Pferdeheilkunde 24 (2008) 1 (Januar/Februar) 96-98

Sperm distribution in the oviduct and uterus of mares within two hours after artificial insemination

Sandra M. Fiala[1], Maria Ines M. Jobim[2], Terttu Katila[3], Ricardo M. Gregory[2] and Rodrigo C. Mattos[2]

Departamento Morfologia, Instituto de Biologia-UFPel, Pelotas-RS[1], REPROLAB, Departamento Medicina Animal, Faculdade de Veterinária-UFRGS, Porto Alegre – RS[2] and Department of Production Animal Medicine, University of Helsinky, Finland[3]

Summary

Our objective was to determine the sperm distribution in the uterus and the number of sperm cells that reach the oviducts after 0.5, 1, 1.5 and 2 h of artificial insemination (AI). Thirty mares with a dominant follicle > 35 mm in diameter and no bacterial growth or neutrophils detected in uterine smears were inseminated with a total dose of 500 x 10^6 cooled sperm (25 x 10^6 ml-1 spermatozoa diluted in skim milk) stored at 4°C for 18 to 22 h until use. The mares were slaughtered 0.5 (n = 3), 1 (n = 10) 1.5 (n = 4) or 2 h (n = 13) after AI. The oviducts were separated from the uterus and flushed with phosphate-buffered saline. Spermatozoa were detected in oviduct flushes from 0.5 h onwards after AI and sperm cells were observed in flushes from oviducts in 66.7% of the mares regardless the time of AI. No differences were observed either in the number of sperm cells between the groups or in the number of mares with sperm cells in the oviducts. No differences were observed in the number of mares presenting spermatozoa in the oviduct ipsilateral to the dominant follicle when compared with the contralateral oviduct or in the number of spermatozoa that reached the tubes. Sperm cells were found in the uterus by histological examination in 88.8% of the mares slaughtered 1 h after AI and spermatozoa were located in the uterine glands in 70% of the mares. It can be concluded that sperm cells reach the tube already 0.5 h of insemination. The uterine glands are a possible reservoir for spermatozoa in addition to the oviductal isthmus and the uterotubal junction.

Keywords: artificial insemination, mare, sperm transport, oviduct, uterus, reproduction

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Verteilung der Spermien in Eileiter und Uterus in den ersten zwei Stunden nach künstlicher Besamung

Ziel der Arbeit war die Bestimmung der Spermienverteilung im Uterus und die Ermittlung der Anzahl derjenigen Spermien, die den Eileiter innerhalb von 30, 60, 90 und 120 min nach instrumenteller Samenübertragung erreichen. Zum Einsatz kamen 30 Stuten ohne Nachweis von neutrophilen Granulozyten im Endometriumausstrich und kulturellem Bakteriumwachstum aus der Tupferprobe. Die Insemination erfolgte, sobald ein dominanter Follikel (>35mm im Durchmesser) vorlag mit einer Gesamtanzahl von 500 x 10^6 gekühlten Spermien (25 x 10^6 ml-1 Spermatozoen in Magermilchverdünner), die vor Gebrauch für 18 bis 22 Stunden bei 4°C gelagert worden waren. Die Stuten wurden 30 min (n=3), 60 min (n=10), 90 min (n=4) und 120 min (n=13) nach Insemination geschlachtet. Die Eileiter wurden vom Uterus abgetrennt und mit Phosphatpuffer gespült. Spermatozoen konnten in Eileiterspülproben ab 30 min nach Insemination nachgewiesen werden. Unabhängig vom Zeitpunkt der Insemination wurden in 66,7% der Proben Spermien ermittelt. Es bestanden keine Unterschiede bezüglich der Spermienanzahl innerhalb der Gruppen, der Anzahl der Stuten, in deren Eileiter Spermien gefunden werden konnten, der Anzahl der Spermien in den zum dominanten Follikel ipsilateralen Ovidukt, verglichen mit dem kontralateralen Organ, oder der Anzahl derjenigen Spermien, die den Eileiter erreicht hatten. In 88,8% der Stuten, die eine Stunde nach Insemination geschlachtet worden waren, konnten histologisch Spermien im Uterus nachgewiesen werden, zu 70% waren sie innerhalb der Uterindrüsen lokalisiert. Zusammenfassend wird festgestellt, dass Spermien bereits innerhalb von 30 min die Eileiter erreichen. Die Uterindrüsen dienen möglicherweise als Reservoir für Spermien, zusätzlich zum Eileiteristhmus und dem uterotubalen Verbindungsstück.

