Effect of liposome-containing diluent and centrifugation on motion parameters and membrane integrity of electroejaculated cooled bovine spermatozoa

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Abstract

The objectives were to evaluate the effect of OptiXcell® diluent and centrifugation on cooled bovine sperm viability. It was hypothesized that motility and membrane integrity of cooled spermatozoa diluted in OptiXcell® would be higher than in frozen semen for at least 48 h, and removing seminal plasma by centrifugation would improve cooled sperm viability. First, semen was collected from nine bulls and extended in OptiXcell®. An aliquot was frozen, while the remaining semen was refrigerated for 96 h. Motility and membrane integrity were higher in spermatozoa cooled for up to 48 h than in frozen semen. Sperm motility, VSL, DSL, STR and BCF did not differ between 0 and 72 h of cooling, but decreased at 96 h. Next, semen was collected from 11 bulls and refrigerated for 72 h with (control) or without (centrifuged) seminal plasma. Motion parameters did not differ with treatment. In non-centrifuged samples, motility, VAP, DSL and BCF decreased at 72 h. In centrifuged samples, motility decreased at 72 h and velocity at 48 h. Viability of cooled bovine semen diluted in OptiXcell® was superior than in frozen semen for 48 h. Removal of seminal plasma by centrifugation did not improve preservation of cooled sperm viability.

Keywords: Cryopreservation, bovine, centrifugation, motility, chilled semen

Introduction

The use of cooled shipped semen for artificial insemination is a routine procedure in the equine and canine industries.[1,2] Maintaining semen at refrigeration temperature lowers the metabolic rate of spermatozoa and decreases overgrowth of bacteria, extending the lifespan of spermatozoa.[3] In general, pregnancy rates achieved with cooled semen are higher than with frozen semen.[4,5] This is because freezing and thawing induce sperm damage associated with plasma membrane reorganization, accumulation of intracellular ions, osmotic stress, cryoprotectant toxicity and formation of intracellular ice crystals, altering sperm viability and function.[6,7] These changes in the sperm plasma membrane decrease the ability of spermatozoa to attach to oviductal epithelial cells and form an oviductal reservoir, decreasing their survival time within the female's reproductive tract.[8] Therefore, to achieve acceptable pregnancy rates with frozen semen, insemination of an appropriate number of spermatozoa and accurate timing of insemination close to ovulation are of utmost importance.[5]

In cattle, frozen semen is almost exclusively used for artificial insemination in most countries, except New Zealand where cooled semen is used in 95% of artificial inseminations.[3] Efficient use of frozen semen requires estrus synchronization and timed artificial insemination (TAI) or accurate estrus detection since timing of insemination with respect to ovulation is critical for fertilization. Unfortunately, ovulatory response to estrus synchronization protocols is variable in terms of percentage of cows ovulating and timing of ovulation.[9] This can have a negative impact on pregnancy rates after TAI with frozen semen. The higher survival of cooled spermatozoa in the cow's tract could allow for higher flexibility in the timing of insemination, improving pregnancy rates after TAI. The improved fertility would also allow for a reduction in

the number of spermatozoa per insemination dose, maximizing the number of doses obtained per ejaculate and optimizing the use of genetically superior bulls.[3] In addition, while a complete semen evaluation is still needed to ensure proper semen handling and packaging, the cost and complexity of semen processing for cooling is substantially reduced compared to freezing. Semen can be collected and processed for shipment at the farm, decreasing the costs associated with transporting, testing, and housing bulls in semen freezing centers. Furthermore, semen cooling could be an alternative for bulls with poor post-thaw sperm survival. On the other hand, bulls in a cooled semen program may need to have semen collected multiple times within a breeding season to meet the needs of producers requesting the shipments. This may increase labor at the farm during the breeding season.

