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—Original Article—

Reconsideration of the evaluation criteria for bull ejaculated sperm motility in the context of rotation

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Abstract. Progressive movement of spermatozoa has conventionally been regarded as a good indicator of motility. However, bull spermatozoa exhibit two types of progressive movement: progressive/planar movement without rotation and progressive/helical movement with rotation. The aim of this study was to reconsider the evaluation criteria of bull ejaculated sperm motility in the context of rotation. Here, we compared the movement patterns of ejaculated spermatozoa with relatively high and low protein kinase A (PKA)-mediated signaling activities, because sperm motility is positively regulated by PKA-mediated signaling activities. We prepared sperm samples with high and low PKA-mediated signaling activities by suspending spermatozoa in media containing either the stimulator ($NaHCO_3$) or inhibitor (KH-7) of adenylyl cyclase 10, and we then investigated movement patterns and relative velocities using a microscopic high-speed camera and recording system. In the control medium without $NaHCO_3$ and KH-7, most spermatozoa exhibited round/planar movement without rotation and asymmetrical bends in the principal pieces. $NaHCO_3$ significantly promoted changes in movement patterns from round/planar movement to progressive/planar movement (without rotation) as well as symmetrization of flagellar bends and increased relative velocities. KH-7 significantly increased spermatozoa exhibiting progressive/helical movement (with rotation), decreased relative velocities, and symmetrized flagellar bends with a reduction in their size. These indicate that progressive/planar movement (without rotation) and fast movement characterize the movement patterns of bull ejaculated spermatozoa with high PKA-mediated signaling activities. A sign of reduced PKA-mediated signaling activity is not only slow movement but also helical movement (with rotation). Thus, it is beneficial to add a new parameter of "rotation" to the evaluation criteria of bull ejaculated sperm motility.

Key words: cAMP, Bicarbonate, Flagellar bend, KH-7, Motility assay

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In bovine reproduction, artificial insemination (AI) with frozen-thawed spermatozoa is commonly used as a well-established biotechnology method for the production of calves. At the selection stage of freshly ejaculated spermatozoa, which are used for the production of frozen spermatozoa in AI stations, sperm motility is first evaluated to determine sperm quality. Furthermore, in the examination for the selection of lot numbers of straws of frozen spermatozoa, some of the testing straws are thawed and subjected to the evaluation of sperm motility. Thereafter, the remaining straws with the same lot numbers as the testing straws that have passed the examination of sperm motility are used for AI [1, 2]. Motility of the spermatozoa is one of the most important characteristics associated with their fertilizing ability. As the current evaluation criteria of bull sperm motility are based on the degrees of progressive movement (linearity and straightness) and movement velocities, progressive and fast movement is usually recognized to be as good motility [3, 4]. Progressive movement of frozen-thawed spermatozoa is characterized mainly by three-dimensional helical movement with rotation around the long axis [5, 6]. Moreover, a detailed investigation indicated that helical waves of the flagellum are balanced by counter-rotation of the head; consequently, bull frozen-thawed spermatozoa are likely capable of moving in a forward direction with relatively high linearity [6]. However, we previously found that bull ejaculated (unfrozen) spermatozoa typically exhibit progressive motility with the two-dimensional planar movement of flagella [7], and there are two types of progressive movement patterns, "progressive/helical movement and progressive/planar movement", in bull spermatozoa. These indicate that there are differences in the main patterns of progressive movement between bull ejaculated (unfrozen) and frozen-thawed spermatozoa, and specific evaluation criteria of motility should be set for each kind of spermatozoa, including ejaculated (unfrozen) and frozen-thawed spermatozoa. Thus, we decided to reconsider the evaluation criteria for motility of bull ejaculated (unfrozen) spermatozoa in the context of rotation.

In the spermatozoa of livestock, a regulatory system to exhibit normal motility is gradually developed during the transit through the epididymis [8–10] by a complicated mechanism including modulation of glycogen synthase kinase-3 activities [11, 12], decrease of the

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sperm mitochondrial Ca^{2+} uptake rates [13], and the interaction with cauda epididymal proteins [14]. In the cauda epididymidis, however, sperm movement is quiescent by the exposure to weakly acidic luminal fluid containing stabilizing factors [10, 15]. Upon ejaculation, the flagellar movement of mammalian spermatozoa is stimulated by bicarbonate from male accessory genital gland secretions and female reproductive fluids. Bicarbonate is absorbed into the sperm cytoplasm through ion transporters and subsequently binds to and activates adenylyl cyclase 10 (ADCY10) to generate cyclic adenosine 3',5'-monophosphate (cAMP) [10, 16]. The importance of cAMP in the regulation of bull sperm motility was previously demonstrated by showing that frequencies of flagellar beating and percentages of motile cells increased in adenosine 5'-triphosphate (ATP)-reactivated detergent-lysed spermatozoa in response to cAMP [17]. The increased cAMP activates protein kinase A (PKA) that binds to A-kinase anchoring proteins and phosphorylates motility-regulatory proteins in the principal piece [7, 18, 19]. Additionally, in marine invertebrate spermatozoa, protein phosphorylation is observed in the 15–22 kDa light chains (including Tctex2-related protein) and heavy chains of axonemal dynein motor proteins at the activation of flagellar movement [20, 21]. Considering the above, we compared movement patterns of spermatozoa with relatively high and low PKA-mediated signaling activities in this study.

