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Progressive motility – a potential predictive parameter for semen fertilization capacity in bovines

Y. Li[2], *D. Kalo*[3], *Y. Zeron*[4] *and Z. Roth*[1]

South China Agricultural University, College of Veterinary Medicine, Guangzhou, China; Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot, Israel; and Sion Artificial Insemination Center, Hafetz-Haim, Israel

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Summary

We examined the association between progressive motility of spermatozoa and *in vitro* fertilization (IVF) competence of bovine ejaculates. Fresh semen was evaluated using a computerized sperm quality analyzer for bulls using progressive motility as the primary parameter. Ejaculates with high progressive motility (HPM; >81%) were compared with those with low progressive motility (LPM; <62%). Semen concentration and sperm velocity were lower ($P < 0.05$) in HPM versus LPM ejaculates. Volume and motile sperm concentration did not differ between groups ($P > 0.05$). Examination of sperm morphology revealed a higher proportion of spermatozoa with abnormal morphology ($P < 0.01$) in LPM versus HPM ejaculates, the predominant abnormal feature being a bent tail ($P < 0.05$). Sperm viability, acrosome integrity and DNA fragmentation did not differ between HPM and LPM samples. Mitochondrial membrane potential was higher ($P < 0.01$) in HPM versus LPM semen. Zinc concentrations in the seminal plasma correlated with progressive motility ($R^2 = 0.463$, $P = 0.03$). In addition, representative ejaculates from HPM and LPM groups were cryopreserved in straws and used for IVF. The proportions of embryos cleaved to 2- and 4-cell stages (88.1 ± 1.1 versus 80.5 ± 1.7, $P = 0.001$) and developed to blastocysts (33.5 ± 1.6 versus 23.5 ± 2.2, $P = 0.026$) were higher for HPM than LPM semen. The total cell number of embryos and blastocyst apoptotic index did not differ between groups. Although sperm progressive motility is associated with IVF competence, further examination is required to determine whether progressive motility can serve as a predictor of semen fertilization capacity *in vivo*.

Keywords: Bovine, Element concentration, Progressive motility, Sperm characteristic

Introduction

Studies in a variety of different species have sought a correlation between semen characteristics, quality and fertility using computerized instruments (Verstegen *et al.*, 2002; Rodriguez *et al.*, 2011). Among the physiological parameters, sperm motility and morphology are the ones most significantly correlated with fertility (Farrell *et al.*, 1998; Al-Makhzoomi *et al.*, 2008). However, motility by itself is limited in predicting semen fertilization capacity (Comhaire *et al.*, 1987; Roudebush & Diehl, 2001; Lewis, 2007), and multiparametric analysis to predict semen quality is suggested (Vincent *et al.*, 2012).

Progressive motility is the sperm's ability to move straight forward in a clearly defined direction; this ability is essential for spermatozoon movement in the female reproductive tract (Hafez & Hafez, 2000). A study in pig revealed significant effects of progressive motility, as well as of curvilinear velocity and beat cross frequency, on farrowing rate (Broekhuijse *et al.*, 2012). Prediction of spontaneous male fertility based on four semen measurements (sperm concentration, total progressive motility, normal morphology and

[1]All correspondence to: Z. Roth. Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel. Tel: +972 8 9489103. Fax: +972 8 9489552. e-mail: roth@agri.huji.ac.il
[2]South China Agricultural University, College of Veterinary Medicine, Guangzhou 510642, China.
[3]Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel.
[4]Sion Artificial Insemination Center, Hafetz-Haim, Israel.

hypo-osmotic swelling test) enabled the correct identification of about 84% of patients as fertile (Jedrzejczak *et al.*, 2008).

Although the mechanism that underlies progressive motility is not yet clear, it might involve mitochondrial activity, which has a key role in spermatozoon motility (Olson & Winfrey, 1992), as documented in humans (Cardullo & Baltz, 1991; Gopalkrishnan *et al.*, 1991; Ruiz-Pesini *et al.*, 1998), equine (Love *et al.*, 2003), rat (Gravance *et al.*, 2001), boar (Spinaci *et al.*, 2005) and ram (Martinez-Pastor *et al.*, 2004). In addition, a positive correlation has been recorded between mitochondrial membrane potential and *in vitro* fertilization (IVF) rate in humans (Kasai *et al.*, 2002).

The concentration of calcium in the female reproductive tract has been suggested to play a role in sperm motility after ejaculation (Lefièvre *et al.*, 2009) and in the modulation of hyperactivated flagellar movement (Suarez & Dai, 1995; Chang & Suarez, 2011). Calcium is also involved in the regulation of other sperm functions, such as the protein kinase family enzymes, and Ca^{2+}-channel and protein phosphorylation activities (Carafoli, 2002) through both capacitation and acrosome reaction.

Taken together, sperm progressive motility seems to be a promising predictor of sperm quality and fertility, at least in combination with other parameters. Here we examined the association of Holstein semen of high or low progressive motility (HPM and LPM, respectively) with physiological and morphological parameters and IVF capacity.