In spite of the potential advantages of this technology, few studies have evaluated the use of cooled semen in cattle. A decrease in sperm motility, membrane integrity, acrosome integrity and pregnancy rate was observed when semen was cooled for 48 h in some studies.[10,11] However, sperm motility and *in vitro* fertilizing ability were preserved for 6 days in another study.[3] Differences in breed, semen extender, cryoprotectant, semen collection method and semen processing protocol may account for the variation in reported results. A noteworthy protocol difference in the later study was the removal of seminal plasma by centrifugation prior to cooling. This practice was shown to improve survival and membrane stability of stallion spermatozoa during cooling.[12,13] Furthermore, semen collection on-farm requires the use of electroejaculation since the facilities and personnel required for semen collection using an artificial vagina are rarely available. Electroejaculation yields samples with variable concentration of seminal plasma, and sometimes contaminated with urine. Therefore, centrifugation and removal of seminal plasma and contaminating fluids could improve quality of bovine cooled semen obtained by electroejaculation, or could prolong storage times. However, to date the effect of centrifugation on survival of cooled spermatozoa has not been critically evaluated in cattle.

Furthermore, the ability of liposome-containing diluents to support viability of cooled bovine spermatozoa has not been tested. Liposome-containing diluents, such as OptiXcell®, have been shown to provide improved cryoprotection during freezing and increased pregnancy rates compared with other semen diluents like Tryladil®, BioXcell® or TRIS-egg yolk.[14,15] Liposomes added to the semen extender can incorporate into the plasma membrane and change the lipid phase transition.[14] This decreases the sensitivity of spermatozoa to cooling and improves their survival.[14] In addition, OptiXcell® is a chemically defined diluent, free of animal proteins. The diluent is clear and free of particles, allowing accurate evaluation of sperm function using modern technologies, such as computer-assisted semen analysis or flow cytometry.

The objectives of this study were to evaluate the ability of the liposome-containing diluent OptiXcell® to support viability of cooled bovine spermatozoa during prolonged storage, to compare viability of cooled and frozen spermatozoa diluted in OptiXcell®, and to evaluate the effect of centrifugation on cooled sperm viability. It was hypothesized that motility and membrane integrity of cooled spermatozoa diluted in OptiXcell® would be higher than in frozen semen for at least 48 h, and that removing seminal plasma by centrifugation would improve sperm motility and membrane integrity during cold storage of electroejaculated bovine semen.

Materials and methods

Experiment 1: Motility and membrane integrity of cooled spermatozoa diluted in OptiXcell® compared with frozen semen

Nine 17-month old Angus bulls were included in this study. A cleanout semen collection was performed one week prior to the study. On the day of the study, one ejaculate was obtained from each bull. The cloudy sperm-rich fraction was collected using electroejaculation (Pulsator IV, Lane Manufacturing Inc., Denver, CO) during penis protrusion or erection. Sperm concentration was evaluated using a densimeter and sperm morphology was evaluated using phase contrast. The mean percentage of morphologically normal spermatozoa was 71.4±3.8%. Immediately after collection, 200 µl of an antibiotic cocktail (CSS Antibiotic Mix, IMV Technologies, Maple Grove, MN) was added per ml of raw semen. Each 200 µl of antibiotic cocktail contained 100 µg of tylosin, 500 µg of gentamycin and 300/600 µg of linco-spectin. Semen was then placed in a water bath at 34°C for 5 min. Pre-warmed (34°C) OptiXcell® (IMV Technologies) was added to a final concentration of 50 $x10^6$ spermatozoa/ml. The extended semen was placed in a refrigerator at 5°C for 5 h. After 5 h, pre-cooled 0.5 ml straws were loaded. The straws were held horizontally in a rack 3 cm above liquid nitrogen for 10 min. The straws were then plunged in the liquid nitrogen and stored at -196°C until evaluation. The remaining semen was maintained in the refrigerator at 5°C for 96 h. At 0, 24, 48, 72 and 96 h of cold storage, an aliquot of each sample was warmed to 38°C for 10 min for evaluation of sperm motility. In addition, membrane integrity was evaluated at 24 and 48 h. Two weeks after freezing, one straw from each sample was thawed in a water bath at 38°C for 30 s, and sperm motility and membrane integrity were evaluated after allowing the semen to warm at 38ºC for 10 min. Statistical analysis was performed using SAS package (SAS Institute, Cary, NC). Distribution of the data was tested for normality using a Shapiro Wilk test. Normally distributed data were expressed as mean ± SEM. Sperm motion parameters and membrane integrity were compared among treatments using ANOVA for repeated measurements. Paired comparisons were performed using a Tukey's test. Differences were considered significant if $P < 0.05$.

