Original Article

The Use of Computer-Assisted Sperm Analysis for Discriminating Series of Motility Pattern of Frozen-thawed Boar Semen

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Abstract

Computer-assisted sperm analysis (CASA) was initiated to reduce subjective bias on the motility assessment and to discriminate a series of motility patterns of boar semen. The objectives of this present study were to evaluate the motility pattern of frozen-thawed boar semen at 0 and 60 min after thawing using CASA. Forty one ejaculates from Landrace (n=14), Yorkshire (n=12) and Duroc (n=15) boars were cryopreserved and included in the experiment. The semen was thawed at 37°C for 30 sec. The post-thawed sperm qualities including subjective motility, plasma membrane integrity and motility pattern were determined immediately after thawing (T_0) and at 60 min (T_{60}) after incubation at 38°C. All motility parameters were recorded. Motion parameters including curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linear coefficient (LIN, %), amplitude of lateral head displacement (ALH, μm) and total motility (MS-CASA, %) were measured. The results revealed that total motility and VSL assessed by CASA after thawing (T_0) differed ($p<0.05$) among breeds. Some motion characteristics of FT boar semen i.e., VSL, VAP, VCL and ALH significantly decreased an hour after post-thawing ($p<0.05$). However, there was no significant difference in MS-CASA, and LIN between T_0 and T_{60} groups.

Keywords: boar, CASA, frozen-thawed semen, spermatozoa

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บทคัดย่อ

การศึกษารูปแบบการเคลื่อนที่ของอสุจิในน้ำเชื้อสุกรที่ผ่านการแช่แข็งโดยการใช้เครื่องตรวจวิเคราะห์อสุจิ (CASA)

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CASA (computer-assisted sperm analysis) ได้มีการนำมาใช้ในการตรวจประเมินคุณภาพการเคลื่อนที่ของอสุจิเพื่อลดความผันแปรของการประเมินค่าคะแนนการเคลื่อนไหวจากผู้ตรวจ และสามารถอธิบายถึงรูปแบบของการเคลื่อนที่แบบต่างๆ ในน้ำเชื้อสุกร จุดประสงค์ของการศึกษาในครั้งนี้ คือ การใช้เครื่อง CASA ตรวจประเมินรูปแบบการเคลื่อนที่ของน้ำเชื้อสุกรแช่แข็งภายหลังทำละลายน้ำเชื้อทันทีและในอีก 60 นาทีถัดมา จากตัวอย่างน้ำเชื้อจำนวน 41 ตัวอย่าง จำแนกเป็นสายพันธุ์ Landrace 14 ตัวอย่าง สายพันธุ์ Yorkshire 12 ตัวอย่าง และ สายพันธุ์ Duroc 15 ตัวอย่าง ได้ถูกนำมาเก็บรักษาโดยวิธีการแช่แข็ง และทำการละลายน้ำเชื้อที่อุณหภูมิ 37°ซ. เป็นเวลา 30 วินาที ทำการตรวจประเมินคุณภาพโดยประเมินผล การเคลื่อนไหว ความสมบูรณ์ของเยื่อหุ้มอสุจิ และรูปแบบการเคลื่อนที่ของอสุจิ โดยได้ทำการตรวจวัดทันทีและภายหลังการเก็บรักษาที่ 38°ซ. จึงทำการตรวจอีกครั้งใน 60 นาทีถัดมา รูปแบบของการเคลื่อนที่จำแนกเป็น วิถีการเคลื่อนที่แบบโค้ง (VCL, ไมโครเมตรต่อวินาที), วิถีเคลื่อนที่แบบตรง (VSL, ไมโครเมตรต่อวินาที), วิถีการเคลื่อนที่แบบเฉลี่ย (VAP, ไมโครเมตรต่อวินาที), linear coefficient (LIN, ร้อยละ), amplitude of lateral head displacement (ALH, ไมโครเมตร), ร้อยละการเคลื่อนที่ ผลการศึกษาพบว่า เมื่อตรวจประเมินด้วยเครื่อง CASA ค่าร้อยละการเคลื่อนที่และวิถีการเคลื่อนที่แบบตรงมีความแตกต่างอย่างมีนัยสำคัญทางสถิติในระหว่างสายพันธุ์ ($p<0.05$) และรูปแบบของวิถีการเคลื่อนที่ยังลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบระหว่างภายหลังการทำละลายทันทีกับการเก็บรักษาต่อที่ 60 นาทีถัดมา ($p<0.05$) อย่างไรก็ตามไม่พบความแตกต่างทั้งในร้อยละการเคลื่อนที่และ LIN เมื่อผ่านระยะเวลาการเก็บรักษาที่ 60 นาที