Materials and methods

Chemicals and media

All chemicals, unless otherwise stated, were purchased from Sigma (Rehovot, Israel). Follicle-stimulating hormone (FSH) isolated from ovine pituitary extract (Ovagen) was from Bioniche Animal Health (Follitropin-V; Belleville, Ontario, Canada). Dulbecco's phosphate-buffered saline (PBS), fetal calf serum (FCS) and RQ1 RNase-free DNase I reagents were purchased from Promega (Madison, WI, USA). The In-Situ Cell-Death Detection Kit (TUNEL assay) was from Roche (Indianapolis, IN, USA). The Mitocapture™ Mitochondrial Apoptosis Detection Kit (JC-1 dye) was from BioVision Research Products (Mountain View, CA, USA). Non-essential and essential amino acids were from Life Technologies (Carlsbad, CA, USA). Paraformaldehyde (16% vol/vol) was from Electron Microscopy Sciences (Hatfield, PA, USA). Culture media, HEPES–Tyrode's lactate (HEPES–TL), sperm–TL (SP–TL) and IVF–TL, were prepared in our laboratory: HEPES–TL was supplemented with 0.3% (wt/vol) bovine serum albumin (BSA), 0.2 mM sodium pyruvate and 0.75 mg/ml gentamicin (HEPES–TALP); SP–TL was supplemented with 0.6% BSA, 1 mM sodium pyruvate and 0.2 mg/ml gentamicin (SP–TALP); IVF–TL was supplemented with 0.6% (wt/vol) essential fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.05 mg/ml gentamicin and 0.01 mg/ml heparin (IVF–TALP) as described by Parrish *et al.* (1986). Oocyte maturation medium was made up of TCM-199 and Earle's salts supplemented with 10% (vol/vol) heat-inactivated FCS, 0.2 mM sodium pyruvate, 50 μg/μl gentamicin, 2.2 g/l sodium bicarbonate, 2 μg/ml 17β-estradiol and 1.32 μg/ml FSH. Potassium simplex optimized medium (KSOM) contained 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4{\cdot}7H_2O$, 0.8% (vol/vol) sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM D(+)-glucose, 25 mM $NaHCO_3$, 0.01 mM phenol red, 1 mM L-glutamine and 0.01 mM EDTA supplemented with 1.7 mM $CaCl_2{\cdot}2H_2O$, 0.1 mg/ml polyvinylalcohol (PVA), 10 μl/ml essential amino acids and 5 μl/ml non-essential amino acids, 100 U/ml penicillin G and 0.1 mg/ml streptomycin.

Experimental design

The study included three parts. The first was performed to define the cutoff value for HPM and LPM groups. Repeated measurement analysis of ejaculates ($n = 332$, only first collections) from 52 working bulls over an entire month (November 2012) revealed a normal distribution pattern with similar median and mean values. The average progressive motility differed between bulls ($P < 0.01$), the maximum, median and minimum progressive motility values were 83.8, 71.3 and 45.5%, respectively (Fig. 1*A*) and the mean ± standard deviation (SD) value was 71.0 ± 7.2%. A similar distribution pattern was found when data were analyzed for ejaculates regardless the bulls. The maximum, median and minimum values were 87.4, 71.9 and 32.5%, respectively (Fig. 1*B*) and the mean ± SD value was 71.5 ± 9.4%. Based on these findings, the experimental unit used in the current study was the ejaculate rather the bull. Ejaculates with progressive motility values >81% (i.e. plus one SD from the mean) were defined as HPM ejaculates and those with progressive motility values <62% (i.e. minus one SD from the mean) were defined as LPM ejaculates. This model fits the commercial routine procedure used in most of the semen-production centres supplying semen to breeders and producers, i.e. ejaculate quality is evaluated objectively on a specific collection day, independent of previous collections from the same bull.

In the second part of the study, using the SQA-Vb, progressive motility served as the primary

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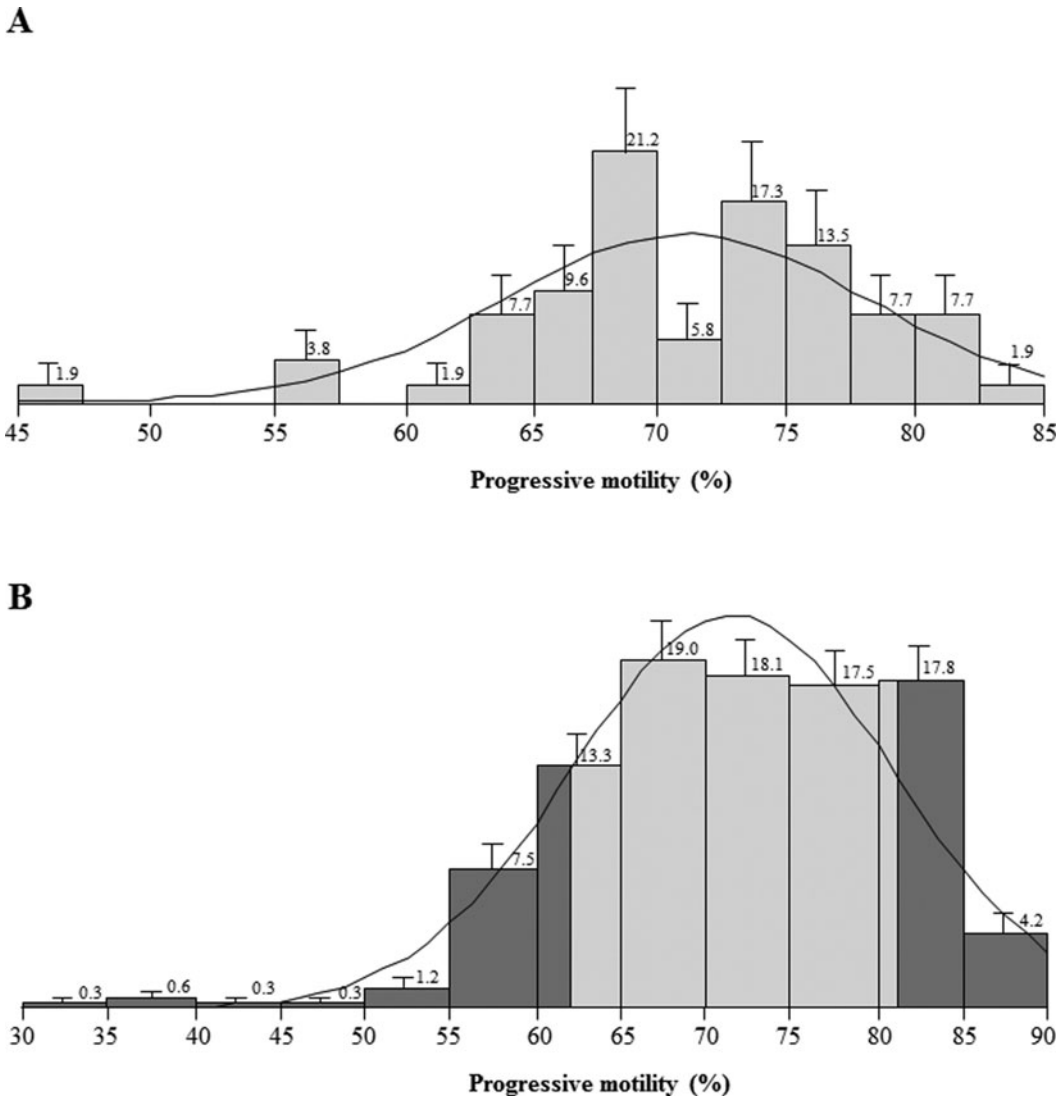

