13).

Unlike common AI programs, stallions under free running conditions mate an estrous mare multiple times for 1 – 4 days, with a frequency up to nine times daily, with very various intervals between matings and as often as every 1 to 2 h (21, 30). Under good conditions, foaling rates in healthy harem females are as high as 86 to 90% per season (7, 21).

In retrospective studies, it has been found that if mares were inseminated twice or more with cooled semen, pregnancy rate was higher compared to mares inseminated only once (e.g. 28). This was also partly demonstrated by Vidament et al. (36) for frozen-thawed semen, but was not confirmed by Kloppe et al. (12) and Sieme et al. (27). It could also be shown that pregnancy rates were highest when mares were inseminated in a “time window” within 24 h (cooled semen) or 12 h prior (frozen-thawed semen) and up to 6 - 12 h after ovulation (3, 27). Therefore, in order to decrease ultrasound examinations for appropriate insemination timing, these findings lead to a common practice of using a double insemination regime, before and after ovulation, in order to achieve equal fertility results with less intensive management (4, 19).

Beside the potential disadvantage of more semen use and transportation costs, multiple insemination may lead to an increased incidence of post breeding induced endometritis with decreased pregnancy rates. Indeed, when inseminating a mare, an endometrial inflammatory reaction usually peaks between 6 to 12 h and regresses 48 to 72 h after insemination (10, 33). It is well documented that these reactions represent a natural immune response to the introduced seminal fluid, sperm as well as bacteria and consist of activation and recruitment of inflammatory cells [e.g. polymorphonuclear cells (PMN-cells)] (13, 34). This temporary inflammation is considered useful because it contributes to elimination of contaminants, such as excess sperm and seminal plasma (34). However, some “susceptible” mares (10 - 15% of the population) (40) develop a persistent breeding induced endometritis (PBIE) that typically lasts longer than 48 h after insemination (1, 10).

Considering the observation of frequent daily mating in horse harems under natural conditions, we investigated if four instead of two inseminations, administered in two different time intervals using only one commercial insemination dose, would increase pregnancy rate

per cycle. In parallel, we investigated the influence of such multiple inseminations on occurrence of breeding induced endometritis.

2. Materials and Methods

2.1 Horses and ethical note

Privately owned, clinically healthy mares of different breeds whose reproductive history was known for a minimum of one year were recruited over two seasons. Exclusion criteria included mares with a history of severe PBIE. Informed owner consent was received before every insemination trial. The experiment was approved by the Animal Health and Welfare Commission of ….. and strictly followed institutional guidelines for humane animal treatment (permit number ……).

2.2 Study design

Mares were allocated randomly (drawn by lot) into three groups: inseminated twice (group DI), inseminated four times in a relatively short interval (group MISI), and inseminated four times in a longer interval (group MILI). The conventional treatment of the reproduction center (as described in 19) was administered to group DI and therefore represented the control group.

2.3 Examination and insemination procedure

Before each insemination trial, the mares were presented regularly to a stallion until they showed clear signs of estrus (5). Their cycles were monitored daily using transrectal ultrasonography [7.5 MHz, 50 mm linear array probe (MyLab™ OneVET, Esaote, Italy)]. Uterine edema, uterine fluid (diameter of fluid appearance measured in mm), the number and size of follicles (in mm), and the presence of a corpus luteum (CL) were recorded. If a follicle was larger than 35 mm in diameter, an intramuscular treatment of 1.25 mg deslorelin (BioRelease® Deslorelin, Caledonian Holdings Ltd, Auckland, New Zealand) was administered at 5.00 pm, aiming to induce ovulation 36 to 48 h later. Twenty-four hours after deslorelin treatment (5.00 pm), mares were examined in order to verify the size of the follicle and to exclude early ovulation.

Mares were prepared for insemination by cleaning the vulva with iodine solution and water. Before insemination, a pre-insemination bacteriological and cytological examination was performed (see section 2.5). For inseminations with frozen semen (exclusively from stallions

with commercial doses of 4 to 9 straws each), an insemination pipette (Universal insemination pipette for equine, Minitüb GmbH, Tiefenbach, Germany) was placed deep into the uterine horn on the side of the ovulating follicle using guided rectal examination. If chilled semen was used, the pipette was placed in the uterine body.