Experiment 2: Effect of centrifugation on motility and membrane integrity of cooled spermatozoa

Eleven Angus bulls (17 m to 3 y old) were included in the study. A cleanout semen collection was performed one week prior to the study. On the day of the study, one ejaculate was obtained from each bull. The cloudy sperm-rich fraction was collected using electroejaculation. Sperm concentration was evaluated using a densimeter, and sperm morphology was evaluated using phase contrast. The mean percentage of morphologically normal spermatozoa was 72.8±3.7%. An antibiotic cocktail (CSS Antibiotic Mix, IMV Technologies; 200 µl/ml of semen) was immediately added to the semen, which was placed in a water bath at 34°C for 5 min. Pre-warmed (34°C) OptiXcell® was added to a final concentration of 50 $x10^6$ spermatozoa/ml. Each ejaculate was divided into two aliquots. One aliquot was refrigerated without centrifugation (NC treatment). Sperm concentration in undiluted semen ranged from 156 to 998 $x10^6$ spermatozoa/ml (492.2±109.9 $x10^6$ spermatozoa/ml, mean±SD). The dilution ratio (semen:extender) in NC semen ranged from 1:2 to 1:19, with an average ratio of 1:8. The other aliquot was centrifuged at 800 x g for 10 min. The supernatant was removed and the pellet was resuspended in OptiXcell® to a final concentration of 50 $x10^6$ spermatozoa/ml (CE treatment).

Both aliquots were placed in a refrigerator at 5°C for 72 h. At 0, 24, 48 and 72 h of cold storage, an aliquot of each sample was warmed to 38°C for 10 min for evaluation of sperm motility. In addition, membrane integrity was evaluated at 24 and 48 h. Sperm motion parameters and membrane integrity were compared among storage times within treatment using ANOVA for repeated measurements. Paired comparisons were performed using a Tukey's test. Comparisons between treatments within storage time were done using a paired T test.

Evaluation of sperm parameters

Sperm motility was evaluated with a computer assisted semen analyzer (SpermVision Professional, Minitube of America, Verona, WI). The settings of the instrument were: field depth of view 20 μm, pixel to μm ratio 130 to 100, cell area 18 to 80 μm, frames acquired 30, frame rate 60 Hz, AOC cut off static cells 5 and DSL cut off 4.5 μm/s (progressive motility). Mean percentage of total (TMOT) and progressively (PMOT) motile spermatozoa was assessed from all spermatozoa present in 7 fields with a 20X phase-contrast objective. Other motion parameters analyzed were average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), wobble (WOB), curvilinear distance (DCL), straight line distance (DSL), and distance of average path (DAP). Membrane integrity was evaluated using fluorescence microscopy under a 40X objective. Spermatozoa were incubated for 10 min at 38°C with the vital stains SYBR14 and propidium iodide (PI; Live/dead kit, Minitube of America) following instructions from the manufacturer. All spermatozoa present in 10 fields were classified as membrane-intact (green fluorescence) or membrane-damaged (red fluorescence) by a built-in software within the CASA system (SpermVision Professional, Minitube of America), and mean percentages were reported. Phase contrast microscopy was used for assessment of sperm morphology. Spermatozoa were diluted 1:10 in formalin buffered solution (Animal Reproduction Systems, Chino, CA). A wet mount was prepared and 100 spermatozoa were classified based on their morphological characteristics under oil immersion at 100X magnification.