Figure 1 Data of ejaculates (n = 332) collected from 52 working bulls throughout November 2012 at the Israeli Artificial Insemination Center 'Sion'. (*A*) The graph presents the distribution of bulls according to their progressive motility. (*B*) The graph presents the distribution of ejaculates according to their progressive motility regardless bulls. Progressive motility lower than 62% [i.e. minus one standard deviation (SD) from the mean] was defined as low (left tail of the curve, marked dark grey); progressive motility higher than 81% (i.e. plus one SD from the mean) was defined as high (right tail of the curve, marked dry grey).

parameter in selecting semen for the experimental groups. Ejaculates were collected every 2–3 days throughout December 2012, for a total of 7 collection days. Ejaculates were selected as LPM (n = 15) or HPM (n = 15) according to the above analysis. To eliminate any potential differences in sperm quality within serial collections, only the first ejaculates of an examined day were taken. Nine ejaculates were used from each HPM and LPM group to determine sperm characteristics, and five ejaculates were used to determine elemental concentrations in the seminal plasma. For each experimental group, an additional representative ejaculate (HPM = 86.3% and LPM = 59.6%) was cryopreserved in straws (n = 50 straws per group). These straws were used for IVF.

In the third part, IVF procedure was repeated 10 times with ~100 oocytes per group per replicate. Before IVF, cryopreserved straws (n = 5 per ejaculate per group) were evaluated by SQA-Vb, to determine the precise progressive motility after freezing and thawing. Cumulus–oocyte complexes (COCs) were matured for 22 h, arbitrarily divided into two groups and fertilized with Percoll-purified HPM or LPM spermatozoa (~1 × 10^6) for 18 h. Percoll purification was performed separately for HPM and LPM cryopreserved straws (n = 3/group per Percoll

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gradient per run). After fertilization, putative zygotes were incubated for 8 days, and cleavage and blastocyst formation rates were assessed at 42–44 h and on day 8 after fertilization, respectively. In addition, TUNEL procedure was performed and total cell count determined for 8-day blastocysts.

Animals

Semen was collected at the Israeli Artificial Insemination Center ('Sion', Hafetz-Haim, Israel) during the winter (November to January), in accordance with the 1994 Israeli guidelines for animal welfare and experimentation. Bulls were fed the same total mixed ration throughout the experiment, containing 7.2% (wt/wt) protein, 36.2% (wt/wt) neutral detergent fibre, 20.0% (wt/wt) acidic detergent fibre, 1.45 Mcal/kg net energy and 3.5 g minerals/kg (NaCl, Ca and P) on a dry matter basis.

Semen collection and evaluation

Mature Holstein-Friesian bulls were mounted on a live teaser and semen was collected into a disposable tube using a heated (38°C) sterile artificial vagina. The ejaculate was immediately transferred to a nearby laboratory and the semen was evaluated by computerized sperm quality analyzer, an analytical veterinary device that combines electro-optics, computer algorithms, and video microscopy calibrated for bull semen (SQA-Vb, Medical Electronic Systems, Caesarea, Israel). Samples were prepared and inserted into testing capillaries according to the SQA-Vb user's guide, and then placed into the SQA-chamber for 40 s. Analysis included the following physiological characteristics: volume (ml), concentration (1×10^6 sperm/ml), total motility (motility, %), progressive motility (%), morphologically normal sperm (%), motile sperm concentration (1×10^6 sperm/ml), progressive motile sperm concentration (1×10^6 sperm/ml) and velocity (μm/s). As per routine procedure at the Artificial Insemination Center 'Sion', samples with a concentration greater than 650×10^6 sperm/ml and motility greater than 70% were defined as being of good quality.

Cryopreservation was performed following the routine procedure at 'Sion' as described by Orgal *et al.* (2012). Briefly, samples were diluted to a final concentration of 90×10^6 sperm/ml. The extender contained 10% (vol/vol) glycerol, 20% (wt/vol) egg yolk, 20 mg lactose, 1000 IU penicillin and 500 mg streptomycin per ml. Diluted semen was chilled for 30 min down to 4°C and inserted into 0.221-ml chilled straws containing 20×10^6 sperm. Straws were then kept at 4°C for 2.5 h, followed by separation on racks and cooling for 10 min down to –95°C in a programmed box in vapour nitrogen-saturated atmosphere followed by plunging into liquid nitrogen.

Evaluation of sperm characteristics

Fresh undiluted ejaculates were purified by Percoll gradient (45/90%). Purified spermatozoa were then stained with Diff-Quick Staining Kit (Jorgensen Laboratories, Inc., Loveland, CO, USA) and subjectively analyzed for morphological characteristics under an inverted microscope (Nikon, Tokyo, Japan) at ×200 magnification. At least 200 spermatozoa were examined for each sample. Abnormal spermatozoon morphology was categorized into detached head, bent or coiled tail, and other.

Viability was assessed by propidium iodide (PI) staining. Purified spermatozoa were resuspended in 0.5 ml SP–TALP supplemented with 2.4 mM PI and incubated at 38.5°C in a 5% CO_2 incubator in the dark. Total cells and dead cells (fluorescing red) were counted under an inverted fluorescence microscope (Nikon). For each sample, at least 200 spermatozoa were analyzed. The ratio of live to dead spermatozoa was calculated for each sample from each group.

Acrosome integrity was assessed by triple-fluorescence test, as described by Orgal *et al.* (2012) with minor modifications. Purified spermatozoa were resuspended in 0.5 ml SP–TALP, and incubated at 38.5°C in a 5% CO_2 incubator with 2 mg/ml Hoechst 33342 diluted in PBS for 8 min. Then 2.4 mM PI and 50 μg/ml fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) were added, and samples were incubated for additional 10 min. Acrosome integrity was determined under an inverted fluorescence microscope at ×200 magnification; 200 spermatozoa were scored per sample. All spermatozoa fluoresced blue in their nuclear area; spermatozoa with bright green fluorescence (FITC–PNA) in the whole acrosomal region were scored as acrosome-damaged; dead spermatozoa were PI positive with red fluorescence in the nuclear area.