We prepared bull ejaculated (unfrozen) spermatozoa with high and low PKA-mediated signaling activities by suspension in the medium containing $NaHCO_3$ and an ADCY10 inhibitor (KH-7), respectively. Next, we investigated their movement patterns and measured their relative velocities in order to reconsider the evaluation criteria for motility of bull ejaculated (unfrozen) spermatozoa.

Materials and Methods

Preparation of sperm samples

All reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan), unless specified otherwise. Collection of ejaculates was performed with the permission of the Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries (Technology Center) for the research project 'Improvement of fertility assay for Japanese Black bull spermatozoa (2016–2019)'. Ejaculated spermatozoa were collected using an artificial vagina from 27 Japanese Black bulls (> 1 year old), which were kept in the Technology Center, and then transported to the laboratory of Kobe University within 3 h at 25–30 °C. After the examination of sperm characteristics, surpluses of ejaculated spermatozoa were used in this study.

We prepared bull ejaculated (unfrozen) spermatozoa with high and low PKA-mediated signaling activities as described below. Ejaculated spermatozoa were washed three times in phosphate-buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA) by centrifugation at 700 × *g* for 5 min each time. They were suspended in the modified Krebs-Ringer Hepes (mKRH) medium (pH 7.4, lacking $CaCl_2$ and bovine serum albumin) that was composed of 94.0 mM NaCl, 1.19 mM $MgSO_4$ $7H_2O$, 1.19 mM KH_2PO_4, 4.78 mM KCl, 25.07 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 27.64 mM glucose, 50 mg/ml streptomycin sulfate (Nacalai Tesque, Kyoto, Japan), and 100 IU/ml penicillin G potassium salt (Sigma-Aldrich). The medium contained either $NaHCO_3$ (5, 10, or 20 mM, a stimulator of ADCY10), Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-cyclic monophosphorothioate (cBiMPS, 50 or 100 μM, a cell-permeable, phosphodiesterase-resistant cAMP analog, Enzo Life Sciences, Farmingdale, NY, USA), or KH-7 (10 or 50 μM, Tocris Bioscience, Bristol, UK, an inhibitor of ADCY10) to adjust the sperm concentration to 2.5×10^7 cells/ml. $NaHCO_3$, cBiMPS, and KH-7 were dissolved in Milli Q water, 10% (v/v) dimethyl sulfoxide (DMSO), and 100% (v/v) DMSO (solvents), respectively, as stock solutions (concentrations; 200 mM, 4 mM, and 25 mM, respectively) and then added to the medium. The final concentrations of the solvents were equalized among all samples within the same experiments. These sperm samples were used for the investigation of sperm movement patterns, trajectory of sperm movement and flagellar bends, and for the extraction of sperm proteins for Western blotting. In addition, all procedures for the sample preparation were performed at room temperature, and all of the prepared samples were used for the following experiments within 13 min.

Investigation of sperm movement patterns and measurement of sperm relative velocities

A drop of the sperm suspension (10 μl) was put on the glass chamber used for the sperm motility assay with the depth of 50 μm (Fujihira Insustry, Tokyo, Japan) that was on a heated stage (38.5˚C) of a bright-field and upright microscope (Olympus, Tokyo, Japan) and covered with coverslips (17 mm × 17 mm, Matsunami Glass Industries, Kishiwada, Japan). Movies of the spermatozoa on the chamber were captured using either a CMOS camera (BU238M, Toshiba Teli, Tokyo, Japan) or CCD camera (CS230B, Olympus) and recorded with a recorder for non-compressed movies (FCR-1, TechnoScope, Saitama, Japan) or HDD & DVD Hi-vision recorder (Regza RD-R100, Toshiba, Tokyo, Japan) at the frame rate of 30 Hz. The focus of the camera was set to the intermediate position between the coverslip and bottom of the glass chamber. The movies were converted into the sequential frame images (JPEG images) using the software "Free Video to JPG Converter" (https://free-video-to-jpg-converter.jp.uptodown.com/windows). Movement patterns (progressive/planar movement, progressive/helical movement, round/planar movement, round/helical movement, other movement including beating only, and no movement) were determined for approximately 100 spermatozoa on sequential images that were played back frame-by-frame using Windows Media Player (Microsoft, Redmond, WA, USA). Moreover, relative velocities of spermatozoa were measured as described in our previous report [7]. Briefly, video frames (#0−5) of the movies (for 15/30 sec) were converted to JPEG-formatted images every 3/30 sec [six frames; time 0/30 sec (#0), 3/30 sec (#1), 6/30 sec (#2), 9/30 sec (#3), 12/30 sec (#4), and 15/30 sec (#5)]. The positions of the sperm connecting piece on the frames (coordinates of the positions of the frames #0–5 were [X#0–5, Y#0–5]; e.g., the coordinates of the position of the frame #3 were [X#3, Y#3]) were obtained by using Microsoft PowerPoint 2013, Japanese Version (Microsoft). Next, migration length (for 15/30 sec) was calculated in each spermatozoon according to the following numerical formula.