Results

Experiment 1: Motility and membrane integrity of cooled spermatozoa diluted in OptiXcell® compared with frozen semen

Total motility ($P<0.0001$), progressive motility ($P=0.0001$), DSL ($P=0.005$), VSL ($P=0.001$), STR ($P=0.009$) and BCF ($P=0.007$) differed significantly among treatments (Fig. 1). Total and progressive sperm motility did not differ for 72 h, but were lower at 96 h than 0, 24 and 48 h. Motility was higher in spermatozoa cooled for up to 48 h than in frozen semen. However, sperm motility did not differ between semen cooled for 72 or 96 h, and frozen. DSL, VSL, STR and BCF were lowest in spermatozoa cooled for 96 h. The percentage of spermatozoa with intact membranes did not differ among cooling times but was higher in semen cooled for 24 h (88.5 ± 3.3%) or 48 h (82.5 ± 3.9%) than frozen semen (42±7.6%) ($P<0.0001$). No other parameters differed with treatment ($P>0.05$).

Experiment 2: Effect of centrifugation on motility and membrane integrity of cooled spermatozoa

There was no difference in any of the parameters between centrifuged and non-centrifuged samples at any time ($P>0.05$). Among non-centrifuged samples, total motility

(P=0.0065), progressive motility (P=0.006), DSL (P=0.0068), VAP (P=0.005), VSL (P=0.003) and BCF (P=0.03) differed with time (Fig. 2). Total motility, progressive motility, VAP and BCF did not change for the first 48 h. However, these parameters decreased at 72 h. DSL was not different between 0 and 24 h, but decreased at 48 h. VSL decreased in all cooled samples compared with the initial value. The percentage of membrane-intact spermatozoa was not different between 24 h (88.5±3.3%) and 48 h (82.5±3.9%) of cooling in the presence of seminal plasma.

Among centrifuged samples, total motility (P=0.013), progressive motility (P=0.007), DCL (P<0.0001), DAP (P<0.0001), DSL (P=0.005), VCL (P<0.0001), VAP (P=0.0001), VSL (P=0.026), WOB (P=0.026) and ALH (P=0.029) differed with time (Fig. 3). Sperm motility did not differ between 0 and 48 h, but was lower at 72 h. DCL and VCL were higher at 24 h than the rest of the treatments. DAP and VAP decreased at 48 h. WOB was not different among cooled samples, but was lower at 24 h than 0 h. The percentage of membrane-intact spermatozoa was not different between 24 h (81.6±5.3%) and 48 h (77.5±4.7%) of cooling in the absence of seminal plasma.

Discussion

Use of frozen semen has become the standard for artificial insemination in cattle. However, the freezing process induces changes in sperm structure and function that alter their viability and longevity in the female reproductive tract. Because cooled semen does not undergo freezing and thawing, sperm damage is minimized resulting in higher viability and fertilizing ability.[3,16] This allows for a reduction in the insemination dose, optimizing the use of genetically superior bulls. It also increases pregnancy rates compared with frozen semen, improving the efficiency of TAI. However, the benefit of storing cooled bovine semen is limited to 24 h.[10,16,17] Extending the storage period would simplify the management of semen shipments and inseminations.

In an attempt to prolong storage time, egg yolk, lecithin or glycerol were added to the semen extender.[11,17] However, a significant decline in sperm motility and membrane integrity was still observed at 48 h. Pregnancy rates were lower after TAI with semen cooled for 48 h than frozen semen.[11] Oxidative stress and accumulation of reactive oxygen species during liquid preservation produced irreversible sperm damage, with a decrease in sperm motility and fertility.[11]

In this study, a liposome-containing diluent (OptiXcell®) provided appropriate cryoprotection to preserve sperm motility during cooling for a longer period than previously reported.[10,11,16,17] Sperm motion parameters and membrane integrity were better preserved in semen cooled for up to 48 h than in frozen semen. While a decline was observed after 72 or 96 h, motion parameters were still comparable with frozen semen. OptiXcell® is a chemically defined commercial diluent that contains liposomes and is free of animal proteins. The diluent is clear and free of particles, allowing accurate evaluation of sperm function using modern technologies, such as CASA or flow cytometry. In previous studies, this extender provided improved cryoprotection during freezing and increased pregnancy rates compared with Tryladil®, BioXcell® or TRIS-egg yolk.[14,15] Liposomes added to the semen extender can incorporate into the plasma membrane and change the lipid phase transition.[14] This decreases the sensitivity of spermatozoa to cooling and improves their survival.[14]