Mitochondrial membrane potential ($\Delta\Psi m$) was evaluated by JC-1 dye. Purified spermatozoa were stained with JC-1 dye in incubation buffer (1:1000 vol/vol) at 38.5°C in a 5% CO_2 in air incubator for 15–20 min. Thereafter, samples were centrifuged (500 *g*), the supernatant was discarded and the pellet was resuspended in 1 ml of prewarmed incubation buffer. Then, 10 μl of sample was placed on a slide, covered with a glass coverslip, and examined for high (red) and low (green) $\Delta\Psi m$ populations under an inverted fluorescence microscope using Nis Elements software (Nikon) at ×200 magnification. From each sample, at least 200 spermatozoa were randomly analyzed. The proportion of high and low $\Delta\Psi m$ spermatozoa was calculated for each sample from each group.

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TUNEL assay was performed using the in-situ cell death detection kit according to the manufacturer's (Roche) instructions with the following modification: the permeabilization step was performed for only 2 min, at room temperature. Samples were purified by Percoll gradient (45/90%) and stained. At least 500 spermatozoa were examined per group and the proportion of TUNEL-positive spermatozoa was calculated. TUNEL assay was monitored under an inverted fluorescence microscope using Nis Elements software.

Elemental concentrations in the seminal plasma

Elemental concentrations in the seminal fluid were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES; 'Genesis' from Spectro GMBH, Kleve, Germany) as described previously (Orgal *et al.*, 2012), using scandium as the internal standard and a blank control in parallel. Briefly, 0.3–0.8 ml of seminal fluid was digested with 2 ml HNO_3 (65% vol/vol) in a polypropylene flask at 90 to 100°C in a water bath for 2 h. The digested seminal fluid was cooled and the volume was brought to 15 ml with deionized water. Elemental concentrations were measured in the clear solution using an End-On-Plasma ICP-AES model 'ARCOS' from Spectro GMBH. Measurements were calibrated with standards for ICP from Merck. Samples with elemental concentrations exceeding the linear dynamic range were diluted and reanalyzed. Dilution was performed using calibrated pipettes. The calibration–verification standard was measured to check instrument stability.

In vitro fertilization

In vitro production of embryos was performed according to Gendelman *et al.* (2010). Briefly, Holstein cow ovaries were obtained from a local abattoir; COCs were aspirated and incubated in humidified air with 5% CO_2 in air for 22 h at 38.5°C. Following maturation, COCs were co-incubated with Percoll-purified spermatozoa (~1×10^6). After fertilization, putative zygotes were denuded of cumulus cells by gentle vortexing in HEPES–TALP containing 1000 U/ml hyaluronidase, and randomly placed in groups of 10 in 25-µl droplets of KSOM. All embryo droplets were overlaid with mineral oil and cultured for 8 days at 38.5°C in an atmosphere of humidified air with 5% CO_2, 5% O_2, and 90% N_2.

TUNEL assay and total cell count in embryos

TUNEL assay was performed according to the manufacturer's (Roche) instructions as described by Kalo & Roth (2011). Total cell number of embryos was determined by nuclear staining (Hoechst 33342) and the proportion of TUNEL-positive blastomeres was used to evaluate embryo quality. Briefly, embryos were fixed in 4% (vol/vol) paraformaldehyde in PBS for 15 min and stored in PBS–PVP at 4°C. During the assay, embryos were washed three times in PBS–PVP, placed in permeabilization solution containing PBS with 1 mg/ml PVP and 0.3% (vol/vol) Triton X-100, and incubated in permeabilization solution containing PBS with 1 mg/ml PVP, 0.1% Triton X-100 and 0.1% (wt/vol) sodium citrate for 20 min at room temperature. For positive and negative controls, samples were incubated in 50-ml drops of 50 U/ml RNase-free DNase at 37°C for 1 h in the dark. Thereafter, samples were incubated in TUNEL reaction mixture (containing FITC-conjugated dUTP and TdT) for 1 h at 37°C in the dark. The negative control was incubated under the same conditions, but without TdT. Finally, samples were stained with 1 mg/ml Hoechst 33342 in PBS–PVP for 15 min at room temperature. The apoptotic cell ratio for each blastocyst was determined by calculating the number of TUNEL-positive blastomeres out of the total cell number.

Statistical analysis

Data were analyzed using JMP-7 software (SAS Institute Inc., 2004, Cary, NC, USA). Repeated measurement model was used to examine differences between bulls. One-way ANOVA (analysis of variance) procedure was used to determine the statistical difference among HPM and LPM groups. Data are presented as means ± SEM. A P-value < 0.05 was considered significant. Linear regression was analyzed to associate progressive motility (independent variables) to sperm physiological parameters and elemental concentrations (dependent variables). The regression model was determined by R^2 values. Regression parameters were considered statistically significant at $P < 0.05$. P-values between 0.05 and 0.1 were also reported as trends that might be real and worthy of note.

Results

Semen physiological traits

In fresh semen, motility, progressive motility, and proportion of sperm with normal morphology were higher ($P < 0.01$) in the HPM versus LPM group. Whereas the sperm concentration was lower in the HPM group than in the LPM group ($P < 0.05$), volume, velocity, motile sperm concentration and progressively motile sperm concentration did not differ between groups ($P > 0.05$, Table 1). In cryopreserved semen, the motility, progressive motility, motile sperm

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Table 1 Traits of fresh semen with high and low progressive motility (HPM and LPM, respectively)

Ejaculates (n)	PM (%)	Motility (%)	Morphology (%)	Concentration ($\times 10^3$/ml)	MSC[a] ($\times 10^3$/ml)	PMSC[b] ($\times 10^3$/ml)	Volume (ml)	Velocity (μm/s)
HPM (15)	85.1 ± 0.3*	97.0 ± 0.1*	90.1 ± 0.1*	1228.6 ± 217.5	1191.7 ± 211.1	1048.9 ± 187.0	3.9 ± 0.5	96.5 ± 2.9
LPM (15)	55.2 ± 1.8	62.1 ± 1.9	78.6 ± 0.9	1943.7 ± 103.9*	1204.8 ± 73.7	1070.4 ± 66.9	4.8 ± 0.4	108.2 ± 4.1

Ejaculates were routinely collected at the Israeli Artificial Insemination Center 'Sion' and evaluated by SQA-Vb, with progressive motility (PM) serving as the primary parameter.
[a]Motile sperm concentration.
[b]Progressively motile sperm concentration.
Data are presented as means ± standard error of the mean (SEM), $^*P < 0.01$.