คำสำคัญ: พ่อพันธุ์สุกร CASA น้ำเชื้อแช่แข็ง อสุจิ

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Introduction

The assessment of semen quality is important for the success of artificial insemination in pigs. It can be routinely assessed by subjective visual examination under a phase contrast microscope e.g. individual progressive motility, concentration and morphology etc., or by evaluation of farrowing performances which remains time-consuming of high cost. Microscopic techniques have limitations including subjectivity, variability, the small number of sperm analyzed and poor correlation with fertilizing potential (Rijsselaere et al., 2005). It has been demonstrated that the assessment of sperm motility using light microscopy resulted in a 30-60% (Amann, 1989; Cancel et al., 2000; Chan et al., 1990) variability caused by the evaluator's skills. Computer-assisted sperm analysis (CASA) provides objective and detailed information on various motility characteristics and morphometric dimensions that cannot be identified by conventional light microscopic semen analysis (Rijsselaere et al., 2005). The use of sperm motion parameters via computer-assisted sperm analysis (CASA) reflects the correlation between sperm motility and fertility results and provides a way of objectively classifying a given population of spermatozoa with respect to their motility (Holt et al., 2007). CASA allows the objective assessment of the sperm cell characteristics including motion, velocity and morphology (Verstegen et al., 2002). Time lapse and multiple exposure photography were the forerunners of sperm motility analysis by CASA. These techniques involved recording the movement of live spermatozoa on microscope slides by opening the shutter for a fixed period so that the cell movements produced continuous tracks on the exposed film. The motion parameters typically derived using automated CASA systems provide information about the velocity, linearity and lateral displacement of sperm heads as they progress along their trajectories (Didion, 2008; Rijsselaere et al., 2004). Our previous study found that the breed of boar and individual boars within the same breed significantly influenced most of the post-thaw sperm parameters (Buranaamnuay et al., 2009). However, the use of CASA to evaluate the FT boar semen was performed. Moreover, a thermal resistance test (38°C for 60 min) for post-thawed semen qualities was not evaluated.

This technique showed itself to be a potential method for evaluating the survival capacity of spermatozoa *in vitro* (Koonjaenak et al., 2007).

The objectives of the present study were to evaluate the motility pattern of frozen-thawed boar semen at 0 and 60 min after thawing in three breeds of boars using CASA.

Materials and Methods

Semen collection, freezing and thawing: Each sperm-rich fraction of forty one ejaculates from 15 purebred boars (5 Landrace; 14 ejaculates, 5 Yorkshire; 12 ejaculates and 5 Duroc; 15 ejaculates) aged between one and three years old were included in the experiment. The boars are routinely used in two commercial swine herds in Nakorn-Pathom province, Thailand. The ejaculates were collected once a week using the gloved-hand technique and were kept in an insulated thermos flask during transport to the laboratory within 40 min after collection. Fresh semen with a minimum of 70% sperm motility was used for freezing with some modifications following Gadea et al. (2004) and Westendorf et al. (1975). The semen was diluted with isothermal Beltsville thawing solution (BTS; Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) extender at a ratio of 1:1 (v/v). The diluted semen was kept at 15°C for 2 h and centrifuged at 800 xg for 10 min. The supernatant was discarded and the pellet resuspended (about 1 to 2:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk). After further cooling to 5°C over a 90-min period, two parts of the semen were mixed with one part of extender III (LEY extender and 9% glycerol with 1.5% Equex-STM®). The final concentration of sperm frozen was approximately $1x10^9$ spermatozoa/ml with 3% glycerol. The straws were sealed with PV powder at the open end of the straws before being placed in liquid nitrogen (LN_2) vapour at 4 cm above the level of LN_2 for 10 min and then plunged into LN_2. The frozen boar semen was stored in LN_2 (-196°C) and thawed using the protocol in a 37°C water-bath for 30 sec and the post-thaw sperm quality evaluated immediately to consider the total motility (Buranaamnuay et al., 2009).

