# Scientific Notes

# Bovine sperm cell motility after incubation in follicular fluid

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Abstract – The objective of this work was to evaluate sperm cell motility after intrafollicular artificial insemination (IFAI) in vivo or after incubation in follicular fluid in vitro. In the in vivo experiment, IFAI was performed, followed by the recovery of follicular content 1 to 4 hours later, in order to assess sperm motility. In the in vitro experiment, spermatozoa from a pool of commercial frozen-thawed semen were evaluated for their kinetics after incubation for 1 or 3 hours, either pure (pool, control group) or in follicular fluid (FF). A low motility of sperm cells was observed in the FF samples, both in vitro and in vivo. In vitro, the main parameters negatively affected in the sperm cells incubated in FF, compared with the control, were: total motility (TM), progressive motility (PM), curvilinear distance, and straightness, after 1 hour of incubation; and TM, PM, average path velocity, and curvilinear velocity after 3 hours of incubation. The ovarian follicle and follicular fluid do not provide a suitable environment to maintain bovine sperm cell motility.

Index terms: infertility, insemination, intrafollicular artificial insemination, spermatozoa.

## Motilidade de espermatozoides bovinos após incubação em fluído folicular

Resumo – O objetivo deste trabalho foi avaliar a motilidade de espermatozoides após inseminação artificial intrafolicular (IAIF) in vivo ou após incubação em fluído folicular in vitro. No experimento in vivo, a IAIF foi realizada com posterior recuperação do conteúdo folicular 1 a 4 horas depois, para avaliação da motilidade espermática. No experimento in vitro, os espermatozoides de um pool de doses comerciais de sêmen congelado/descongelado foram avaliados quanto à sua cinética após incubação por 1 ou 3 horas, puros (pool, controle) ou em fluído folicular (FF). Observou-se baixa motilidade dos espermatozoides nas amostras de FF, tanto in vitro como in vivo. In vitro, os principais parâmetros negativamente afetados nas células espermáticas incubadas em FF, em comparação ao controle, foram: motilidade total (MT), motilidade progressiva (MP), distância curvilínea e retilinearidade, após 1 hora de incubação; e MT, MP, velocidade média da trajetória e velocidade curvilínea, após 3 horas de incubação. O folículo ovariano e o fluído folicular não proporcionam ambiente adequado para manutenção da motilidade dos espermatozoides bovinos.

Termos para indexação: infertilidade, inseminação, inseminação artificial intrafolicular, espermatozoides.

Intrafollicular artificial insemination (IFAI) is the introduction of sperm cells inside a pre-ovulatory follicle through a transvaginal intrafollicular injection (Lucena et al., 1991). This assisted reproductive technique was first performed in women, as an alternative infertility treatment, when there was no response to in vitro fertilization (Lucena et al., 1991; Lucchini et al., 2012). In domestic animals, IFAI was initially carried out in mares to overcome uterine abnormalities and potentiate the use of frozen-thawed semen (Eilts et al., 2002). In cattle, there is only one known report of IFAI increasing the pregnancy rate of cows with low fertility records due to heat stress (López-Gatius & Hunter, 2011). Since this previous study did not investigate the interaction between bovine sperm cells and follicular fluid (FF) after IFAI, the hypothesis that sperm deposition inside pre-ovulatory follicles allows the maintenance of sperm cell viability still needs to be confirmed.

The objective of this work was to evaluate sperm cell motility after intrafollicular artificial insemination in vivo or after incubation in follicular fluid in vitro.

The in vivo experiment was performed in Pelotas, in the state of Rio Grande do Sul, Brazil, and was

approved by the experimental animal ethics committee of Universidade Federal de Pelotas. Six adult cyclic, non-lactating Jersey cows, grazing on natural pasture, were subjected to the following synchronization protocol: on day 0, intramuscular injection of 2 mg estradiol benzoate (Gonadiol, Zoetis Brasil, Campinas, SP, Brazil) and insertion of an intravaginal device (IVD), containing 1 g progesterone (DIB, Zoetis Brasil, Campinas, SP, Brazil), which was kept for eight days. When the IVD was removed, 0.5 mg sodium cloprostenol (Ciosin, MSD Saúde Animal, São Paulo, SP, Brazil) was administered intramuscularly to all cows, and the evaluation of follicular dynamics was initiated. Follicle diameter was assessed daily through transrectal ultrasonography until the largest, healthy, dominant follicles reached a diameter of at least 10 mm. Then, pre-ovulatory follicles were injected with 100 µL cooled semen collected from a bull on the same day of IFAI and were diluted 1:1 in Tris-egg yolk extender. Semen was preheated at 37°C prior to injection, and $20x10^6$ viable sperm cells were injected per follicle, with initial and final motility/vigor of 60%/3 and 40%/2, respectively. All semen evaluations were performed according to the standards of Colégio Brasileiro de Reprodução Animal (CBRA, 2013).