Table 2 Traits of freeze–thawed straws with high and low progressive motility ejaculates (HPM and LPM, respectively)

Ejaculates (n)	PM (%)	Motility (%)	MSC[a] (M/ml)	PMSC[b] (M/ml)	Velocity (μm/s)
HPM (5)	36.5 ± 1.8*	48.1 ± 1.1*	53.9 ± 1.5*	40.9 ± 1.9*	32 ± 1.8*
LPM (5)	14.7 ± 1.4	39.7 ± 0.5	47.5 ± 0.6	17.5 ± 1.5	14.4 ± 1.2

A representative ejaculate was cryopreserved in 0.221 ml chilled straws ($n = 50$/group), each containing 20×10^6 sperms. Before *in vitro* fertilization, straws ($n = 5$/ejaculate) were evaluated by SQA-Vb to determine the precise progressive motility (PM) after freezing and thawing.
[a]Motile-sperm concentration.
[b]Progressively motile sperm concentration.
Data are presented as means ± standard error of the mean (SEM), $^*P < 0.01$.

concentration, progressively motile sperm concentration and velocity post-thawing were higher ($P < 0.01$) in HPM versus LPM semen (Table 2).

Sperm characteristics

Based on abnormal sperm morphology characteristics such as detached head and bent/coiled or double tail, the proportion of spermatozoa with abnormal morphology was higher ($P < 0.01$) in LPM than in HPM ejaculates (Table 3). The main morphological alteration, in both experimental groups, was bent or coiled tail, with a higher proportion ($P < 0.05$) in LPM than in HPM semen (Fig. 2*A* and Table 3).

Sperm viability did not differ between HPM and LPM ejaculates ($P > 0.05$, Table 3). The proportion of spermatozoa with damaged or intact acrosomes did not differ between HPM and LPM ejaculates. This was true for both dead and live spermatozoa (Table 3 and Fig. 2*B*).

Spermatozoa with high ΔΨm were characterized by accumulation and aggregation of JC-1 dye in the mitochondria, which fluoresces bright red. In spermatozoa with low ΔΨm, JC-1 dye remained in the cytoplasm in its monomer form and did not enter into the mitochondria (green fluorescence; Fig. 2*C*). The proportion of spermatozoa with high ΔΨm was higher ($P < 0.01$) in HPM than LPM spermatozoa (Table 3). Conversely, the proportion of TUNEL-positive spermatozoa did not differ between groups and was less than 1% (Fig. 2*D*).

Correlation between sperm physiological parameters and progressive motility

Correlations between semen parameters and progressive motility are presented in Table 4. In particular, motility, normal morphology parameters were highly correlated with progressive motility ($R^2 > 0.98$, $P < 0.001$). ΔΨm was moderately correlated with progressive motility ($R^2 > 0.5$, $P < 0.002$). In contrast, the correlation between concentration and progressive motility was relatively low ($R^2 = 0.3$, $P = 0.002$).

Elemental concentration in the seminal plasma

Data for the major elements in the seminal plasma are presented in Table 5. In general, elemental concentrations did not differ between HPM and LPM ejaculates. While not significant, the concentration of zinc in the seminal plasma was numerically higher in LPM versus HPM ejaculates ($P < 0.09$). Further analysis revealed a moderate correlation ($R^2 = 0.463$, $P = 0.03$) between zinc concentration and progressive motility.

IVF competence and embryo quality

The proportion of cleaved oocytes at 42 to 44 h postfertilization was higher for HPM than LPM ejaculates (88.1 ± 1.1 versus 80.5 ± 1.7, respectively, $P = 0.001$). Blastocyst formation on day 8 post fertilization was higher in the HPM versus LPM group (33.5 ± 1.6 versus 23.5 ± 2.2, respectively, $P = 0.002$).

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Table 3 Sperm characteristics in high and low progressively motile ejaculates (HPM and LPM, respectively)

	Abnormal morphology (%)						Intact acrosome (%)		
Ejaculates (*n*)	Total	Detached head	Bent/coiled	Other	Viability (%)	High ΔΨm (%)	Total	Dead[a]	Viable[a]
HPM (9)	11.4 ± 1.6	2.0 ± 0.8	8.7 ± 2.2	0.7 ± 0.3	82.0 ± 2.4	74.7 ± 1.9**	90.1 ± 1.9	15.7 ± 2.5	74.4 ± 3.8
LPM (9)	20.4 ± 1.7**	2.9 ± 1.8	16.6 ± 2.7*	0.9 ± 0.3	73.6 ± 3.6	60.3 ± 2.7	88.1 ± 0.6	15.1 ± 1.7	73.0 ± 1.6

Fresh undiluted samples were purified by Percoll gradient (45/90%). Purified spermatozoa were stained with Diff-Quick Staining Kit and subjectively analyzed for morphology. Viability was assessed by propidium iodide dye; membrane potential (ΔΨm) was characterized by JC-1 dye, and acrosome integrity was assessed by triple-fluorescence test.

[a]Dead or viable spermatozoa with intact acrosome.

For each assay, at least 200 spermatozoa per ejaculate were randomly analyzed.

Characteristics were evaluated under an inverted microscope (Nikon, Tokyo, Japan) at ×200 magnification.

Data are means ± standard error of the mean (SEM), $^{*}P < 0.05$, $^{**}P < 0.01$.

The proportion of blastocysts developed from cleaved embryos was higher in the HPM than LPM group (37.5 ± 1.9 versus 29.0 ± 2.6, respectively, $P = 0.016$, Fig. 3).