Mares assigned to group DI were inseminated with half of the semen dose 24 h post deslorelin treatment (post-des), and re-inseminated 38 h post-des with the other half of the semen dose if they had ovulated. If ovulation had not occurred, they were rechecked at 42 h and 48 h post-des, and inseminated as soon as ovulation could be confirmed. Group MISI mares were inseminated with a quarter of the semen dose at 24, 25, and 26 h post-des, and then rechecked 38 h post-des and inseminated post-ovulation. In the case of no ovulation, they were rechecked 42 h and 48 h post-des and inseminated post ovulation. Group MILI mares were inseminated with a quarter of the semen dose at 24 h, 30 h, and 36 h post-des and then rechecked for ovulation at 38 h post-des and inseminated post ovulation. In case of no ovulation, they were also rechecked 42 h and 48 h post-des, and inseminated when ovulation was detected using the last quarter of the semen dose. All mares ovulating later than 48 h post-des were excluded from the study.

Twenty-four hours after the last insemination, the mares underwent ultrasound examination to monitor for the occurrence of uterine fluid and to confirm the presence of a CL. Furthermore, a second bacteriological and cytological sampling was conducted. Ultrasonographic examination was repeated 48 h after the last insemination. If the uterine fluid diameter after 24 or 48 h was larger than 5 mm, a postovulatory treatment was undertaken (see section 2.6). A transrectal ultrasound was performed 14 to 19 days after ovulation either at the reproduction center or at the breeder's farm to confirm pregnancy.

2.4. Semen Analysis

Pre-insemination semen quality and quantity was evaluated by calculating the total sperm number using a nucleocounter (AP-100, ChemoMetec, Allerod, Denmark). After dilution with INRA 96^{TM} (IMV, L'Aîgle, France) to a concentration of 30 x 10^6 sperm/mL, sperm motility was assessed with a computer-assisted sperm analyzer (HTM-IVOS, Version 12, Beverly, MA, USA). For that purpose, semen was placed into pre-warmed 20 µL standard count analysis chambers (SC 20-01-C, Leja, Nieuw-Vennep, Netherlands) and assessed in ten fields.

Cells moving faster than 10 µm/sec were considered motile. Only insemination doses containing at least 50 x 10^6 motile sperm cells were included in the study.

2.5. Uterine bacteriology and cytology

Pre- and post-insemination bacteriological and cytological samples were collected in the corpus uteri at the bifurcation of the horns. For the bacteriological examination, a double guarded swab (Uterus culture swab, Minitüb GmbH, Tiefenbach, Germany) was used, which was smeared after collection on a sheep-blood agar as well as on a McConkey agar (Columbia III Agar with 5% Sheep Blood, and MacConkey II Agar, Axon Lab AG, Les Monts-sur-Lausanne, Switzerland). The agars were incubated at 37°C. After 24 and 48 h, they were evaluated and if substantial growth of bacterial colonies (≥ 10 colonies) of the same species was detectable, the culture was considered positive. If < 10 colonies or a mixed culture were detected after 48 h, the bacteriological result was considered negative (38).

For cytological analysis, a cytobrush (Minitüb GMBH, Tiefenbach, Germany), used at the same time as the uterine swabbing was smeared on a slide (Micro Slide assistant®, Glaswarenfabrik Karl Hecht Gmbh, Sondheim/ Rhön, Germany) and stained (Diff-Quick, Medion Grifols Diagnostics AG, Düdingen, Switzerland). After drying, PMN-cells and endometrial cells were counted in ten fields under 400x magnification. A positive cytological result was defined as presence of > 0.5% PMNs of all cells in the sample (as described by 22).