Migration length of each spermatozoon
= square root $[(X\#0 - X\#1)^2 + (Y\#0 - Y\#1)^2]$ + square root $[(X\#1 - X\#2)^2 + (Y\#1 - Y\#2)^2]$ + square root $[(X\#2 - X\#3)^2$

+ (Y#2 – Y#3)^2] + square root [(X#3 – X#4)^2 + (Y#3 – Y#4)^2] + square root [(X#4 – X#5)^2 + (Y#4 –Y#5)^2]

Then, the relative velocity was calculated in each spermatozoon according to the following numerical formula.

The relative velocity of spermatozoon = migration length (for 15/30 sec) of each spermatozoon / the average migration length (for 15/30 sec) of control samples.

The obtained results were shown as the average of relative velocities.

Investigation of trajectory of sperm movement

The sperm suspension (100 µl), to which SYBR14 (final concentrations, 0.3–0.6 µM, Molecular Probes, Eugene, OR, USA) was added for the staining of sperm nuclei, was put on pre-warmed silicon-coated glass slides (Matsunami) and covered with coverslips (50 mm × 24 mm, Matsunami) very gently in order to maintain the space for sperm swimming. The sperm preparations were observed on a heated stage (38.5˚C) of an upright microscope equipped with epifluorescence (U-FBW mirror unit composed of BP460-495 excitation filter, DM505 dichroic mirror, and BA510IF emission filter, Olympus), and trajectories of sperm movement were imaged using a CCD camera (DP73, Olympus) for the exposure time of 1 sec.

Investigation of flagellar bends

The sperm suspension (25 µl) was put on the pre-warmed silicon-coated glass slides, covered with coverslips (50 mm × 24 mm), and the sperm preparation was put on a heated stage (38.5˚C) of a bright-field and upright microscope. Movies of the spermatozoa were captured with a CMOS camera and recorded with a recorder for non-compressed movies at the frame rate of 100 Hz. The movies were converted into the sequential frame images (JPEG images) in order to determine types of flagellar bends (symmetrical bend and asymmetrical bend). The principal bend direction was defined by the side of the beat where the flagellum made the greatest excursion and where it developed the greatest extreme of curvature; the opposite direction of the principal bend was the reverse bend direction, as previously described by Schmitz *et al.* [22].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Procedures of polyacrylamide gel electrophoresis under reducing conditions and Western blotting were described previously [23, 24]. In this study, we prepared extracts from the sperm suspensions that were not pre-warmed. In electrophoresis and Western blotting, we used 12.5% polyacrylamide gels, pre-stained standards (Precision plus protein standards, dual color) (BioRad Laboratories, Hercules, CA, USA), rabbit anti-serine/threonine-phosphorylated protein kinase A substrate polyclonal antibody (1:1000, Cat#9621, Cell Signaling Technology, Danvers, MA, USA), and horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin polyclonal antibody (1:2500, Cat#NA934V, GE Healthcare UK, Buckinghamshire, UK). Western blots were imaged in grey scale using an AE-9300H Ez-Capture MG chemiluminescence imaging system (ATTO, Tokyo, Japan), and the obtained images were used for densitometric analyses with CS Analyser Version 3.00.1022 (ATTO). After immunodetection, the membranes were washed thoroughly with methanol and MilliQ water to strip bound antibodies and blocking agents, and the sperm protein bands were visualized by staining with Quick-CBB PLUS. Images of CBB-stained were taken with the scanner.

Statistical analyses

Data were subjected to one-way analysis of variance (ANOVA) after arc-sine transformation. When F-test results were significant in ANOVA, individual mean values were further tested by Tukey's multiple range tests [25]. All statistical analyses were performed using BellCurve for Excel (Version 2.14, Social Survey Research Information, Tokyo, Japan), which was add-in software for the Japanese version of Microsoft Excel 2013 (Microsoft). The level of significance was set at $P < 0.05$.

Results

Phospho-PKA substrate proteins in the spermatozoa suspended in the medium containing $NaHCO_3$ or KH-7

In order to confirm the effects of $NaHCO_3$ and KH-7 on the PKA-mediated signaling activities of bull ejaculated spermatozoa, we detected phospho-PKA substrate proteins of the spermatozoa by Western blotting (Fig. 1-a). Effects of sperm suspended in the medium containing either 20 mM $NaHCO_3$ or 50 µM KH-7 on the Western blotting patterns could be observed clearly in 16-kDa bands, the molecular size of which was equivalent to that of the light chain of the axonemal dynein [21]. Average densities of the 16-kDa band detected in three experiments [relative densities to the densities of the band in the control samples (0 mM $NaHCO_3$ and 0 µM KH-7)] were 131 ± 25% (means ± standard deviations) for the spermatozoa suspended in the medium containing 20 mM $NaHCO_3$ and 60 ± 19% for the spermatozoa suspended in the medium containing 50 µM KH-7.