Schlüsselwörter: instrumentelle Samenübertragung, Pferd, Spermientransport, Eileiter, Uterus, Reproduktion

Introduction

Spermatozoa are deposited directly into the uterus of the mare during mating or artificial insemination (AI). Frequent uterine contractions after AI carry sperm back and forth between the uterine body and the horn tips (*Katila* et al. 2000). It is very likely that some of these sperm gain access to the oviducts very soon after AI, but the time of the first appearance of sperm in the oviducts of the mare has not been documented. Within 1 h after breeding, seminal compounds were found in the oviduct of the mare (*Mann* et al. 1956) and sperm were detected in the oviducts 2 h after insemination with fresh or frozen-thawed semen (*Bader* 1982). Large numbers of sperms were present in the oviducts 4 h after insemination, but there was a reduction in the number of sperms recovered from the oviducts 6 h after insemination (*Bader* and *Krause* 1980). These observations suggest a gradual elimination of sperms from the oviduct and the cessation of further sperm transport through the uterus (*Bader* 1982). Pregnancy rates were significantly lowered when mares were flushed within 2 h after AI, but uterine lavage performed at 4 h had no adverse effect on fertility (*Brinsko* et al. 1990 and 1991).

Sperm reservoirs are sites within the female tract that accumulate and maintain a population of viable spermatozoa for an extended period. An intimate contact of sperm with the luminal epithelium in those locations is regarded as important for maintenance of sperm viability and function (*Smith* and

Yanagimachi 1990). After breeding, spermatozoa from the different mammals are transported through the uterus, forming a sperm reservoir in the uterotubal junction (UTJ) and the adjacent 1–2 cm of the isthmus (*Viring* 1980, *Rodríguez-Martínez* 2001, *Brandt* et al. 2004, *Chatdarong* et al. 2004, *Rijsselaere* et al. 2004, *Steinhauer* et al 2004, *Suarez* and *Pacey* 2006). In the cat and the bitch, the uterine glands were described as a sperm reservoir before ovulation (*Chatdarong* et al. 2004, *Rijsselaere* et al. 2004). In the mare, during the preovulatory period, the oviductal isthmus (*Thomas* et al. 1994) and the uterotubal junction (*Scott* et al. 2002) have been suggested as sperm reservoirs.

This study aimed to examine sperm distribution in the uterus and to determine the earliest time of the entrance of spermatozoa into the oviducts. Also the numbers of sperm cells that will reach the oviducts after 0.5, 1, 1.5 and 2 h of AI with cooled stored semen were determined.

Materials and methods

Animals

Thirty mixed-breed mares in estrus were selected from a population of horses sent to slaughter in an abattoir located at parallel 32o south in southern Brazil. Semen was collected from a fertile stallion that was housed 20 km from the abattoir.

Clinical examinations and insemination

Candidate mares were examined for reproductive soundness and only clinically normal mares with negative cytology (absence of PMNs) and negative cultures (no growth or insignificant contaminant isolates) were used in this study. Soon after collection using an artificial vagina, semen was diluted in skim milk (Molico, Nestlé, São Paulo, Brazil) to 25x 10^6 ml^{-1}. The AI volume was 20 ml and sperm numbers 500 x 10^6. Semen was kept in a cooling transport container (Equitainer, Hamilton Research Inc., Hamilton, MA, USA) at 4 °C for 18 to 22 h until its use.

Experimental procedures

Mares were slaughtered 0.5 (n = 3), 1 (n = 10) 1.5 (n = 4) or 2 h (n = 13) after AI. Internal reproductive tracts were recovered within 10 min after slaughter. The uterus was sectioned and a portion from uterine body, each horn and each uterotubal junction (UTJ) was obtained after macroscopic examination. The samples were fixed in Bouin's solution and processed for histological examination. The slides were stained with hematoxylin-eosin and analyzed under a light microscope (400x). The slides were evaluated for spermatozoa in the luminal epithelium, uterine glands and UTJ.

Oviducts were separated from the uterus, placed in a dish and flushed with 1 ml phosphate-buffered saline (PBS) from the infundibulum toward the isthmus. The flush was stirred and a sample (50 μl) of each tubal flushing was placed in a Neubauer chamber. Each sperm counted in the chamber represented 5 sperm/mm^3.

Statistical analysis

To normalize the data, the number of sperm cells in flushes was transformed to log10 (y + 10). Data were analyzed using ANOVA. Numbers of spermatozoa in the flushes of the oviducts were considered as dependent variables and time before artificial insemination as independent variable. The Tukey-'s test was used to compare the means. The number of mares with and without sperm cells in the oviducts was evaluated by Chi-Square analysis. Values were considered to be statistically significant at $P < 0.05$.

