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Summary
One insemination with $400x10^6$ total sperm into the uterine body close to the cervix (BOD) results in higher fertility than:
1) 4 inseminations within 24 h, in BOD, with $100x10^6$ total sperm at each insemination,
2) 1 insemination with $50x10^6$ total sperm in BOD or at the tip of the horn, ipsilateral to the preovulatory follicle, by a rectally-guided approach.

Introduction

We demonstrated that fertility with equine frozen semen was improved when the mares were inseminated more than once in a cycle before ovulation and that doses with $300x10^6$ total sperm resulted in higher fertility than doses of $150x10^6$ total sperm per AI (Vidament et al., 1997). The explanation of the positive effect of multiple inseminations is still unclear.

Numerous studies with either low dose of spermatozoa and/or deep uterine AI have been conducted recently with equine semen (reviews: Ball 2004, Morris 2004). Unfortunately, some of them are inconclusive because: 1) the comparison between groups is confounded with too many factors, 2) number of cycles and stallions are insufficient, 3) freezing technique is unspecified or invalid. There is a lack of comparison between these techniques and well established standard procedures (Sieme et al., 2004).

Three studies were carried out: 1) to compare multiple inseminations during 24 h versus once, and 2) to compare 2 sites of insemination: the uterine body close to the cervix (BOD), and the tip of the horn ipsilateral to the preovulatory follicle by a rectally-guided approach (TIP).

Material and Methods

If not specified, sperm numbers are expressed in total number.

Semen from saddle stallions was frozen (freezing extender: INRA82 + 2% egg yolk + 2.5% glycerol, final concentration of semen in 0.5 ml straws: $100x10^6$ spermatozoa per ml) (Vidament et al., 2000). Post-thaw rapid and progressive motilities of used ejaculates was 55% and 40%, respectively.

In experiment 1, mares were inseminated in BOD, 1 or 4 times, with semen from 4 different stallions. Two pairs of stallions were used: AB or CD. Mares were injected with hCG at 10.00 a.m. on Day 0. In the “1x400” group, mares were inseminated once with $400x10^6$ spermatozoa ($200x10^6$ from one stallion and $200x10^6$ from the second stallion of the pair) at 10.00 a.m. on Day 1. In the “4x100” group, mares were inseminated 4 times with $100x10^6$ spermatozoa at each AI: at 5.00 p.m. on Day 0 and at 7.00 a.m., 1.00 p.m. and 5.00 p.m. on Day 1, either in the AABB order or in the BBAA order. Paternity of delivered foals was determined.

In experiment 2, mares were inseminated twice with $400x10^6$ spermatozoa per AI, 24h apart, with the last AI 12 h before ovulation. Semen of two stallions was used. The inseminations were performed either in BOD or at TIP with a special catheter (Minitüb, Germany).

In experiment 3, mares were inseminated once, 12h before ovulation, with either $400x10^6$ spermatozoa in BOD, or $50x10^6$ spermatozoa in BOD or $50x10^6$ spermatozoa at TIP. Semen of 4 stallions was used. The inseminations in the two groups "50" were performed with a special catheter (IMV, France).

Differences between fertilities were tested with a categorical data analysis, considering effects of treatment and stallion (CATMOD, SAS, USA). Statistical significance was considered at $P < 0.1$ (Amann, 2005).

Results

Experiment 1. Per-cycle fertility was significantly higher in the "1x400" group (57%, 16/28) than in the "4x100" group (34%, 8/24) (P=0.08). Among pair of stallions AB, per-cycle fertility was 60% (9/15) in the "1x400" group, 33% (2/6) and 29% (2/7) in the "4x100" group for the AABB and the BBAA order, respectively. Among pair CD, per-cycle fertility was 54% (7/13) in the "1x400" group, 33% (2/6) and 40% (2/5) in the "4x100" group for the CCDD and the DDCC order, respectively. In the "1x400" group, the resulting foals were mainly from stallion A (7/8) in the AB pair and from stallion D (6/6) in the CD pair. The foals from the "4x100" group were very few in each pair (n=4 and 3) but all the stallions have sired foals.

Experiment 2. Per-cycle fertility was similar: 63% (17/27) in the "2x400 BOD" group versus 59% (16/27) in the "2x400 TIP" group (P>0,1). For stallion E, it was 67% (10/15) and 39% (5/13), respectively. For stallion T, it was 58% (7/12) and 79% (11/14). No embryonic loss was observed between 14 and 35 days post-ovulation.

Experiment 3. Due to the overall low fertility of this experiment, the more fertile stallion was used more intensively to produce foals for the next breeding season. So the results had to be adjusted by stallions. Per-cycle fertility were significantly different between groups (P=0.06) (Table 1), with higher significance if the two groups "50" were pooled and compared to the "400" group (26% (78) versus 46% (39), respectively, P=0.02).

Discussion

The negative effect of 4 AI versus 1 AI in experiment 1 could be explained by the high number of interventions in the uterus during 24h that could have resulted in a more acute inflammation post-AI. Another explanation could be mechanical with losses of spermatozoa due to the contractions of the uterus after each AI and to losses in the 4 catheters used, resulting in a total lower number of spermatozoa. It was striking to observe that the foals from the "1x400" group were mostly from only one stallion per pair. This heterospermic experimental scheme could be very powerful to compare fertility between 2 stallions (Amann, 2005).

In experiment 2, fertility with $400x10^6$ spermatozoa was similar, whatever insemination site used. In experiment 3, fertility with $50x10^6$ was similar, whatever insemination site used. These results agree with some recent papers in which no improvement in fertility was found

for TIP versus BOD with different doses of frozen semen (Squires et al., 2003; Sieme et al., 2004; review in Ball, 2004; Morris, 2004).

AI with 50.10^6 resulted in much lower fertility than AI with $400x10^6$ which is consistent with our previous results.

Discrepancies between publications could result from factors like: proper fertility of stallions used, fertility of freezing technique used, number of AI, number of spermatozoa per AI and interval between AI and ovulation.

Table 1 : Per-cycle fertility in Experiment 3 [a]

	Insemination site and number of sperm per AI		
	Uterine body (BOD) $400x10^6$ spz	Uterine body (BOD) $50x10^6$ spz	Horn tip (TIP) $50x10^6$ spz
Stallions			
BAL	3/8 (38%)	2/8 (25%)	2/8 (25%)
DOL	9/15 (60%)	6/15 (40%)	4/15 (26%)
INT	2/8 (25%)	1/8 (13%)	2/8 (25%)
HUR	5/8 (63%)	2/8 (25%)	2/8 (25%)
Total	19/39	11/39	10/39
Adjusted %	46% a	26% b	25% b

[a] Different letters (a, b) within the row denote differences (P=0.06)

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