The IFAI procedure was carried out using a two-needle system, adapted from Gasperin et al. (2012). A 25 G x 3 ½ (0.6x90 mm) inner needle (Becton, Dickinson and Company, Franklin, NJ, USA) was attached to an embryo transfer gun containing a 250-µL semen straw. This inner system was introduced into a 35-cm metal cannula to which a 20 G x 1½ (0.8x40 mm) needle (Becton, Dickinson and Company, Franklin, NJ, USA) was attached. This two-needle system was inserted in a probe holder for ovum pick-up with a 5/7.5 MHz micro-convex transvaginal transducer attached to an Aquila Vet ultrasound (Esaote Europe B.V., AJ Maastricht, Netherlands). After caudal epidural anesthesia, the ovaries were handled by transrectal manipulation, in order to introduce the external needle close to the target follicle through the ovarian stroma. When the follicle was in the adequate position, the outer needle was advanced until its tip became visible 2 mm away from the follicle wall. Then, the inner needle was pushed forward by a second operator, who performed IFAI. Follicles were aspirated using the same equipment, but without the inner system, 1 to 4 hours (2.5±0.8 hours) after IFAI; a 10 µL aliquot of FF containing sperm cells

was analyzed using the CHK2-F-GS optic microscope at 40x magnification (Olympus Optical do Brasil, São Paulo, SP, Brazil), to assess sperm motility on a scale from 0 to 100% (CBRA, 2013).

The in vitro experiment was performed with a pool of different bovine semen from eight commercial straws (Maya Genética Sêmen e Embriões Ltda., Bagé, RS, Brazil); incubation was carried out in the extender (pool, control group) or diluted in FF obtained from five pre-ovulatory follicles of cyclic cows that were grazing on natural pasture during follicular aspiration (Galli et al., 2014). The pool was kept in microcentrifuge tubes on a heated plate at 37°C. Then, $5x10^6$ sperm cells were added to 400 µL FF in four-well dishes. The experiment was carried out in quadruplicates. At each moment (0, 1, and 3 hours) four wells were evaluated for each treatment. Sperm kinetics was examined under the Axio Scope A1 optic microscope at 200x magnification (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA), using the SpermVision, version 3.5, computer-assisted sperm analysis (Casa) system (Minitübe GmbH, Tiefenbach, Germany). Data with normal distribution before or after arcsine transformation were compared by the analysis of variance and Tukey's test. For non-normal data, the Kruskal-Wallis non-parametric analysis of variance was used.

In the in vivo experiment, two follicles from three cows and only one follicle from another three cows were inseminated, totaling nine injected follicles from six cows. Due to the difficulty of taking the Casa equipment to the animal handling facility, FF samples containing sperm cells were analyzed under an optic microscope. It should also be noted that the presence of blood cells in FF recovered after the intrafollicular insemination and follicular aspiration procedures would have also affected the Casa evaluation. Live spermatozoa were obtained at all time points, but in a low concentration and with low motility. From nine samples, only five presented live sperm cells with at the most 10% motility, while the semen kept in a thermal box, diluted 1:1 in the extender, had a final motility of 40%. Because of the reduced number of recovered sperm cells, no statistical analysis was performed.

In the in vitro experiment, sperm kinetic parameters were compared between the control and FF groups after incubation for 1 or 3 hours. At the beginning of the incubation period, i.e., 0 hour, no statistical

analysis was performed. After 1 hour of incubation, total motility (TM), progressive motility (PM), and curvilinear distance were lower whereas straightness was higher (p<0.05) for sperm cells incubated in the FF group (Table 1). After 3 hours, the FF group presented reduced TM, PM, average path velocity, and curvilinear velocity, compared with the control. It is important to highlight that sperm cells incubated in FF presented less than 10% motility after just 1 hour, whereas cells from the pool still showed motility greater that 20% after 3 hours of in vitro incubation.

Although $20 \times 10^6$ sperm cells were injected inside the pre-ovulatory follicles in the in vivo experiment, a reduced number of cells was recovered after follicular aspiration. A possible explanation for this low cell recovery may be the phagocytic activity of cumulus, as previously reported by Pijnenborg et al. (1985), who found that spermatozoa phagocyted by corona cells were incorporated into digestive vacuoles. Granulosa cells may also have this phagocytic activity against spermatozoa, which, under natural circumstances, do not come into contact with these types of cells. In equine species, using electronic microscopy, Eilts et al. (2006) observed that spermatozoa were bound to granulosa cells, suggesting phagocytosis.