Prolonged storage of cooled semen for 6 days was reported using a catalase containing semen extender.[3] While the longer preservation of sperm function could be attributed to semen

extender composition, semen was also centrifuged and seminal plasma was completely removed prior to cooling.[3] High concentrations of seminal plasma in cooled stallion semen can be detrimental to sperm quality and fertility.[12,13,18-20] Centrifugation and removal of seminal plasma improved equine sperm motility after storage times of more than 24 h,[12] and decreased production of reactive oxygen species and degradation of DNA.[19,21] In bulls, a high molecular weight fraction of seminal plasma has been shown to reduce sperm motility and viability.[22] Therefore, seminal plasma was removed in the second part of this study in an attempt to improve sperm motility and membrane integrity during cooling. However, removal of seminal plasma did not improve semen quality during cold storage under the conditions of this study.

Sperm velocity (DCL, VCL, DAP, VAP) in centrifuged samples was generally higher at 24 h of cooling than at any other time points, including initial values. In a previous study, addition of glycerol to the medium increased sperm velocity during cooling.[17] It was thought that glycerol induced cellular dehydration, making spermatozoa lighter and increasing velocity.[17] It could be speculated that incubation of spermatozoa for 24 h in OptiXcell®, which contains glycerol, allowed for a more prolonged equilibration time with subsequently more dehydration and lighter spermatozoa.[23] Also speculative, the prolonged equilibration could have allowed for more incorporation of glycerol into the cell, which could then be metabolized acting as an additional energy source.[24,25] More prolonged cooling may result in cell damage, possibly explaining why the same increase in velocity was not observed at 48 or 72 h. This initial increase in velocity was not observed when semen was cooled in presence of seminal plasma. It is possible that seminal plasma proteins adsorbed to the plasma membrane stabilized the membrane and decreased its permeability to water or glycerol.

This was the first study evaluating use of OptiXcell® for bull semen cooling, and the effect of removing seminal plasma on motility of cooled spermatozoa. Cooled bovine semen diluted in OptiXcell® remained suitable for artificial insemination for up to 48 h, as assessed by sperm motion parameters and membrane integrity. During this period, sperm motility and membrane integrity were superior in cooled semen than frozen semen. Removal of seminal plasma by centrifugation did not improve preservation of sperm motion or membrane integrity in cooled semen, and therefore is not necessary. The effect of these findings on pregnancy rate, and the performance of semen processed using the protocol tested here in a TAI program need to be evaluated.