Movement patterns of the spermatozoa suspended in the medium containing $NaHCO_3$ or KH-7

Fig. 1-b (movies, see the movie files which can be downloaded from the journal homepage) and Fig. 1-c (trajectory of sperm movement) show examples of movement patterns of the spermatozoa suspended in the medium containing either 20 mM $NaHCO_3$ or 50 µM KH-7. Figures 2–4 show results of classification of movement patterns and measurement of relative velocities in the spermatozoa suspended in the medium containing $NaHCO_3$ (5–20 mM) or KH-7 (10 or 50 µM). In addition, Fig. 2 also includes the data of the spermatozoa suspended in the medium containing the cAMP analog cBiMPS. As shown in the upper panels of Fig. 2, 84.0% of spermatozoa exhibited round/planar movement (without rotation) in the control samples (0 mM $NaHCO_3$). Interestingly, the direction of movement was clockwise in almost all spermatozoa (when they were observed using the upright microscope) (Table 1). The addition of $NaHCO_3$ (5–20 mM) to the medium significantly promoted changes in the movement patterns from round/planar movement to progressive/planar movement (without rotation), decreased the degree of curve in the round movement of many spermatozoa, enlarged the size of trajectories of round movement, and increased the relative velocities in a dose-dependent manner [Figs. 1-b, 1-c, and 2 (upper panels)]. These changes were also induced by the addition of cBiMPS (50 and 100 µM) to the medium [Fig. 2 (lower panels)]. Although the