2.6. Postovulatory Treatment

Postovulatory treatments were based on clinical observations and performed until a maximum of 96 h after ovulation. Postovulatory uterine flushing, using a minimum of two liters of physiological saline solution (NaCl 0.9% steril, Laboratorium Dr. G. Bichsel AG, Interlaken, Switzerland), was performed daily in the event of more than 20 mm intrauterine fluid on ultrasound. A dose of 20 IU Oxytocin (Oxytocin, Werner Stricker AG, Zollikofen, Switzerland) was applied intramuscularly every 4 to 8 h if more than 5 mm intrauterine fluid was present and after every flushing therapy. Antibiotic treatments with intrauterine instillation of 10 x 10^6 IU benzylpenicillin sodium (Penicillin®, Natrium Streuli, Streuli Pharma AG, Uznach, Switzerland) and 2 g gentamicin (Vetagent®, Veterinaria AG, Freienbach, Switzerland) were added after flushing therapy if positive postovulatory bacteriological results were obtained (15).

2.7. Statistical analysis

Statistical analysis was carried out using the software NCSS (NCSS 10, LLC, Utah, USA). Only the first cycle of each mare was included in the statistical analysis. To evaluate uterine inflammatory reactions, the results of the cytological and bacteriological examination as well as uterine fluid findings were coded binomially. All possible associations between variables were assessed with a Spearman correlation matrix. A univariable logistic regression model was run for the statistical outcomes: pregnancy, bacteriology and cytology. The model considered age, mare status, semen type, postovulatory uterine fluid presence and postovulatory treatment as explanatory variables in order to determine possible confounding factors. Additionally, two different treatment groupings were considered. First, the three original treatment groups were analyzed separately and second, groups MISI and MILI combined with group DI as a reference. For uterine fluid, two separate models were run that first compared group MISI to group DI and then group MILI to group MISI. Explanatory variables that showed a significant association and were not correlated, were combined into multivariable logistic regression models (for each of the above-mentioned outcomes). All *p*-values were considered significant if ≤ 0.05. Data are presented as mean ± standard deviation (range).

3. Results

A total of 82 mares (22 maiden, 27 barren, 33 foaling) with an average age of 13 ± 5 (3 − 25) years were recruited. Results of 32 mares allocated to group DI, 25 mares to group MISI and 18 mares to group MILI group were analyzed. Mares that did not ovulate within 48 h after deslorelin treatment ($n = 6$) or that were inseminated with a total dose less than 50 x 10^6 total motile sperm cells ($n = 1$) were excluded. The semen used originated from 54 stallions selected by the mares' owners and was available in chilled ($n = 12$) or frozen ($n = 42$) form. Total chilled semen doses included an average 1320 ± 2043 x 10^6 (71 − 8600 x 10^6) motile sperm cells and had a mean volume of 21.5 ± 12.6 (10.0 − 50.0 mL). Frozen semen doses had 388 ± 223 x 10^6 (59 − 901 x 10^6) motile sperm cells and a mean volume of 3.3 ± 0.9 (2.0 − 4.5) mL. The number of inseminated motile sperm cells and volume per total dose did not differ between mares that became pregnant or not (Mann-Whitney U tests: $p = 0.17$ and $p = 0.6$).

A total of 33 mares ovulated within 38 h post-des, 21 mares within 42 h post-des, and 4 mares ovulated within 48 h post-des. The time point of ovulation after induction with deslorelin did not have a significant effect on pregnancy rate (univariable logistic regression test; 38 h: p = 0.93, 42 h: p = 0.89, 48 h p = 0.65). Postovulatory oxytocin was administered to 20 mares (11 mares in group DI, 8 in group MILI and 1 in group MISI). Postovulatory uterine flushing was performed in ten of these mares (3 in group DI, 6 in group MILI and 1 in group MISI) and four of these were flushed additionally with antibiotics (0 in group DI, 3 in group MILI and 1 in group MISI).

3.1 Pregnancy rate

In the univariable logistic regression models, only age was found to have a significant influence on pregnancy rate ($p < 0.001$) and was therefore included in the multivariable models. The status of the mare was not associated with the pregnancy rate in the univariable model but confounded the association of pregnancy rate to treatment groups and was therefore included in the multivariable models.