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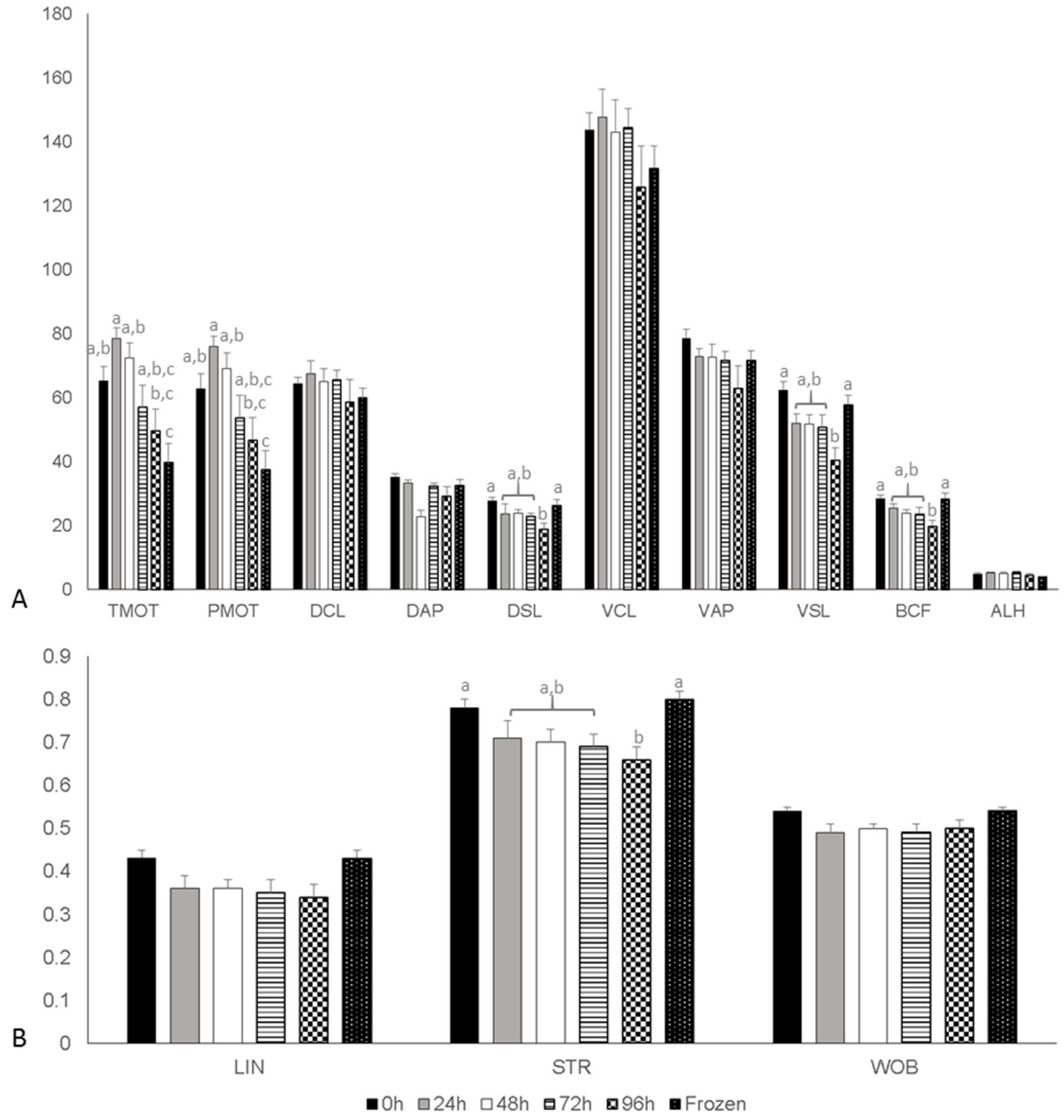

Fig. 1. Motion parameters of bovine spermatozoa cooled for 96 h or frozen (n=9). [a,b,c]P<0.05. TMOT=total motility (%), PMOT=progressive motility (%), DCL=curvilinear distance (μm), DAP=distance of average path (μm), DSL=straight line distance (μm), VCL=curvilinear velocity (μm/sec), VAP=average path velocity (μm/sec), VSL=straight line velocity ((μm/sec), LIN=linearity, STR=straightness, WOB=wobble, BCF= beat cross frequency (hertz), ALH= amplitude of lateral head displacement (μm).

