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Effect of different types of artificial insemination and semen dose on reproductive efficiency in mares

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Summary: The present study was designed to assess the effect of conventional artificial insemination (CAI) and deep intrauterine artificial insemination (DAI) using different concentrations of spermatozoa on the production of embryos in mares during two breeding season (BS). Seventy-four estrus cycles from 13 crossbred mares (Criollo/English Thoroughbred/Quarter horses) were evaluated, considering the location of semen deposition and doses of fresh semen. The animals were assigned to two treatments: treatment CAI (AI on the uterine body; n = 38) and treatment DAI (AI at the apex of the uterine horn, ipsilateral to an ovary with a preovulatory follicle (POF); n = 36). The animals in estrus were evaluated by ultrasonography to measure the dimensions of the POF. It was observed that POF was larger than 33 mm in diameter than uterine edema and a cervical opening grade of 3, 750 mg of deslorelin acetate (IM) was injected. Twenty-four hours later, AI was performed. The CAI mares received 500×10^6 progressively mobile spermatozoa (PMS) and the DAI mares received 250×10^6. Embryo collection occurred on days 7 or 8. The embryo recovery rate (ERR) was 68.4% (26/38) for CAI and 72.2% (26/36) for DAI. No significant difference was found for the ERR for either of the two AI methods or the sperm doses used. In conclusion, both AI methods can be used successfully in horses and it is possible to reduce the dose of PMS by 50% when using DAI without decreasing the fertility rate. The production of embryos may be performed several times during the breeding season in the same mare.

Keywords: deep intrauterine insemination, fresh semen, horse, fertility, reduced semen dose, reproduction

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Introduction

The use of biotechnologies in equine reproduction has significantly increased in recent years. Among the various biotechnologies, artificial insemination (AI) has been shown to result in reliable results, and is now the one used most (*Vianna* 2000). One of the major advantages of AI is a reduced demand in the stallion since a number of mares can be fertilized by a single ejaculate. The technique also reduces contamination of the mare's reproductive tract and the possibility of trauma at mating. The technique also favors the use of superior genetic material to improve the genetics of equine herds (*Vianna* 2000, *McCue* 2012).

The most frequently used method for AI wuth fresh semen in the horse is semen deposition in the uterine body. This is usually performed with a semen dose of 500×10^6 progressively motile spermatozoa (PMS) per insemination (*Squires* et al. 2000, *Sieme* et al. 2004, *Brinsko* 2006).

Technical advances in equine semen processing and in AI techniques have generated technologies capable to reduce the volume and the quantity of PMS used in the inseminating dose, by changing the semen deposition site in the uterus (*Sieme* et al. 2004, *Xavier* et al. 2009). In this case, the AI catheter is passed through the cervical canal but then transrectally guided to the apex of the ipsilateral to uterine horn ipsilateral to the ovary with the preovulatory follicle (POF), allowing reduction of the insemination dose (*Samper* 2001, *Sieme* et al. 2004, *Katila* 2005, *Lyle* and *Ferrer* 2005, *McCue* 2012). With these approach a semen dose of 100×10^6 PMS per insemination is used (*Xavier* et al. 2009), the minimum quantity being 50×10^6 (*Householder* et al., 1981). Another approach is AI by hysteroscopy, where deposition of semen is performed at the junction of the uterus-tubal papilla (*Blinsko* et al. 2003, *Sieme* et al. 2004, *Hayden* et al. 2012).

The development of this technique was motivated by the need to optimize AI (increase the number of inseminated animals) when a reduced number of spermatozoa are available (*Brinsko* 2006). However, this practice is recommended in situations requiring reduced insemination doses or where low-quality semen from elderly or subfertile stallions is used (*Squires* et al. 2000, *Katila* 2005, *Lyle* and *Ferrer* 2005, *Brinsko* 2006, *Xavier* et al. 2009). The use of deep intrauterine AI (DAI) with a reduced dose has also been indicated to reduce endometritis after insemination, a critical need for mares that have a history of reproductive problems (*Morris* et al. 2000, *Sieme* et al. 2004).