Group DI showed lower pregnancy rates compared to both MI groups (multivariable model: p = 0.05) after having adjusted for age and status of the mare. When comparing pregnancy rates of group DI to groups MISI and MILI individually, no difference in pregnancy rate was found between group DI and group MISI (Fig 1; p = 0.17), nor between group DI and group MILI (Fig 1; p = 0.23).

When considering mares inseminated with frozen semen only (n = 58), 12/24 (50.0%) mares of group DI and 25/34 (73.5%) of the MI groups [group MISI: 15/21 (71.4%); group MILI: 10/13 (76.9%)] were diagnosed as pregnant. A multivariable model, that included age and status of the mare, showed that the pregnancy rate of mares inseminated with frozen semen was higher in the MI groups compared to group DI (p = 0.03, OR ± 95% CI = 4.5 ± 1.1−17.9).

Regarding the mares inseminated with chilled semen (n = 17), no significant differences in pregnancy rate were found (DI group: 37.5% *versus* MI group: 62.5%; multivariate model: p = 0.76) after having adjusted for age and status of the mare.

3.2. Uterine fluid accumulation

In the univariable logistic regression models, age (p = 0.01) and the status of the mare (p = 0.009) were identified as possible confounding factors with respect to the presence of uterine fluid. Results of the multivariable model are illustrated in Table 1. In total, 20/75 (27.7%) of mares accumulated uterine fluid 24 h after insemination. Having adjusted for age and status of the mares, mares in group MISI were 10 times less likely to have fluid after insemination than mares of group DI (multivariate logistic regression model: p = 0.03, OR ± 95% CI = 0.1 ± 0.0−0.6) and mares of group MILI were 15 times more likely to have fluid after insemination than mares of group MISI (p = 0.01, OR ± 95% CI = 15.3 ± 1.5−150.4). No significant difference was found between groups DI and MILI (p = 0.9, OR ± 95% CI = 1.08 ± 0.27−4.3)

3.3. Uterine bacteriology and cytology

Having adjusted for age and status of the mare, the evolution of pre- to post-insemination bacteriological results (Table 1) did not render any evidence of a difference between the mares in group DI and group MISI (p = 0.90) nor group MILI (p = 0.48). Likewise, the cytological results were similar between group DI and group MISI (p = 0.67) as well as between group DI and group MILI (p = 0.39) (See Table 1). In detail, post-examination cytology analysis of the mares of group DI revealed 19 ± 32% PMNs *versus* 16 ± 54% in group MISI and 50 ± 109% in group MILI. A total of 18 mares showed more than 10% PMNs in their post-insemination cytology; 8 of the mares belonged to group DI, 4 to group MISI and 6 to group MILI, respectively.

4. Discussion

In the present study, mares inseminated four times within 24 h, using one divided commercial semen dose, showed a significantly higher pregnancy rate compared to mares inseminated twice in the same time period. Significantly less mares experienced intrauterine fluid accumulation 24 h after the last insemination when inseminated at an hourly interval *versus* a 6 h interval.

These findings support some previous reports which demonstrated that more than one pre- or periovulatory insemination lead to higher fertility compared to one insemination per estrus cycle (fresh semen: 27, 28, 29, 37; frozen semen: 36). However, when comparing their results after two *versus* more than two inseminations, contrary to our results, Vidament et al. (36) did not find different pregnancy rates between the chosen insemination regimes (two *versus* more

than two inseminations in 24 h intervals, and two *versus* more than two inseminations in 48 h intervals) using frozen semen. Sieme et al. (27) demonstrated a significantly higher pregnancy rate when inseminating mares more than three times per cycle with chilled and frozen semen *versus* once per cycle, but no difference when comparing results after two *versus* more than two inseminations. An explanation for these divergences might be that, contrary to our study and to the frequent daily matings observed in the wild, these two studies used much longer time intervals (24 to 48 h) between inseminations, which were not always in close time frame to ovulation. The latter was the case in our study, where compared to group DI, groups MISI and MILI had their last preovulatory insemination 2 h and 12 h closer to estimated ovulation, respectively. Conversely, the postovulatory insemination regime in our study was standardized in all cases and performed within a few hours after ovulation. In addition, pregnancy rates in our study were not influenced by the time points of detected ovulations. This and the generally low preovulatory insemination timing differences between the three procedures suggest that the interval between the last preovulatory insemination and ovulation time might not have played a significant role in our study.