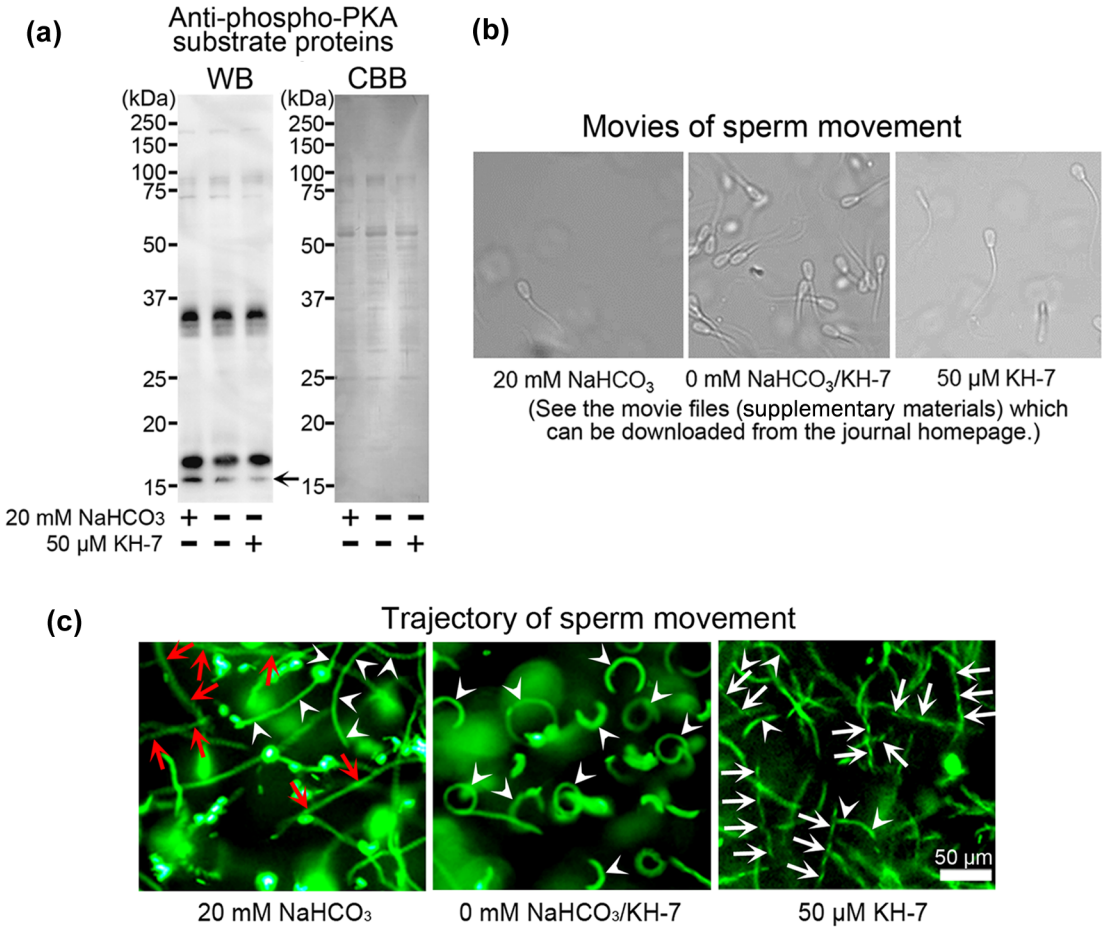

Fig. 1. Phospho-PKA substrate proteins and motility in bull ejaculated spermatozoa that were suspended in the medium containing either $NaHCO_3$ or KH-7. Bull ejaculated spermatozoa were washed and suspended in the medium with either 20 mM $NaHCO_3$, 50 μM KH-7, or without $NaHCO_3$ and KH-7. These samples were used for the immunodetection of phospho-PKA substrate proteins by Western blotting (panel a, n = 3), observation of movement patterns (panel b, n = 4), and trajectory of sperm movement (panel c, n = 3). Each panel is a representative result of repeated experiments. Red arrows in panel c indicate the trajectories of spermatozoa exhibiting progressive/planar movement. White arrow heads indicate the trajectories of spermatozoa exhibiting round/planar movement. White arrows in panel c indicate the trajectories of spermatozoa exhibiting progressive/helical movement.

addition of $NaHCO_3$ was accompanied by increased ion levels (osmotic pressure) and pH in the medium, these side-effects, which were mimicked by the addition of NaCl (Supplementary Fig. 1: online only) and NaOH (Supplementary Fig. 2: online only), had no effect on the changes in the movement patterns. Moreover, the effects of $NaHCO_3$ (20 mM) on the movement patterns were reduced by the addition of KH-7 to the medium (10 and 50 μM, Fig. 3). These results suggest that the effects of adding $NaHCO_3$ to the medium on the movement patterns are primarily dependent on the enhancement of PKA-mediated signaling activities (Fig. 1-a).

The addition of KH-7 (50 μM) to the medium significantly decreased the spermatozoa exhibiting round/planar movement (without rotation), decreased the degree of curve in the round movement of many spermatozoa, and increased the spermatozoa exhibiting progressive/helical movement (with rotation). Relative velocities were significantly decreased (Figs. 1-b and 4). Furthermore, trajectories of spermatozoa that exhibited progressive/helical movement were observed as the straight and short dashed lines (Fig. 1-c). These results indicate that changes of movement patterns from round/planar movement (without rotation) to progressive/helical movement (with rotation) are induced by the reduction of PKA-mediated signaling activities (Fig. 1-a), and progressive movement is not always a sign of good motility.

Flagellar bends of spermatozoa suspended in the medium containing $NaHCO_3$ or KH-7

Figure 5 shows examples of frame images (JPEG images, every 2/100 sec or 1/100 sec) into which movies of motile spermatozoa were divided. In the spermatozoa that exhibited progressive/planar movement in the medium containing 20 mM $NaHCO_3$, the principal piece of the flagellum bent almost symmetrically with a relatively large amplitude. However, in the spermatozoa that exhibited round/planar movement in the control medium without $NaHCO_3$ and KH-7, one-side bends (reverse bends) became smaller and resultantly flagellar