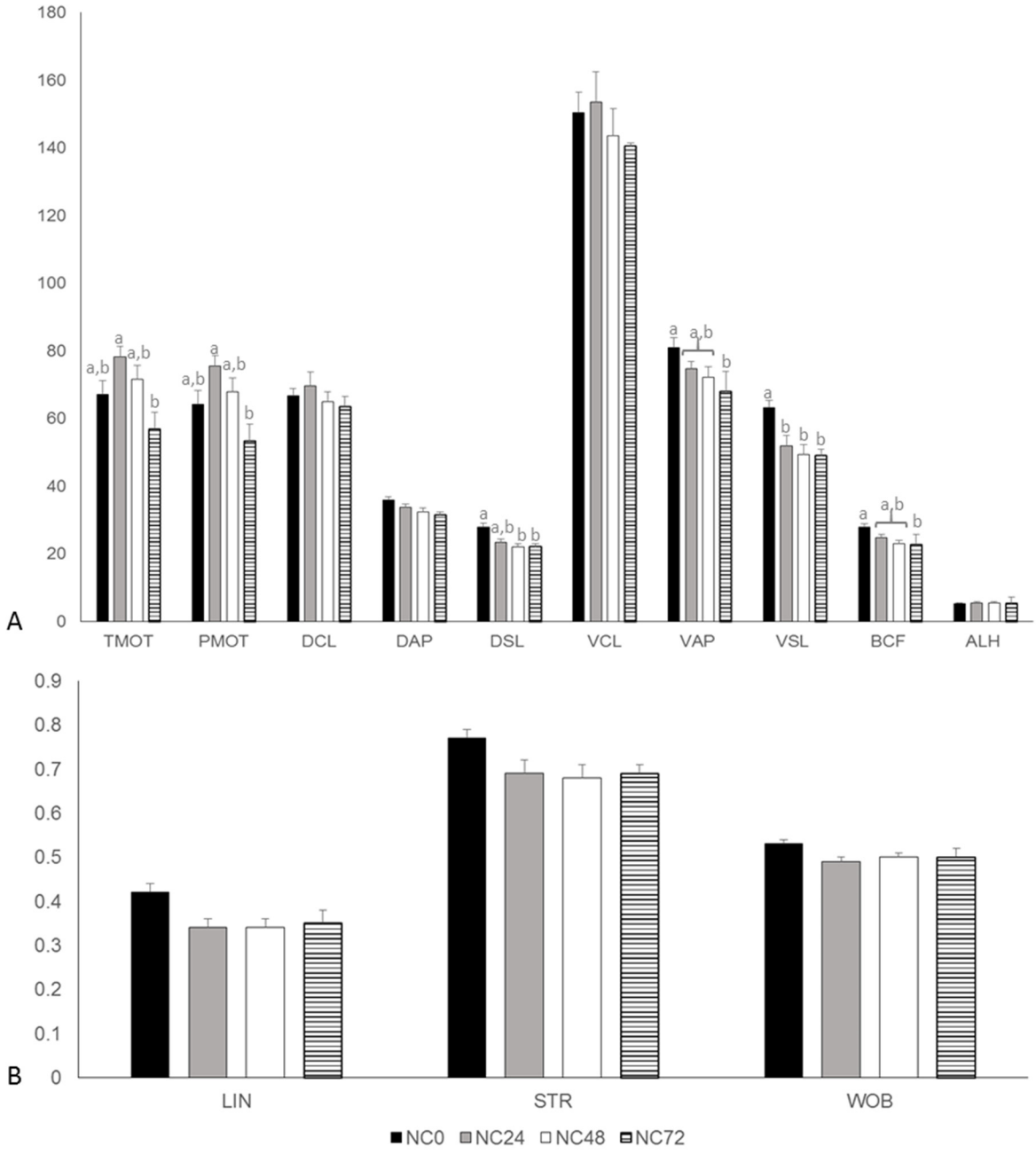

Fig. 2. Motion parameters of bovine spermatozoa cooled for 72 h without centrifugation (n=11). [a,b]P<0.05. TMOT=total motility (%), PMOT=progressive motility (%), DCL=curvilinear distance (μm), DAP=distance of average path (μm), DSL=straight line distance (μm), VCL=curvilinear velocity (μm/sec), VAP=average path velocity (μm/sec), VSL=straight line velocity ((μm/sec), LIN=linearity, STR=straightness, WOB=wobble, BCF= beat cross frequency (hertz), ALH= amplitude of lateral head displacement (μm).