Hypothetically, the semen deposition in apex of the uterine horn (deep intrauterine AI) may result in similar embryo recovery rate by use of half dose semen compared to AI in the uterine body. The objective of the present study was to compare two techniques of conventional AI (CAI, semen deposition in the uterine body) and deep intrauterine AI (DAI, semen deposition in the apex of uterine horn) using different concentrations of sperm cells and determining subsequent fertility as embryo recovery rates (ERRs).

Materials and Methods

The study took place during two breeding seasons, October 2014 to 2015 and October 2015 to March 2016. During this period, 74 estrous cycles from 13 crossbred mares (Criollo/English Thoroughbred/Quarter horses) at the Experimental Farm of Pontifícia Universidade Católica do Paraná (25°39'31'' South, 49°18' 32'' West) were assessed. The mares were, on average, 7.9 years old (range 4 – 18), with an average body condition score (BCS) of 3.0 (1 = thin, 5 = obese; *Henneke* et al. 1983), and an average weight of 388 kg (300 – 450). The animals grazed on Tifton forage grass during the summer and Lolium multiflorum during the winter. In addition, the animals were supplemented with mineral salt (Nutron Pro Horse Sal 60, São Paulo, Brazil) and were provided water ad libitum. Only clinically normal mares (with good reproductive background) and with ovarian cyclicity were used. The mares were monitored for 3 days after AI to check for the presence of possible fluid mating induced endometritis. After 72 hours the mares were free of AI fluids. No medication was needed. Daily, during the BS, the mares were submitted to transrectal examination of the genitalia and to transrectal ultrasonography with the aim to determine the presence of follicles, and endometrial edema and the degree of cervical opening (1–3). Mares were assigned to two different treatments. CAI (n = 38; with deposition of 500×10^6 PMS in the uterine body) and DAI (n = 36, with deposition of 250×10^6 PMS in the apex of the ipsilateral side of the POF). The mares were inseminated alternately, using either CAI or DAI for each BS. At the detection of a preovulatory follicle with ≥ 33 mm in diameter (*Gastal* et al. 2008), uterine edema (2nd or 3rd degree; *Ley* 2006), and a cervical opening grade of 3 [by touch across the floor of the ampulla rectalis, with cervical opening grade 1 indicating a closed cervix (thickness of a finger and rigid), 2 indicating intermediate opening (thickness of two fingers and less rigid), and 3 indicating open (three fingers and relaxed)], the animal was injected with 750 mg of deslorelin acetate (Sincrorrelin, Ourofino Saúde Animal, Cravinhos, São Paulo, Brazil) to induce ovulation (only this treatment). Twenty-four hours after the deslorelin acetate injection, mares were inseminated (*Sieme* et al. 2004) and 24 hours after AI, ovulation was monitored by ultrasonography. Mares that had not ovulated were used in the next estrus. Ovulation was detected (ultrasonography) in the absence of previously monitored POF and presence of Corpus luteum. Mares On day 8 after AI, recovery of the embryo by uterine lavage was performed. After uterine flushing, the mares was injected with 5 mg of dinoprost trometh amine (IM) (Lutalyse, Zoetis Industria de Produtos Veterinarios Ltda, São Paulo, Brazil) for induction of luteolysis and restart of the estrous cycle.

Stallion, semen collection and evaluation, and AI

A 17-year-old Appaloosa stallion (BCS 3.5, 500 kg, with tested fertility) was used. Semen was collected twice a week, using a Botucatu artificial vagina, using a water temperature around 42 °C. The filling temperature of the artificial vagina was around 50 °C, so that at the time of semen collection reached a temperature between 42 to 45 °C. After collection of the ejaculate, it was diluted at a 1:1 ratio with Botu-Semen (Botupharma, Botucatu, São Paulo, Brazil). Sperm motility in raw semen was between 70 and 80 %, the concentration of the raw semen ranged between 150 and 250×10^6/mL, and vigor was 3 to 4 (1 = poor; 5 = strong). For conventional AI (long and sterile gloves, via transvaginal), were used. In AIC, the dose of 500 million spermatozoa per mare was used. The semen volume varied according to quality and concentration of the semen in that day. The inseminante volume ranged from 10 to 20 mL with the addition of milk-based diluent at the ratio of 1:1. Semen was deposited in the uterine body by a rigid and specific catheter for insemination (64 cm in length), coupled a 20 mL syringe. For deep AI, 3 mL syringes coupled to a 75 cm long flexible AI pipette (Minitube, Tiefenbach, Germany) was repeated as for conventional AI, except that after passage of the catheter through the cervix, transrectal palpation was performed to direct the catheter to the apex of the ipsilateral uterine horn of the POF. For DAI the semen was centrifuged and resuspended with 2 ml of milk-based diluent with 250 million spermatozoa.