The total cell number in blastocysts ($n = 20$ embryos per group) did not differ between HPM and LPM groups (88.7 ± 9.3 and 92.4 ± 7.6, respectively, $P = 0.798$). The proportion of apoptotic cells ($n = 7$ embryos per group) did not differ between groups and was 5.6 ± 0.7% and 8.6 ± 1.4% for HPM and LPM groups, respectively ($P = 0.087$; Fig. 4).

Discussion

Reproductive management on modern dairy farms is based on artificial insemination using semen from highly fertile bulls. Therefore, an accurate evaluation of the ejaculated semen is highly important. Physiological parameters such as motility and morphology are commonly used to evaluate semen from bulls (Al-Makhzoomi *et al.*, 2008; Kumar *et al.*, 2012), stallions (Love, 2011) and boars (Waberski *et al.*, 1990). The findings of the current study suggest progressive motility is a reliable parameter for predicting Holstein bull semen quality and fertilization capacity, at least when combined with other parameters, such as IVF competence – HPM semen expressed higher IVF capacity, as indicated by increased developmental rate of preimplantation embryos. In support, Jedrzejczak *et al.* (2008) demonstrated that progressive motility can be an important parameter to be considered for IVF since IVF has a significantly better results with HPM semen. More fertilization studies are needed to determine the correlation between HPM and *in vivo* fertilization, as well as to clarify whether HPM and LPM values differ between breeds (*Bos taurus* versus *B. indicus*), seasons (winter versus summer) or upon exposure to stress.

It is well accepted that various parameters should be taken into account to evaluate semen quality (Kastelic & Thundathil, 2008; Kumar *et al.*, 2012; Vincent *et al.*, 2012), because any single parameter cannot accurately predict fertilization capacity (Kondracki *et al.*, 2011). For instance, ejaculate concentration has been suggested to influence the morphometric characteristics of boar sperm (Wysokińska *et al.*, 2009; Kondracki *et al.*, 2011). Similarly, bull ejaculate with 1000×10^3 sperm/mm^3 or less has a high proportion of morphologically malformed spermatozoa (Kondracki *et al.*, 2012). Conversely, morphologically normal sperm has been shown to be positively correlated with fertility (Al-Makhzoomi *et al.*, 2008). Here we report that LPM ejaculate is characterized by a higher concentration of sperm than HPM ejaculate, but with an increased proportion of morphologically

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Table 4 Correlations[a] between progressive motility and other sperm parameters

	Concentration[b]	Motility[b]	Objective morphology[b]	Subjective morphology[c]	ΔΨm[d]
R^2	0.304	0.997	0.989	0.495	0.569
P-value	0.002	0.001	0.001	0.002	0.002

High and low progressively motile ejaculates ($n = 9$/group) were routinely collected at the Israeli Artificial Insemination Center 'Sion' and submitted to objective and subjective evaluations.
[a]Correlation analysis included data from both high (>81%) and low (<62%) progressively motile ejaculates ($n = 15$/group).
[b]Data were from SQA-Vb.
[c]Data were from visual (microscopic) examination at ×200 magnification.
[d]Mitochondrial membrane potential, evaluated by JC-1 dye.

Table 5 Elemental concentrations (mg/l) in the seminal plasma as determined by inductively coupled plasma-atomic emission spectrometry

Ejaculates (n)	K	Na	Mg	Ca	Fe	Zn	Cu
HPM (5)	1169.0 ± 271.3	2190.4 ± 328.7	63.4 ± 6.3	367.4 ± 45.6	0.38 ± 0.13	2.9 ± 0.2	0.28 ± 0.03
LPM (5)	1322.8 ± 151.3	1889.4 ± 115.0	76.5 ± 7.7	279.2 ± 54.8	0.64 ± 0.12	3.8 ± 0.4	0.24 ± 0.05
P-value	0.634	0.413	0.495	0.251	0.171	0.088	0.518

High and low progressively motile ejaculates (HPM and LPM, respectively) were centrifuged and the seminal plasma was collected.
Data are means ± standard error of the mean (SEM).