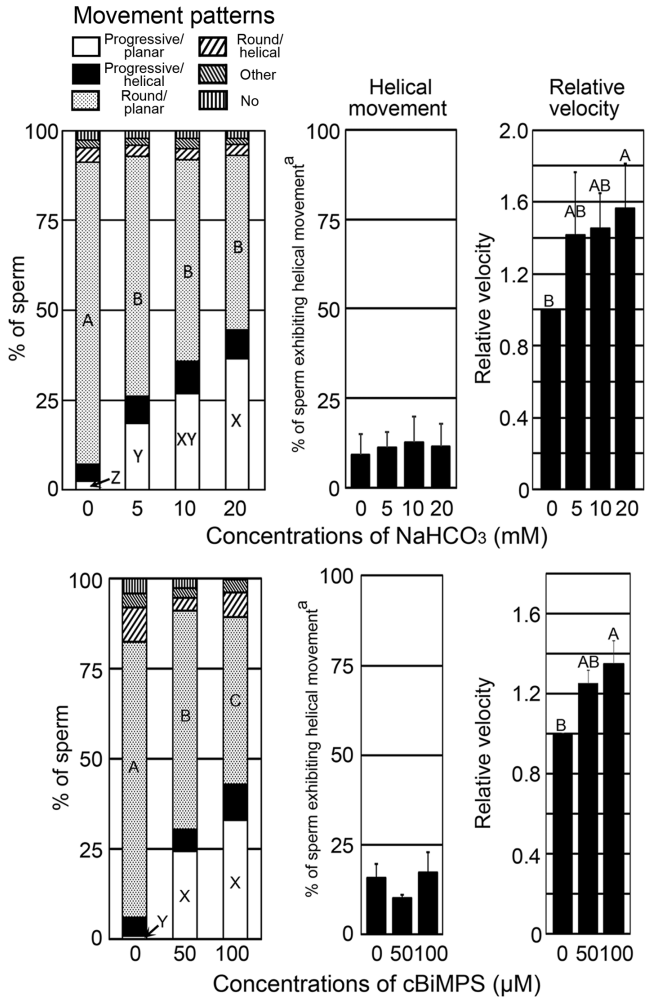

Fig. 2. Movement patterns and relative velocity of bull ejaculated spermatozoa that were suspended in the medium containing either $NaHCO_3$ or cBiMPS. Bull ejaculated spermatozoa were washed and suspended in the medium containing either $NaHCO_3$ (5−20 mM, upper panels, n = 5) or cBiMPS (50 or 100 μM, lower panels, n = 3). These samples were used for the observation of movement patterns and relative velocities. [a] This index was calculated by using the following formula "{[(the number of spermatozoa exhibiting progressive/helical movement) + (the number of spermatozoa exhibiting round/helical movement)] / [(the number of spermatozoa exhibiting progressive/planar movement) + (the number of spermatozoa exhibiting progressive/helical movement) + (the number of spermatozoa exhibiting round/planar movement) + (the number of spermatozoa exhibiting round/helical movement)]} × 100. "A–C", "X–Z" Values with different letters within the same graphs were significantly different (P < 0.05).

bends were anti-symmetrized. Furthermore, in the spermatozoa that exhibited progressive/helical movement in the medium containing 50 μM KH-7, other-side bends (principal bends) also became smaller and differences in the amplitude between principal and reverse bends were reduced. Consequently, it was difficult to distinguish principal bends from reverse bends.

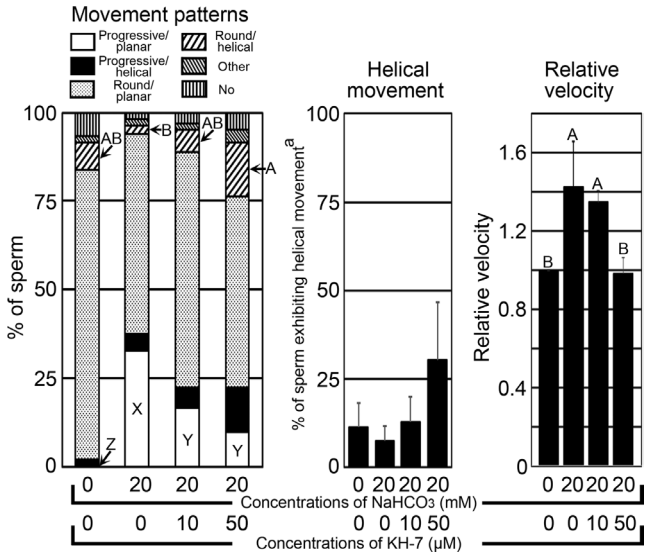

Fig. 3. Movement patterns and relative velocity of bull ejaculated spermatozoa that were suspended in the medium containing $NaHCO_3$ and KH-7. Bull ejaculated spermatozoa were washed and suspended in the medium containing $NaHCO_3$ (20 mM) and KH-7 (10 or 50 μM). These samples were used for the observation of movement patterns and relative velocities (n = 4). [a] See the footnote of Fig. 2. "A, B", "X–Z" Values with different letters within the same graphs were significantly different (P < 0.05).

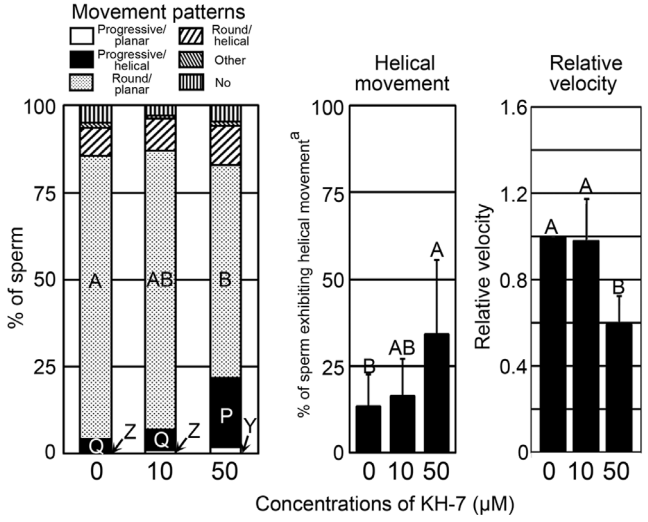

Fig. 4. Movement patterns and relative velocity of bull ejaculated spermatozoa that were suspended in the medium containing KH-7. Bull ejaculated spermatozoa were washed and suspended in the medium containing KH-7 (10 or 50 μM). These samples were used for the observation of movement patterns (n = 8) and relative velocities (n = 4). [a] See the footnote of Fig. 2. "A, B", "P, Q", "Y, Z" Values with different letters within the same graphs were significantly different (P < 0.05).