Results

Spermatozoa were detected in oviductal flushes at 0.5 h after insemination. Differences between groups were observed neither in the number of sperm cells (P = 0.27) flushed nor in the number of mares with sperm cells in the oviducts (P = 0.26) (Table 1). Sperm cells were observed in flushes from oviducts in 66.7% of the mares regardless the insemination moment. No differences were observed in the number of mares presenting sperm cells (P = 0.41) in the oviduct ipsilateral to the dominant follicle when compared with the contralateral oviduct or in the numbers of spermatozoa that reached (P = 0.27) the tubes (Table 2). Sperm cells were observed in the uterus by histological examination in 88.8% and 76.9% of the mares slaughtered 1 and 2 h after insemination, respectively. One or two hours after AI, spermatozoa were

Table 1 The number and percentage of mares presenting sperm cells in the oviducts and mean and standard deviation of sperm cells flushed from the oviducts 0.5, 1, 1.5 and 2 h after artificial insemination (AI).
Anzahl und prozentuale Häufigkeit derjenigen Stuten, bei denen Spermien in den Eileitern nachgewiesen werden konnten, und Mittelwert mit Standardabweichung der Spermien in Eileiterspülproben 30, 60, 90 und 120 min nach Insemination.

Time after AI	Mares		Sperm cells [log (y+10)]
	n	n (%) with sperm	Mean ± SD
0.5 h	3	2^a (66.6)	3.05^b ± 1.82
1.0 h	10	8^a (80.0)	3.69^b ± 1.46
1.5 h	4	1^a (25.0)	1.79^b ± 1.58
2.0 h	10	7^a (70.0)	3.32^b ± 1.64

$x^2 = 3.975$; P= 0.26, values with common subscript letter (a) represent no significant difference. Values with a common subscript letter (b) represent no significant difference (P = 0.27).

Table 2 The number and percentage of ipsi- and contralateral oviducts presenting sperm cells and mean and standard deviation of sperm cells flushed from the oviducts after artificial insemination.
Anzahl und prozentuale Häufigkeit von zum dominanten Follikel ipsi- und kontralateralen Eileitern, in denen Spermien gefunden wurden, und Mittelwert mit Standardabweichung der Spermien in der Eileiterspülprobe nach Insemination.

Oviduct	Total n	with sperm		Sperm cells [log (y+10)] Mean ± SD
		n	%	
Ipsilateral	27	14^a	51.8	4.26^b ± 0.40
Contralateral	27	11^a	40.7	4.10^b ± 0.29

located in just a few uterine glands, without PMN's presence in 70% and 61.5% of the mares, respectively.

Discussion

This study demonstrated the presence of spermatozoa in the oviducts already 0.5 h after AI. It is possible that the spermatozoa enter the oviducts even earlier as in the swine (*Baker* and *Degen* 1972), but we did not have the possibility to examine mares immediately after AI.

In the present report, the number of sperm cells observed 2 h after AI in the oviducts and at the UTJ junction was similar to the observation by *Fiala* et al. (2007) using the same sperm concentration and the same examination time after insemination.

The number of sperm cells delivered into the tubes was similar within 0.5 and 2 h after insemination. The number of mares with spermatozoa and the sperm population observed into the contra- and ipsilateral tubes of the dominant follicle were similar in the first two hours after insemination. These findings demonstrate that probably a first wave of sperm transport occurred immediately after seminal deposition aided by contractions of the myometrium associated with AI (*Katila* et al. 2000). Sperm cells seem to be directed independently of a hormonal guidance or a thermotaxis effect (*Bahat* and *Eisenbach* 2006). It is possible that this wave is responsible for the sperm population in the reservoirs in addition to the presence of the first spermatozoa in the oviducts.

The mares used in this study were clinically normal but their reproductive history was unknown. Spermatozoa were detected in more than 66% of the mares. Probably the mares without sperm cells in the oviducts can belong to a subfertile group, with impaired uterine contractility and sperm transport.

Spermatozoa were located in uterine glands 1 h after the insemination. Maybe also in the mare, the uterine glands can act as sperm reservoirs like the oviductal isthmus (*Thomas* et al. 1994) and the uterotubal junction (*Scott* et al. 2002). In the queen, the UTJ and the uterine crypts acted as sperm reservoirs before ovulation whereas the isthmus was a sperm reservoir around the time of ovulation (*Chatdarong* et al. 2004). In the bitch, histology revealed that the spermatozoa were mainly located in the uterine glands and at the UTJ, while very few spermatozoa were detected in the uterine tube. The uterine glands and the UTJ might act as sperm reservoirs in the bitch and sperm transport in the genital tract is affected by the time of AI in relation to ovulation. They may also play an important role as an initial selection mechanism for the spermatozoa that will reach the fertilization site (*Rijsselaere* et al. 2004).

It is concluded that in the mare sperm cells reach the oviductal tubes 30 minutes after semen deposition. The uterine glands are a possible reservoir for spermatozoa like the oviductal isthmus and the uterotubal junction.

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Dr. Sandra Fiala
Departamento Morfologia
Instituto de Biologia-UFPel
Pelotas-RS
Brazil
sandrafiala@yahoo.com.br