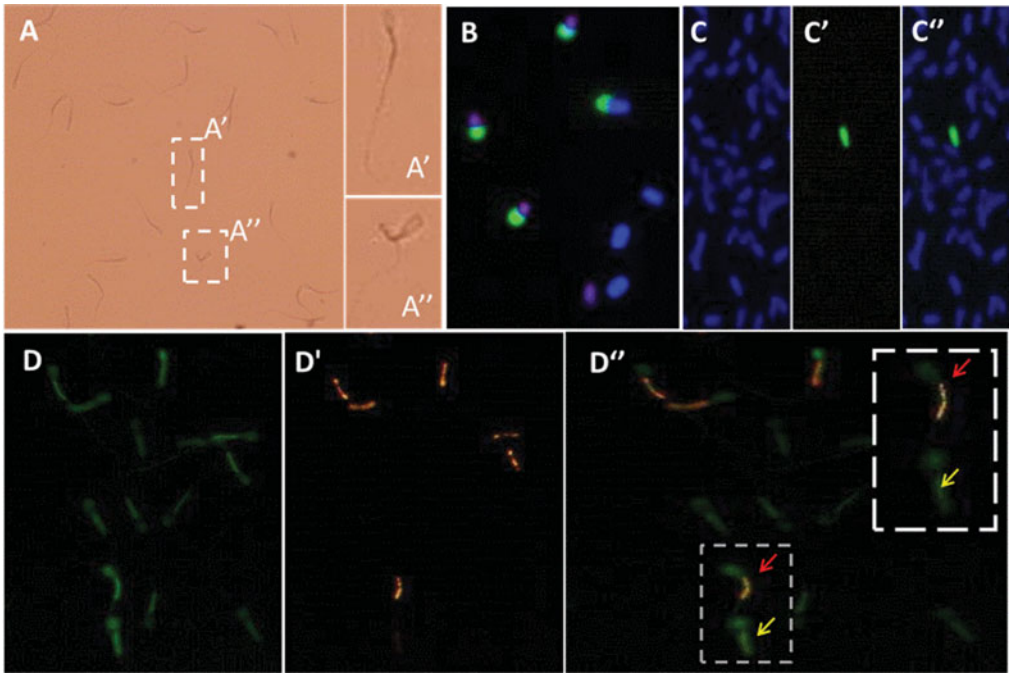

Figure 2 (*A*) Morphology of low progressive motility (LPM) spermatozoa stained with Diff-Quick dye and evaluated microscopically: (*A′*) bent tail, (*A″*) coiled tail. (*B*) Triple-fluorescence photomicrography of spermatozoa stained with Hoechst 33324, propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC–PNA). Presented are dead spermatozoa with intact acrosome (purple); dead sperm with damaged acrosome (purple + green); live spermatozoa with intact acrosome (blue); live spermatozoa with damaged acrosome (blue + green). (*C*) Representative picture of spermatozoon nuclei stained with Hoechst 33324 (blue fluorescence); (*C′*) TUNEL-positive (i.e. apoptotic) spermatozoon (green fluorescence); (*C″*) pictures *C* and *C′* merged. (*D*) Representative picture of mitochondrial membrane potential in spermatozoa stained with JC-1 (green fluorescence, monomer form); (*D′*) red JC-1 fluorescence (accumulated and aggregated form); (*D″*) merged picture of spermatozoa with high ΔΨm (red arrow) and low ΔΨm (yellow arrow).

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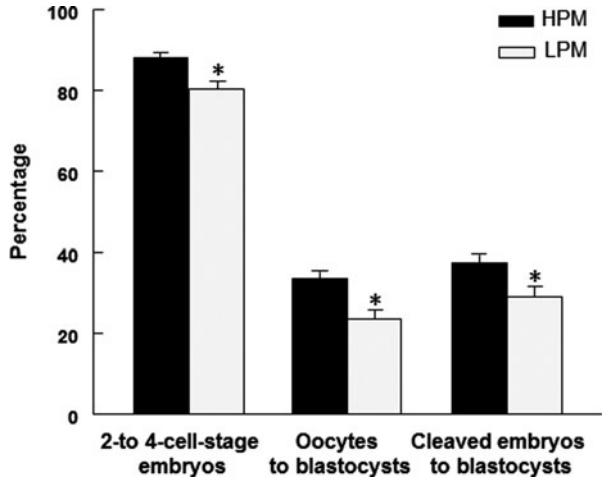

Figure 3 Embryonic development following fertilization with high and low progressively motile semen (HPM and LPM, respectively). Holstein cow ovaries were obtained from a local abattoir. Oocytes were aspirated, matured for 22 h and fertilized for 18 h with HPM or LPM Percoll-purified spermatozoa (~1 × 10^6). Putative zygotes were denuded of cumulus cells and cultured for 8 days. The experiment was repeated 10 times with ~100 oocytes per group per replicate. Presented are cleavage rate of oocytes that developed to 2- to 4-cell-stage embryos 42 h post fertilization and the percentage of oocytes that developed to the blastocyst stage 8 days postfertilization. Data are presented as means ± standard error of the mean (SEM), $^*P < 0.05$.

abnormal sperm, in particular with bent or coiled tails, suggesting that concentration alone is not a good predictor of semen quality. Given the high correlation between motility, progressive motility and morphology found in the current study, it seems that progressive motility might serve as a good predictor for semen quality before and after thawing, at least when combined with others parameters.

According to the bull breeding soundness evaluation (BBSE) of the Society for Theriogenology (SFT), a progressive motility of 30% is suggested as to be the threshold for potentially satisfactory breeder bulls (Alexander, 2008). Vincent *et al.* (2012) reported an average motility of 60.5% and progressive motility of 29.5% for bulls, without any difference between bulls with poor and average fertility. Assessment of sperm quality parameters in bulls of high (>60%) and low (20–35%) IVF outcome revealed a good correlation between motility, progressive motility and normal morphology after sperm thawing and IVF capacity, assessed by pronucleus formation; however, the regression parameters were not significant (Tanghe *et al.*, 2002). In the current study, a retrospective analysis of ejaculates collected for an entire month indicated that he highest and lowest values of progressively motile sperm per ejaculate were 87.4 and 32.5%, respectively. A comparison between the high and low tails of the distribution curve explored the association between progressive motility and IVF, expressed by higher cleavage rate and higher blastocyst formation for HPM. In support of this, both motility and normal morphology were relatively lower in LPM ejaculates. It should be noted however that differences between the above studies might be due to different cutoff values for high and LPM, different culture conditions and/or the type of analysis used for quality control (Lenz *et al.*, 2011).

Given that fertilization is a process that requires several sperm functions, it was reasonable to associate cellular semen characteristics rather than morphology with progressive motility. The findings of the current study revealed that ΔΨm is significantly higher in HPM than LPM groups, and correlated with progressive motility. A similar positive correlation between motility and ΔΨm has been reported by Paoli *et al.* (2011). In humans, spermatozoa with high ΔΨm are associated with high fertilization capacity, suggesting the importance of mitochondrial functionality for successful fertilization (Gallon *et al.*, 2006). Nevertheless, ΔΨm by itself cannot serve as a criterion for sperm quality because it is only moderately correlated with IVF outcome (Tanghe *et al.*, 2002).

Various elements are involved in bull spermatozoon function (Massányi *et al.*, 2004). For example, calcium plays a pivotal role in sperm's main functions – maturation, motility, and acrosome reaction (Darszon *et al.*, 2011). However, a previous study reported no significant differences in calcium concentration in semen of high versus low quality (López Rodríguez *et al.*, 2013). Similarly, in the current study, the seminal calcium concentration was only numerically higher and did not differ significantly between HPM and LPM seminal fluids. Nevertheless, given that the machinery is highly sensitive, these data are worthy of note. Calcium concentrations ≥0.22 mM are sufficient to induce hyperactivated motility, whereas concentrations ≥0.58 mM are required to induce an acrosome reaction and to obtain adequate binding of the sperm to the oocyte zona pellucida (Marin-Briggiler *et al.*, 2003). Moreover, an influx of extracellular and intracellular calcium is required for motility and velocity regulation (Alavi *et al.*, 2011), as well as acquisition of hyperactivated motility (Suarez *et al.*, 1993). Although not examined here, it is possible that intracellular calcium rather than seminal plasma calcium concentration underlies the differences in fertilization capacity between HPM and LPM ejaculates.