The follicular environment of pre-ovulatory follicles, therefore, does not allow sperm cell viability to be maintained for prolonged periods in cows. The exact reasons for this low viability after incubation in FF in vivo or in vitro are still unknown; however, there are some possible explanations. Hamdi et al. (2010) showed that the high-density lipoprotein (HDL), a component of follicular fluid, triggers sperm hyperactivation as early as 1 min after incubation. Although crucial for fertilization, when premature, sperm capacitation can induce sperm cell death before oocyte nuclear maturation. In addition, the glycosaminoglycan hyaluronic acid, present in the expanded cumulus cells, especially before ovulation, induces sperm capacitation and acrosome reaction (Kim et al., 2013).

According to Leemans et al. (2015), FF has a detrimental effect on stallion sperm viability, which can be prevented by heat inactivation or filtration. When previously heated at 56°C, for 30 min, follicular fluid from women efficiently induced human sperm motility for up to 12 hours in vitro (Getpook & Wirotkarun, 2007). However, in the present study, in both the in vivo and in vitro experiments, FF samples were not subjected to any procedure.

In disagreement with the results of the present work, studies conducted in humans showed that IFAI is a promising tool to overcome primary infertility. Tocci & Lucchini (2011), after performing IFAI, diagnosed four (44.4%, 4/9) pregnancies via positive beta-hCG on day 16, of which three (33.3%, 3/9) were confirmed six weeks later by ultrasonography. In another study, higher pregnancy rates of 29% were reported after

**Table 1.** Sperm kinetic parameters after in vitro incubation at 37°C, for 1 or 3 hours, in own extender (pool, control group) or in bovine follicular fluid (FF)[1].

| Parameter | 0 hour | 1 hour | | 3 hours | |
| --- | --- | --- | --- | --- | --- |
| | Pool | Pool | FF | Pool | FF |
| Total motility (TM) | 38.4±4.3 | 38.3±3.7a | 5.4±3.7b | 26.3±3.7a | 4.1±3.7b |
| Progressive motility (PM) | 27.8±4.2 | 28.7±2.3a | 2.4±2.3b | 20.9±2.3a | 2.7±2.3b |
| Average path distance (DAP) | 28.0±3.1 | 25.1±5.9 | 19.4±5.9 | 28.1±5.9 | 18.5±5.9 |
| Curvilinear distance (DCL) | 46.8±6.6 | 42.2±9.1a | 28.6±9.1b | 48.3±9.1 | 30.2±9.1 |
| Straight-line distance (DSL) | 21.2±2.7 | 17.4±5.4 | 17.0±5.4 | 19.6±5.4 | 16.2±5.4 |
| Average path velocity (VAP) | 62.9±6.4 | 57.8±12.6 | 48.1±12.6 | 64.5±12.6a | 39.8±12.6b |
| Curvilinear velocity (VCL) | 104.7±13.7 | 96.8±20.0 | 71.6±20.0 | 110.2±20.0a | 65.1±20.0b |
| Straight-line velocity (VSL) | 47.7±5.6 | 40.2±11.7 | 42.5±11.7 | 44.9±11.7 | 34.7±11.7 |
| Straightness | 0.8±0.01 | 0.7±0.2a | 0.9±0.2b | 0.7±0.2 | 0.6±0.2 |
| Linearity | 0.4±0.1 | 0.4±0.1 | 0.6±0.1 | 0.4±0.1 | 0.4±0.1 |
| Wobble | 0.6±0.01 | 0.6±0.1 | 0.7±0.1 | 0.6±0.1 | 0.4±0.1 |
| Amplitude of lateral head displacement (ALH) | 2.9±0.2 | 3.4±0.6 | 2.2±0.6 | 3.6±0.6 | 1.8±0.6 |
| Beat-cross frequency (BCF) | 30.5±0.5 | 28.7±6.5 | 35.2±6.5 | 28.4±6.5 | 24.2±6.5 |

[1]Means followed by different letters indicate significant differences by Tukey's test, at 5% probability. The experiment was performed in four replicates.

IFAI, in comparison with those of 11% for intrauterine insemination (Lucchini et al., 2012). However, in both studies, women were subjected to a stimulation protocol to ensure strict control of ovulation, and IFAI was performed 36 hours after hCG, i.e., immediately before ovulation. Based on the results of the present study, it is possible to infer that IFAI is suitable when carried out just before ovulation, since the follicular environment and FF are not suitable for maintaining sperm viability for long periods.