Embryonic recovery

Embryo recovery was always performed on day 8 after AI, with a two-way uterine catheter (Equine Folley, Minitub, Germany, 28 or 32 FR). At one end, it was coupled to a flask with Ringer's lactate solution (Fresenius Kabi Brazil Ltd., Barueri, São Paulo State, Brazil; Fresenius Kabi F0312; batch 0214; 273.20 mosmol/L; pH 6.0 – 7.5). At the other end, it was coupled to a filter for the collection of the embryo (Minitub, Tiefenbach, Germany). For embryo recovery, 1 L of Ringer's lactate solution were infused for three times of 3 L. After uterus washing, the embryo in the filter was retrieved. The embry-

Table 1 Embryo recovery rate, number of cycles and collections evaluated in mares inseminated with fresh semen diluted according to the semen

Treatments	Evaluated Cicles (n)	Embryo Recovery n (%)	Embryo stage n (%)	Embryo quality grade n (%)	Uterine washes for embryo recovery n (%)
Convencional AI (with 500x10^6 sptzs)	38	26/38 (68.4)^a	Bex=24/26(92.3) Mo=2/26(7.7)	Grade 1= 24/26(92.3) 2= 1/26(3.8) 3= 1/26(3.8)	1°. 12/26(46.1)^a 2°. 11/26(42.3)^a 3°. 3/26(11.5)^b
Deep AI (with 250x10^6)	36	24/36 (66.6)^a	Bex=24/24 (100,0)	Grade 1= 23/24(95.9) 2= 1/24(4,1)	1°. 12/24(50.0)^a 2°. 5/24(20.8)^a 3°. 7/24(29.1)^a
Total	74	50/74 (67.5)	Bex=48/50(96.0) Mo=2/50(4.0)	Grade 1=47/50(94.0) 2=2/50(4.0) 3= 1/50(2.0)	1°.24/50(48.0)^a 2°.16/50(32.0)^{ab} 3°. 10/50(20.0)^b

Different letters in the same column show statystical difference (P<0.05)

os were identified using a stereoscopic zoom lens (70–270) (Nikon, Japan), and evaluated related to quality [grade 1 = excellent, 2 = good, and 3 = poor] (*McCue* and *Squires* 2015), cryopreserved, and stored in liquid nitrogen.

Statistical Analysis

The ERRs between groups were calculated and the differences between the groups were compared by a SAS-assisted chi-square test (SAS Institute, Cary, NC, USA, 2014). For all analyses, $P < 0.05$ was considered statistically different.

Results and Discussion

Because of equideoculture growth (around US 4.9 billion/year in Brazil), embryo production has received special attention in recent years. Because embryo transfer is less used in horse than in cattle, improvement of the methods is still required. The present study addressed two methods of AI, varying in location of semen deposition in the uterus and the dose of PMS, aiming to optimize the production of equine embryos.

No difference in ERR between the CAI or DAI groups (Table 1; $P > 0.05$) was observed. CAI resulted in a 68.4% embryo recovery rate, only 1.8% higher than the DAI method (66.6%). On one hand, CAI proved to be easier and less laborious to perform when depositing the semen in the body of the uterus, requiring only transvaginal palpation. The DAI methodology required a longer catheter and more professional skill, more work at the inseminatory stage and skilled manipulation of the long catheter to reach the apex of the ipsilateral horn of the POF, while taking care to not hurt the endometrium.