Hypo-osmotic swelling test (Hos-test): The plasma membrane integrity (PMI) of the spermatozoa was assessed using the short hypo-osmotic swelling test (Hos-test) described by Perez-Llano et al. (2001), with some modifications (Koonjaenak et al., 2007). Briefly, aliquots of each semen sample (100 µl) were incubated at 38°C for 10 min, with 1,000 µl of either hypo-osmotic (75 mOsm/kg) or iso-osmotic (300 mOsm/kg) solution. The solutions were prepared with fructose and Na-citrate in distilled water and final osmolarity was measured by freezing point depression. Following the 10-min incubation, 200 µl of the semen-hypo-osmotic solution was fixed in 1000 µl of hypo-osmotic solution plus 5% formaldehyde (Merck Co. Ltd., Boeco, Germany) for later evaluation. Sperm coiling was assessed by placing 20 µl of well-mixed sample on a warm slide, which was covered with a cover slip before being observed under a light microscope (x 1,000) and 200 spermatozoa per slide were counted. To determine the percentage of sperm with intact membranes, the proportion of coiled tail sperm from the control sample (300 mOsm/kg) was subtracted from the results of the hypo-osmotic condition.

Computer-assisted sperm analysis: The subjective motility of fresh and frozen-thawed semen was evaluated using a light microscope at 400x magnification (Dott and Foster, 1979). The motility of diluted frozen-thawed semen was assessed using the CASA system (Halminton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, USA). A 80 µl aliquot of the thawed semen was re-extended with 920 µl of pre-warmed BTS (37°C) to obtain a final concentration of $40x10^6$ spermatozoa/ml. A 5 µl aliquot was placed in a pre-warmed container (37°C). The cell motion analyzer provided a stage warmer to allow for sample distribution upon the sample chamber and to pre-warm samples. A waiting period of 1 min preceded each measurement (Iguer-Ouada and Verstegen, 2001; Smith and England, 2001). After this primary assessment (T_0), the thawed semen was placed in an incubator at 38°C for 60 min (T_{60}) (Thermal resistance test) before being assessed again by CASA (Koonjaenak et al., 2007). To select cells from debris, the camera recognized the position of the sperm heads in successive frames. Spermatozoa heads were marked with a different color to enable the observer and the analyzer to differentiate between the different motility parameters. In addition, all the motility parameters were recorded for a single sperm cell.

Determination of the motility pattern of frozen-thawed boar semen: Each semen sample was measured twice, 3 fields were evaluated per sample and 100 cells per field were evaluated. Motion parameters consisted of (1) Curvilinear velocity (VCL, µm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time,(2) linear velocity (VSL, µm/s), the straight trajectory of the spermatozoa per unit of time (= straight line distance from the beginning to the end of the track divided by time taken), (3) mean velocity (VAP, µm/s), the mean trajectory of the spermatozoa per unit of time, (4) Linear coefficient (LIN, %), the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement which is defined as (VSL/VCL) x 100, (5) Amplitude of lateral head displacement (ALH, µm), which is the mean width of sperm head oscillation (Schäfer-Somi and Aurich, 2007) and (6) total motility (MS-CASA), expressed as the percentage of spermatozoa with VCL >15 µm/s.

Statistical analyses: The statistical analyses were performed using SAS (SAS version 9.0, Cary, NC, USA). Descriptive statistics were used to describe semen quality after thawing. Pearson's correlation was used to evaluate the correlation among sperm parameters. The differences of post-thawed sperm qualities between 0 and 60 min were analyzed using a paired t-test. The difference between breeds was evaluated using the General linear model procedure

(GLM). The least-square means were obtained and were compared using Student's *t* test. p <0.05 was regarded as a significant difference.