In addition, considering that each insemination in the aforementioned studies consumed one commercial semen dose, the results from our study model are particularly interesting because we used only one commercial dose with a minimum of 50×10^6 motile sperm cells per cycle. Despite using less semen per insemination, there was a 20% increase in pregnancy rate when comparing four *versus* two inseminations. This difference remained when evaluating only the frozen semen results which generally have lower semen numbers than fresh semen. An insufficient sample size could be the reason a significantly improved pregnancy rate between groups MISI and MILI compared individually to group DI was not found in the chilled semen results. Further studies with larger group sizes would be required to confirm this.

Our experiments attempted to mimic the natural conditions of feral horses, which belong to the group of polygynous mammals (18, 21). Multiple mating per estrus and not necessarily with the same stallion potentially generates direct material benefits and indirect genetic advantages for the mares. These seem to overshadow direct risks associated with multiple mating (2, 9) i.e. each copulation absorbing and distracting the stallion and representing a potential menace to the whole harem. However, and especially in the horse, detailed mechanisms of repeated uterine semen infusions and potentially linked sperm competition (8, 24) are not fully understood.

Our findings might be partly explained by the fact that mares of the MISI group showed less uterine fluid accumulation than those of the DI and MILI group, suggesting a better uterine clearance in mares inseminated several times in relatively short intervals. This phenomenon might well be the result of increased myometrial activity by way of oxytocin release. It is well known, from previous studies, that oxytocin is not only present in the semen of stallions (39), but increased pituitary oxytocin secretion is also found after mechanical stimulation of the vaginal walls, cervix and uterus as well as other sexual stimuli (e.g. stallion call, visual contact with stallion, active teasing) (20, 23). This remains true for AI, where a rapid onset of myometrial contractions has been observed (23). Indeed, Taverne et al. (32) also found the frequency of uterine contractions increased after a third insemination in the same estrus cycle.

Our decision to evaluate inseminations over a relatively long interval of 6 h was based on studies that had demonstrated ejaculates build semen reservoirs on the epithelial cells of the isthmus tubae uterinae (6, 16, 31, 35). It can be hypothesized that with longer intervals, fresh loads of spermatozoa would arrive in an oviduct already containing sperm cell reservoirs and conditions would allow selected sperm cells to participate in fertilization. This assumption could not be confirmed in our experiments.

Various studies have demonstrated the effects of inseminations on the intrauterine environment including a significant increase of PMNs (6, 11, 13, 34). It was also found that mares inseminated twice *versus* once did not develop increased incidence of uterine fluid accumulation as a clinical expression of endometritis (19, 26, 29).To the authors' knowledge, this is the first study investigating the influence of more than two inseminations per cycle on cultural and cytological alterations in the uterine body. Interestingly, we found no significant increase of positive culture and cytology results in mares inseminated four times compared to two, which might be the result of increased myometrial stimulation and other uterine defence mechanisms. Initially, we hypothesized that mares inseminated four times would present more inflammatory reactions and uterine fluid than those inseminated only twice. Such results would support the outcome of studies where "susceptible" mares retained more liquid in the uterus 2 h after insemination than resistant mares (e.g. 14). In other studies, an association was found between the failure of the chemotactic pathway in susceptible mares and lack of uterine contractions (e.g. 25), also leading to the conclusion that multiple inseminations should not be performed in susceptible mares. Results of these studies were not confirmed in

our experiments, probably due to selection before the study, where historically susceptible mares were excluded, and the effectiveness of the post-insemination management (e.g. only four mares required antibiotic therapy). Further investigations in mares without any treatment after multiple inseminations and in susceptible mares would be of interest.