Buchanan et al. (2000), tested concentrations of 5, 25, and 500×10^6 PMS in AI with fresh, diluted semen. Spermatozoa (5 and 25×106) were deposited at the apex of the ipsilateral uterine horn of the POF and 500×10^6 in the uterine body. They obtained 90.0% PR when the PMS were deposited in the uterine body and 57.0% when at the apex of the horn ($P < 0.05$); when they compared doses of 5×10^6 versus 25×10^6, no difference was found in the PR. In comparison with our data, there is a large difference in the PR after CAI. We suggest that the different results may be because in our study was used many reproductive cycles (two breeding seasons), where were included (evaluated) good results obtained in the most favorable months for light, forage, temperature, and other factors with results obtained during less favorable months. In the present study, the mares were subjected to successive cycles of embryo recovery, restarting of the estrous cycle, and re-insemination. We believe that the continuous use of these animals during the BS may have led to lower fertility of the animals. *Aurich* et al. (2011) reported significant changes in the ERR in successive collection procedures, but there was no increase or reduction in ERR as the number of embryo collections increased. In a study similar to ours, *Xavier* et al. (2009) compared 72 cycles of crossbred mares and found conception rates of 42.9% and 45.9% ($P > 0.05$) for CAI and DAI, respectively, using doses of 100×10^6 and 20×10^6 PMS, respectively, lower rates than in our study. It is possible that the higher temperatures in the southeast region of Brazil may have exerted some influence on the ERR because the temperatures under which our study was conducted were cooler. Our study was conducted in southern Brazil, under a temperate climate with four well-defined seasons of the year and where the temperature does not exceed an average of 22 degrees Celsius in the warmer months. On the other hand, *Sieme* et al. (2004), using CAI and DAI with 50×10^6 and 300×10^6 PMS/dose (fresh semen), demonstrated no differences in PR. They concluded that artificial insemination techniques (CAI, DAI, or hysteroscopic AI) or semen parameters (volume, inseminant dose, PMS concentration) did not significantly influence PR. The reports of *Sieme* et al. (2004), corroborated the findings of the present study, where there were no differences in CAI with a high number of PMS compared with a lower number (50% less PMS) in DAI (Table 1). The report of *Sieme* et al. (2004) further addresses that even in mares with problems associated with AI, the PMS concentration or AI method had no influence on the results. Our data allow us to conclude that the semen deposition site (CAI or DAI) and the concentration do not affect the fertility rates, but CAI is easier and faster to perform. However, when the availability of semen is restricted, DAI should be used because it is efficient even at low doses of PMS and allows for insemination of more mares to the same stallion (*Hayden* et al., 2012).

In the present study, embryo development stage, embryo quality, the number of uterine washes per embryo recovery process, and ovulation hours (after injection of ovulation inducer) were also evaluated. A high percentage of embryos were of the highest degree quality (94.0%) (according to International Embryo Transfer Society) and were at the expanded blastocyst stage (96.0% of the total) when recovered. On the first wash, 48.0% of the embryos were recovered, compared to the subsequent attempts ($P < 0.05$). Interestingly, 22.8% of the embryos were recovered only after the third wash, indicating that even for professionals it was difficult to recover all embryos in the two previous washes. (Table 1).

From total 74 cycles, 43 cycles generated 27 positive collections (62.8%) in the first BS (2014–2015), and 31 cycles (23 positive, 74.1%) (2015–2016) in second. In the first BS, 32.6% of embryos were obtained by CAI (n = 14) and 67.4% by DAI (n = 29) and in the second BS there were 24 positive (CAI) and 7 (DAI). There was 5.69 cycles per mare (variation of 1–20 cycles) and ovulation rate was 90%; the ovulation rate within 48 hours after the deslorelin injection resulted in 85.0% and the AI/ovulation interval was 24 hours. The ERR difference between two stations was 62.8% in favor to first.

It can be concluded that the AI methods used in the present study can be applied successfully for artificial insemination of horses; deep intrauterine AI allowed the use of lower numbers of spermatozoa, while maintaining a good embryo recovery rate; it is also possible to use mares for embryo production repeatedly during the same breeding season.

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