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## References

CBRA. Colégio Brasileiro de Reprodução Animal. **Manual para exame andrológico e avaliação de sêmen de animal**. 3.ed. Belo Horizonte: CBRA, 2013.

EILTS, B.E.; PINTO, C.R.F.; PACCAMONTI, D.L.; NIEMANSTVERDREIT, A.S.; GARCIA, E.; GODKE, R.A. Transvaginal intrafollicular sperm cell injection with concomitant artificial insemination in the cyclic mare. **Theriogenology**, v.58, p.631-633, 2002. DOI: 10.1016/S0093-691X(02)00798-7.

EILTS, B.E.; LYLE, S.K.; BORKHSENIOUS, O.N.; HENK, W.G.; PACCAMONTI, D.L.; FERRER, M.S. Spermatozoal binding and phagocytosis by granulosa cells after intrafollicular insemination. **Animal Reproduction Science**, v.94, p.326-330, 2006. DOI: 10.1016/j.anireprosci.2006.05.010.

GALLI, C.; DUCHI, R.; COLLEONI, S.; LAGUTINA, I.; LAZZARI, G. Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. **Theriogenology**, v.81, p.138-151, 2014. DOI: 10.1016/j.theriogenology.2013.09.008.

GASPERIN, B.G.; FERREIRA, R.; ROVANI, M.T.; SANTOS, J.T.; BURATINI, J.; PRICE, C.A.; GONÇALVES, P.B.D. FGF10 inhibits dominant follicle growth and estradiol secretion *in vivo* in cattle. **Reproduction**, v.143, p.815-823, 2012. DOI: 10.1530/REP-11-0483.

GETPOOK, C.; WIROTKARUN, S. Sperm motility stimulation and preservation with various concentrations of follicular fluid. **Journal of Assisted Reproduction and Genetics**, v.24, p.425-428, 2007. DOI: 10.1007/s10815-007-9145-6.

HAMDI, S.M.; VIEITEZ, G.; JASPARD, B.; BARBARAS, R.; PERRET, B.; MIEUSSET, R.; PARINAUD., J.; COLLET, X. Effects of human follicular fluid and high-density lipoproteins on early spermatozoa hyperactivation and cholesterol efflux. **Journal of Lipid Research**, v.51, p.1363-1369, 2010. DOI: 10.1194/jlr.M000679.

KIM, E.Y.; NOH, E.H.; NOH, E.J.; PARK, M.J.; PARK, H.Y.; LEE, D.S.; RIU, K.Z.; PARK, S.P. Effect of glycosaminoglycans on *in vitro* fertilizing ability and *in vitro* developmental potential of bovine embryos. **Asian-Australasian Journal of Animal Sciences**, v.26, p.178-188, 2013. DOI: 10.5713/ajas.2012.12406.

LEEMANS, B.; GADELLA, B.M.; STOUT, T.A.; NELIS, H.; HOOGEWIJS, M.; VAN SOOM, A. An alkaline follicular fluid fraction induces capacitation and limited release of oviduct epithelium-bound stallion sperm. **Reproduction**, v.150, p.193-208, 2015. DOI: 10.1530/REP-15-0178.

LÓPEZ-GATIUS, F.; HUNTER, R.H.F. Intrafollicular insemination for the treatment of infertility in the dairy cow. **Theriogenology**, v.75, p.1695-1698, 2011. DOI: 10.1016/j.theriogenology.2011.01.006.

LUCCHINI, C.; VOLPE, E.; TOCCI, A. Comparison of intrafollicular sperm injection and intrauterine insemination in the treatment of subfertility. **Journal of Assisted Reproduction and Genetics**, v.29, p.1103-1109, 2012. DOI: 10.1007/s10815-012-9836-5.

LUCENA, E.; RUIZ, J.A.; MENDOZA, J.C.; LUCENA, A.; LUCENA, C.; ARANGO, A. Direct intrafollicular insemination: a case report. **The Journal of Reproductive Medicine**, v.36, p.525-526, 1991.

PIJNENBORG, R.; GORDTS, S.; ONGKOWIDJOJO, R.; BROSENS, I. Sperm phagocytosis by corona cells in a human *in vitro* fertilization system. **Annals of the New York Academy of Sciences**, v.442, p.310-317, 1985. DOI: 10.1111/j.1749-6632.1985.tb37534.x.

TOCCI, A.; LUCCHINI, C. Feasibility and results of a modified intrafollicular insemination technique for treating primary infertility. **Journal of Assisted Reproduction and Genetics**, v.28, p.821-824, 2011. DOI: 10.1007/s10815-011-9593-x.