A limitation of this clinical field study was the use of mares of different breeds, age and status, that were treated individually after insemination and ovulation, and owned by different private owners with variable management routines. Different types of semen (chilled *versus* frozen-thawed) were used with a high variance of semen quality and quantity. However, the investigators made substantial efforts to standardize test conditions and solid statistical methods were applied. Therefore, the results of this study warrant further investigation, i.e. confirmation of results with a higher number of homogenous animals and semen, as well as research on the underlying mechanisms that contributed to the findings.

5. Conclusion for practice

In conclusion, multiple periovulatory inseminations in close time frame to ovulation, an approach to natural conditions, resulted in significantly higher pregnancy rates along with significantly less uterine fluid accumulation compared to a standard periovulatory double insemination regime. In addition, in normal mares, multiple inseminations seemed to not have any clinically measurable negative effects on cytological or bacteriological results. Therefore, inseminations up to four times can be recommended without restriction in normal mares. Management of susceptible mares is more complex and a single insemination followed by treatment suiting the clinical symptoms should remain the gold standard in such cases until further similar studies on multiple inseminations demonstrate otherwise.

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Conflict of Interest Statement:

The authors declare no conflict of interest.

Author contributions:

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6. References

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Figures and Tables

Figure 1 Pregnancy outcome (in %) in mares inseminated at different intervals with one total dose of either chilled or frozen semen. Group DI (double insemination) was inseminated twice (*n* = 32). Group MISI (multiple insemination short interval) was inseminated four times in relatively short intervals (*n* = 25), and group MILI (multiple insemination long interval) four times in relatively long intervals (*n* = 18). There was no significant difference in pregnancy rates regarding semen type used.

Abb. 1 Trächtigkeitsresultate (in %) von Stuten, die in unterschiedlichen Intervallen mit jeweils total einer Portion Kühl- oder Gefriersperma besamt wurden. Stuten der Gruppe DI wurden zweimal besamt (n = 32), diejenigen der Gruppe MISI viermal in relativ kurzen Intervallen (n = 25) und Stuten der Gruppe MILI viermal in relativ langen Intervallen (n =

18). Bezüglich des verwendeten Samentyps (gekühlt oder tiefgefrorenen) gab es keine signifikanten Unterschiede der Trächtigkeitsraten.

Table 1 Numbers of examinations (*n*) and proportions (%) of 75 mares with positive ultrasonographic [presence of uterine fluid accumulation and mean fluid accumulation (mm ± SD)], bacteriological (presence of colonies) and cytological (presence of PMN-cells) evidence of endometrial symptoms after double insemination (group DI), multiple inseminations in a short interval (group MISI) or multiple inseminations in a long interval (group MILI).

Tabelle 1 Gesamtanzahl Stuten pro Untersuchungsgruppe (n) sowie Anzahl (n) und prozentualer Anteil (%) von Stuten mit positivem Ultraschallbefund (Vorliegen einer Flüssigkeitsansammlung, mittlere Flüssigkeitsansammlung in mm ± Standardabweichung), von Stuten mit bakteriologischem (Vorliegen von Kolonien) und zytologischem Hinweis (Vorliegen von PMN-Zellen) auf endometriale Symptome nach Doppelbesamung (Gruppe DI), Mehrfachbesamungen mit Kurzzeit-Intervallen (Gruppe MISI) oder Mehrfachbesamungen mit Langzeit-Intervallen (Gruppe MILI).

Parameter		**Double inseminations**	**Multiple inseminations**		
			Total	**MISI**	**MILI**
Uterine fluid accumulation (ultrasonographic evolution)	mares (n)	32	43	25	18
	positive mares (n)	11	9	1	8
	(%)	34.4	20.9	4.0	44.5
	mean fluid accumulation (mm ± SD)	6.2 ± 11.5	4.7 ± 10.7	0.8 ± 4.0	10.2 ± 14.4
Bacteriological evolution	mares (n)	21	39	22	17
	positive mares (n)	2	7	1	6
	(%)	9.5	18.0	4.6	35.3
Cytological evolution	mares (n)	22	38	21	17
	positive mares (n)	18	32	16	15
	(%)	81.8	81.6	76.2	88.2

Data are presented as number of horses (*n*) or means ± standard deviations (SD)