In boar semen, several seminal plasma elements are related to semen quality. In particular, sodium and chloride concentrations are higher in semen

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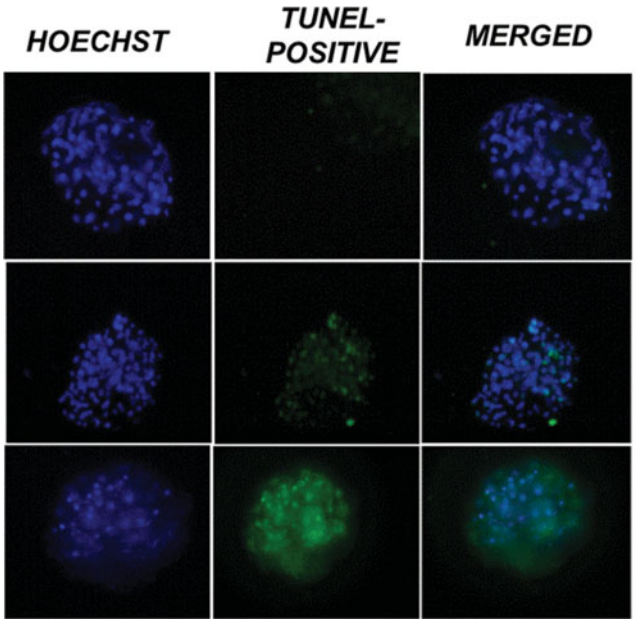

Figure 4 Representative pictures of 8-day blastocysts examined by TUNEL assay. Nuclei stained in blue, Hoechst 33324; TUNEL-positive nuclei stained in green. Presented are embryos with no (upper panel), low (middle panel), or high (lower panel) proportion of TUNEL-positive blastomeres.

defined as low quality, whereas zinc concentration is higher in semen of high quality. In support of this, zinc concentrations in the current study correlated with sperm progressive motility. The microelement zinc is closely associated with spermatogenesis and sperm's physiological functions (Bedwal & Bahuguna, 1994). In spermatozoa, zinc is predominantly localized in the outer dense fibres of the flagellum (Calvin, 1979). Zinc in the seminal plasma originates mainly from the prostate gland (Mann & Lutwak-Mann, 1981) and its concentration is correlated with motility parameters (Henkel *et al.*, 1999). Among its biological properties (Prasad, 1991), zinc is also a beneficial antioxidant factor (Prasad *et al.*, 2004), antibacterial agent (Fair *et al.*, 1976) and co-activator in DNA damage repair mechanisms (Ho & Ames, 2002), and it plays a role in sperm membrane stabilization (Lewis-Jones *et al.*, 1996). Nevertheless, the association between zinc concentration in the seminal plasma and sperm motility is still controversial. In humans, seminal zinc concentration is positively correlated with semen motility. High levels have been found in normozoospermia whereas low levels are found in asthenozoospermia (Dissanayake *et al.*, 2010; Atig *et al.*, 2012). In contrast, increased zinc level in the seminal plasma decreases sperm motility (Khan *et al.*, 2011). Similarly, high zinc concentration inhibits progressive motility of healthy human spermatozoa, with no influence on the percentage of motile spermatozoa (Sørensen *et al.*, 1999). Conversely, positive associations between zinc concentrations and motility (Kumar *et al.*, 2006) or progressive motility (Alavi-Shoushtari *et al.*, 2009) have been reported for bull semen. Taken together, it seems that seminal plasma zinc concentration by itself is not a good predictor of bull semen quality (Lewis-Jones *et al.*, 1996; Wiwanitkit, 2011).

Bovine semen is highly sensitive to cryopreservation, expressed by reduced viability, motility and velocity post-thawing (Lemma, 2011). In the current study, the proportion of progressively motile spermatozoa decreased during the freeze–thaw process for both HPM and LPM samples, by 2.4- and 3.7-fold, respectively, relative to fresh semen. These samples were used to examine fertilization capacity *in vitro*. Although spermatozoon movement and its interaction with the female genital tract cannot be mimicked *in vitro*, a fertilization assay based on IVF techniques seems to be a reliable method to test fertilization competence, in particular when combined with other semen evaluations (Larsson & Rodríguez-Martínez, 2000). The findings of the current study indicated a higher fertilization capacity for HPM versus LPM semen, reflected by a high proportion of oocytes that cleaved and developed to blastocysts. One explanation for these differences is that HPM spermatozoa successfully penetrate the cumulus cells surrounding the oocyte, presumably due to their ability to move straight forward. Further tests based on spermatozoon penetration (Gadea *et al.*, 1998) or binding to the zona pellucida (Zhang *et al.*, 1998) might confirm this assumption.

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Alternatively, the higher IVF capacity of HPM samples might be due to higher mitochondrial function (i.e. ΔΨm) as found in fresh HPM semen. Tollner *et al.* (2011) reported that post-thaw macaque spermatozoa exhibit only a slight decrease in progressive motility but a marked decrease in the ability to penetrate cervical mucus, presumably due to low energy. It is therefore reasonable to assume that in the current study, the ΔΨm remained higher in the post-thaw HPM semen relative to the LPM samples. This assumption is supported by the finding that not only the rate of first cleavages but also the proportion of cleaved oocytes that developed to the blastocyst stage were higher in the HPM versus LPM samples. It should be noted, however, that the correlations between *in vitro* and *in vivo* fertilization vary among studies. Some studies in cattle have reported a positive correlation between IVF capacity and field fertilization competence (Zhang *et al.*, 1997; Ward *et al.*, 2003) but others do not (Ohgoda *et al.*, 1988; Schneider *et al.*, 1999). Spermatozoa from bulls with superior field fertility display an increased ability to fertilize oocytes *in vitro* (Al Naib *et al.*, 2011). Conversely, it has been reported that the bull has no effect on IVF results when high spermatozoon concentration is used, but a clear effect at lower concentration (Kroetsch & Stubbing, 1992).

Conclusions

In bull, semen motility and morphology correlate with progressive motility. Spermatozoa with HPM exhibited a higher ΔΨm and zinc concentration in the seminal plasma and higher fertilization capacity than those with LPM *in vitro.* Further examination of whether progressive motility is also a good predictor for *in vivo* fertilization capacity is required.

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