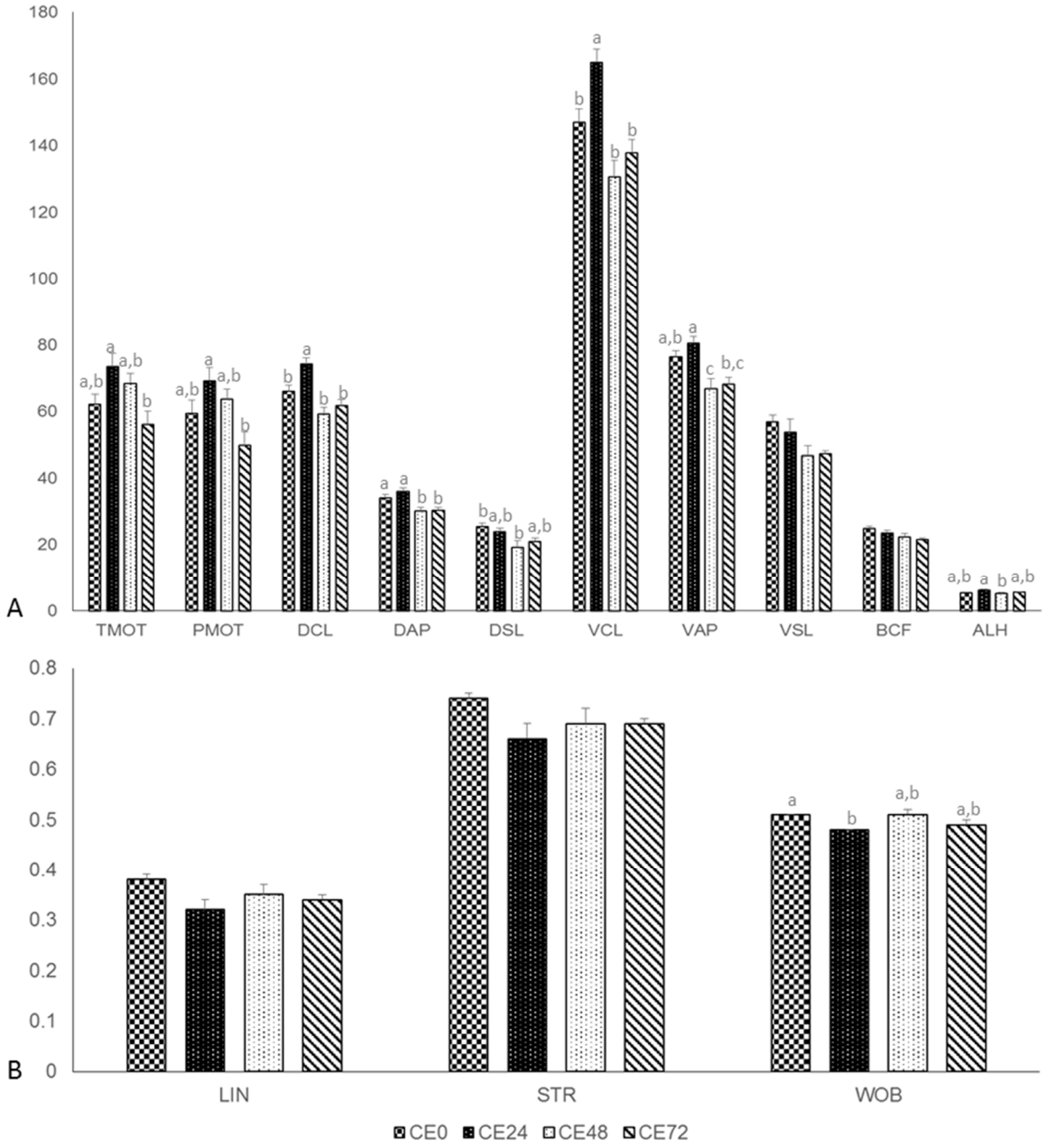

Fig. 3. Motion parameters of centrifuged bovine spermatozoa cooled for 72 h (n=11). [a,b]P<0.05. TMOT=total motility (%), PMOT=progressive motility (%), DCL=curvilinear distance (μm), DAP=distance of average path (μm), DSL=straight line distance (μm), VCL=curvilinear velocity (μm/sec), VAP=average path velocity (μm/sec), VSL=straight line velocity ((μm/sec), LIN=linearity, STR=straightness, WOB=wobble, BCF= beat cross frequency (hertz), ALH= amplitude of lateral head displacement (μm).