Results

On average, the subjective motility of FT boar semen was 28.2% (range 5% to 45%), while PMI was 18.5% (range 3% to 45%). The subjective motility was 29.0±2.4% in Duroc (n=15), 30.3±2.4% in Landrace (n=14) and 25.3±2.4 in Yorkshire (n=12) (p=0.05). PMI was 19.4±2.9% in Duroc, 18.1±2.9% in Landrace and 17.9±2.9% in Yorkshire (p=0.05). The LIN, VSL, VAP, VCL and ALH at 0 and 60 min after thawing are presented in Table 1. Sperm parameters determined by CASA differed between T_0 and T_{60}. A thermal resistance test revealed that there were no significant differences in all parameters among the three breeds. The CASA assessment is presented in Table2.

The post-thawed motion parameters varied among individuals and among breeds (Table 1 and 2). On average, the VSL in Landrace was significantly higher than Duroc (30.3 versus 40.0 µm/sec (p<0.05). VAP, VCL and the ALH significantly decreased in all breeds after incubation (Table 2). There was no significant difference for the subjective motility and PMI among breeds. The progressive motility and VSL of Landrace was higher than Duroc and Yorkshire (Table 1). No significant difference in motility (%), LIN (%), VSL, VAP, VCL and ALH at T_{60} (p>0.05) was found among the breeds.

Table 1 Least square means (LSM) ± standard error of the mean (SEM) of sperm parameter post-thaw (PT) in semen collected from boar. Samples examined for sperm motility were examined immediately by CASA (n = numbers of ejaculation)

Parameter	After thawing(T_0)			After thermal resistance test (T_{60})		
	Duroc (n=15)	Landrace (n=14)	Yorkshire (n=12)	Duroc (n=15)	Landrace (n=14)	Yorkshire (n=12)
MS-CASA (%)	4.7±2.9[a]	15.4±3.0[b]	20.9±3.2[b]	7.7±3.0[a]	10.3±3.1[a]	10.1±3.4[a]
LIN (%)	39.1±4.9[a]	44.4±5.0[a]	49.8±5.5[a]	48.7±5.6[a]	44.2±5.8[a]	52.8±6.3[a]
VSL (µm/sec)	30.3±2.9[a]	40.0±3.0[b]	35.9±3.2[a,b]	27.7±3.1[a]	32.2±3.3[a]	29.1±3.5[a]
VAP (µm/sec)	59.1±5.9[a]	61.0±6.1[a]	59.0±6.6[a]	38.3±3.6[a]	41.3±3.7[a]	36.5±4.0[a]
VCL (µm/sec)	115.3±13.3[a]	114.1±13.7[a]	109.92±14.8[a]	75.2±7.1[a]	82.8±7.4[a]	63.3±8.0[a]
ALH (µm)	5.9±0.8[a]	8.1±0.8[a]	7.2±0.9[a]	3.8±0.9[a]	4.4±0.9[a]	4.7±1.0[a]

[a, b] Means with different superscripts in the row are significantly between breed (p<0.05)
MS-CASA: Total motility, VAP (µm/s): Velocity average path, LIN (%): Linearity (VSL divided by VCL), VCL (µm/s): Velocity curved line, VSL (µm/s): Velocity straight line, ALH (µm): Amplitude of lateral head displacement

Table 2 Least square means (LSM)±standard error of the mean (SEM) of sperm parameter post-thaw (PT) in semen collected from boar. Samples examined for sperm motility were examined immediately T_0 and after a thermal resistance test (T_{60}). (n = 41; numbers of ejaculation)

Parameter	T_0	T_{60}
MS-CASA	13.1±1.9[a]	9.3±1.9[a]
VAP	59.7±2.9[a]	38.8±2.9[b]
VSL	35.2±1.9[a]	29.6±1.9[b]
VCL	113.3±6.3[a]	74.3±6.3[b]
ALH	7.0±0.5[a]	4.3±0.5[b]
LIN	44.1±3.2[a]	48.4±3.2[a]