Table 1. Movement direction of bull ejaculated spermatozoa exhibiting round movement

Samples	% of the spermatozoa exhibiting: [a]			
	Round/planar movement in the clockwise direction	Round/helical movement in the clockwise direction	Round/planar movement in the anti-clockwise direction	Round/helical movement in the anti-clockwise direction
#1	85.0	0.0	15.0	0.0
#2	94.0	0.0	5.0	1.0
#3	96.0	0.0	3.0	1.0
#4	97.0	1.0	2.0	0.0
Means ± standard deviations	93.0 ± 5.48 [b]	0.25 ± 0.50 [d]	6.25 ± 5.97 [c]	0.50 ± 0.58 [d]

[a] One hundred spermatozoa of each sample that exhibit round movement in the medium without $NaHCO_3$ and KH-7 were osberved using the upright microscope, and then classified into four categories of the above-mentioned movement patterns. In these four samples examined, most of the spermatozoa exhibited round movement (see Fig. 2). [b–d] Values with different superscript letters within the same line were significantly different ($P < 0.05$).

Discussion

In the samples with relatively high PKA-mediated signaling activities (the samples suspended in the medium containing 20 mM $NaHCO_3$ or 100 μM cBiMPS), the number of spermatozoa exhibiting progressive/planar movement (without rotation) as well as the relative velocities of sperm movement significantly increased while the degree of curve in the round movement of many spermatozoa decreased compared to the samples with relatively intermediate PKA-mediated signaling activities (the control samples suspended in the medium without $NaHCO_3$ and cBiMPS). These parameters declined in parallel with the decrease in the concentration of $NaHCO_3$ or cBiMPS in the medium. In the samples with relatively intermediate PKA-mediated signaling activities, the main movement pattern became round/planar movement (without rotation) (Figs. 1 and 2). As described in the Introduction, sperm motility is positively regulated by PKA-mediated signaling activities [10]. These indicate that good motility of bull ejaculated (unfrozen) spermatozoa is characterized by progressive/planar movement (without rotation) and fast movement. In addition, progressive/helical movement (with rotation) and slow movement appeared in the samples with relatively low PKA-mediated signaling activities (the samples suspended in the medium containing 50 μM KH-7) (Figs. 1 and 4), suggesting that not only slow movement but also helical movement (occurrence of rotation) are signs of poor motility of bull ejaculated (unfrozen) spermatozoa.

Symmetrical and large bends of flagella are required for sperm to exhibit progressive and fast movement, respectively. However, when flagellar bends are anti-symmetrized, spermatozoa decrease movement linearity and exhibit round movement. Moreover, the size of flagellar bends is positively correlated with movement velocities. Thus, movement patterns of the spermatozoa are determined by the types of flagellar bends [26, 27]. In this study, we compared flagellar bends among bull spermatozoa with different movement patterns and relative velocities. Bull ejaculated spermatozoa that exhibited progressive/planar and fast movement in the medium containing 20 mM $NaHCO_3$ were characterized by flagellar symmetrical and relatively large bends, whereas symmetrical bends were converted to asymmetrical bends owing to the reduction of one-side (reverse) bends in ejaculated spermatozoa that exhibited round/planar and intermediate-speed movement in the medium without $NaHCO_3$ and KH-7 (Figs. 2 and 5). Moreover, in ejaculated spermatozoa exhibiting progressive/helical and slow movement in the medium containing 50 μM KH-7, both principal and reverse bends were reduced; consequently, it was difficult to distinguish principal bends from reverse bends (Figs. 4 and 5). These observations of flagellar bends allow us to explain how movement patterns of bull ejaculated spermatozoa are changed by suspension in the medium containing either $NaHCO_3$ or KH-7 and indicate that symmetry and size of flagellar bends in bull ejaculated spermatozoa are dependent on PKA-mediated signaling activities. Flagellar bends arise from the integrated sliding movements among outer doublet tubulins that are controlled by the axonemal dynein [26, 27]. The axonemal dyneins are motor proteins as well as ATPases whose activities are controlled by the PKA-dependent phosphorylation in the light chain proteins [21] and interaction with ATP [28]. The synthesis of ATP in the spermatozoa is dependent on the ADCY10-cAMP-PKA signaling cascade [29]. This suggests that the above-mentioned changes of flagellar bends in bull ejaculated spermatozoa may be linked to the activities of axonemal dyneins that are modulated by the PKA-mediated activities.