[a,b] Means with different superscripts in the row are significantly between breed (p<0.05)

Discussion

The present study described the post-thaw quality of FT boar semen from different breeds and evaluated its quality using computerized assessed sperm. CASA motility was assessed by computer program to detect the size and movement of sperm which could reduce the variations of sperm motility estimation by evaluators and the morphology parameters of the same or different ejaculates assessed by different observers. It is suggested that the system offers an accurate and rapid calculation of different sperm parameters, such as total and progressive motility, slow, medium and rapid moving sperm, linearity of sperm movement, the beat cross frequency, the amplitude of the lateral head displacement, and several velocity parameters (Rijsselaere et al., 2005). The CASA system is also indicative of sperm capacitation or hyperactivation.

The thermal resistance tests were used to depict the ability of spermatozoa to sustain incubation at temperatures close to the female body temperature

with the assumption that they would describe the vitality of the spermatozoa (Koonjaenak et al., 2007). It appears that spermatozoa change motility, becoming less linear and progressively less vigorous, a process known as a ''hyperactivated movement'' (Mortimer, 1997). Hyperactivated motility occurs in parallel with the attainment of the capacitated state in the female reproductive tract (Yanagimachi, 1970). Kaul et al. (2001) studied capacitation in buffalo and bull spermatozoa and indicated that the percentage of spermatozoa that exhibit capacitated characteristics increases following incubation, which is in agreement with the present findings.

In pigs, AI by frozen semen is not popular due to a high sensitivity to cold shock (Holt, 2000). There are many factors influencing boar semen freezing namely breed, individual, ejaculate manipulation, extenders and freezing techniques (Eriksson et al., 2000). It was found that there are variations in sperm quality among boars and ejaculations in the same boar. Individuality seems to be main factor influencing ejaculated variability in sperm cryosurvival (Barbas and Mascarenhas, 2009).

In present study we found that sperm motility varied among the breeds and there was higher percentage of motility in Yorkshire than in Duroc and Landrace breeds. Most parameters, except LIN, significantly decreased after the thermal resistance test. According to results of Kaeoket et al. (2008[a,b]) it was found that there is no significance in breed difference among Landrace, Pietrain, Duroc and Yorkshire. This might come from the male-to-male differences in sperm cryo-tolerance after freezing and thawing. Although the motility (MS-CASA) of Duroc is increased in T_{60} when compared with T_0, it has also composition in different breeds of boars which can explain the major differences in post-thaw survival and fertility breeds (Waterhouse et al., 2006). In addition, Jame et al. (1999) reported the composition of sperm plasma membrane presents highly specific lipid composition, which has a very high level of phospholipids, sterol, saturated and PUFA. The composition of the sperm membrane or the factors influencing post-thawed boar motility spermatozoa among breeds boars spermatozoa and seminal plasma should remain to be investigated in the future. Saravia et al. (2007) revealed a significant difference among breeds in the size of morphologically normal spermatozoa, which were significantly larger and more elliptic in the Duroc breed. In all species, differences among individuals seem to be of genetic origin and it has been suggested that there are differences in specific DNA sequences identified among boars in which thawed semen quality was classified poor or good (Thurston et al., 2001). An increase in LIN has been previously observed when motility was assessed in fresh semen and then later after undergoing freeze-thaw procedures.

Peña et al. (2003) suggested that capacitation-like changes occur in sperm motility after freeze-thaw procedures. Thawed sperm is characterized by an increase in LIN and a decrease in VCL and ALH. However VSL and VAP do not increase after freeze-thaw procedures.

In conclusion, some motion characteristics of FT boar semen i.e., VAP, VCL and ALH significantly decreased after an hour post-thawing, while the LIN remained unchanged. Conventional microscopic methods for sperm evaluation in combination with CASA have allowed us to obtain precise information about sperm quality in pigs.

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