As reviewed by Inaba [21], the central apparatus (central pair microtubule, central pair projection, and central bridge) and radial spokes (spoke stalk and spoke head) of the flagellum are likely involved in the maintenance of two-dimensional planar movement (without rotation). This is supported by the observations that eel spermatozoa, which are lacking in the central apparatus and radial spokes, exhibit three-dimensional helical movement (with rotation), and treatment with antibodies to the radial spoke induces the change from two-dimensional planar movement to three-dimensional helical movement [21, 30, 31]. These results indicate the existence of systems regulating the maintenance of planar movement in the flagellar central apparatus and radial spokes. In this study, helical movement (with rotation) appeared in bull ejaculated spermatozoa with relatively low PKA-mediated signaling activities (in the spermatozoa suspended in the medium containing 50 μM KH-7), while planar movement (without rotation) was maintained in the spermatozoa with relatively intermediate PKA-mediated signaling activities (in the spermatozoa suspended in the medium without KH-7) (Figs. 1 and 4). These results may be interpreted as potentially showing that PKA-mediated signaling activities of the central apparatus and radial spokes are linked to the occurrence of rotation in bull ejaculated spermatozoa.

Bull ejaculated spermatozoa with relatively intermediate PKA-

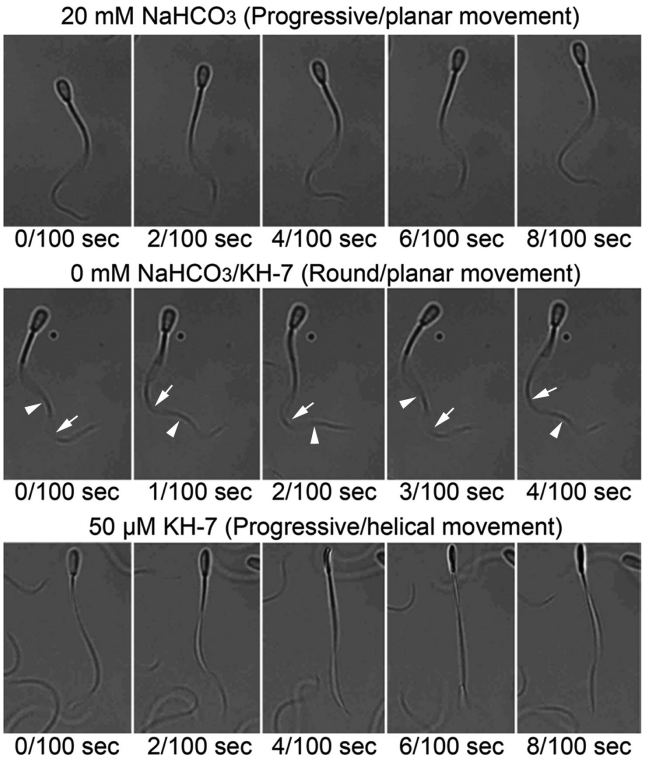

Fig. 5. Typical examples of flagellar bending in bull ejaculated spermatozoa that were suspended in the medium containing either $NaHCO_3$ or KH-7. Bull ejaculated spermatozoa were washed and suspended in the medium with either 20 mM $NaHCO_3$, 50 μM KH-7, or without $NaHCO_3$ and KH-7. Movies of flagellar bending were recorded for the spermatozoa exhibiting progressive/planar movement in the medium with 20 mM $NaHCO_3$ (the first row of photographs, a representative results of repeated observations for more than 50 spermatozoa), the spermatozoa exhibiting round/planar movement in the medium without $NaHCO_3$ and KH-7 (the second row of photographs, a representative results of repeated observations for more than 50 spermatozoa), and the spermatozoa exhibiting progressive/helical movement in the medium with 50 μM KH-7 (the third row of photographs, a representative results of repeated observations for more than 30 spermatozoa) at the frame rate of 100 Hz. The recorded movies were divided into frame images (JPEG images, every 2/100 sec or 1/100 sec). Arrows and arrow heads indicate principal bends and reverse bends, respectively.

mediated signaling activities (spermatozoa suspended in the medium without $NaHCO_3$ and KH-7) exhibited round movement with the preferential circling direction (when observing sperm samples using the upright microscope), which was clockwise (Figure 1-b, Table 1). This is in agreement with the previous observation using bull frozen-thawed spermatozoa (round movement with the preferential anti-circling direction when observing sperm samples using the inverted microscope) [6]. In our previous experiments [32], similar round movement was observed in bull hyperactivated spermatozoa (nonfull-type hyperactivated spermatozoa), but the circling direction (when observing sperm samples using the upright microscope) appears to be preferentially anti-clockwise. Thus, in the evaluation of sperm motility, the observation of the circling direction may allow us to distinguish the non-hyperactivated spermatozoa (with relatively intermediate PKA-mediated signaling activities) from the hyperactivated spermatozoa, which both exhibit round movement.

The millimolar concentration of extracellular Ca^{2+} is indispensable for the induction of hyperactivated spermatozoa by the incubation in mKRH medium containing cBiMPS [7, 33, 34]. The increase in intracellular Ca^{2+} levels can induce the flagellar asymmetrical bends by the reduction of one-side (reverse) bends at the initiation of hyperactivation in the capacitated spermatozoa with considerably high PKA-mediated signaling activities [10, 35]. In this study, we observed that bull ejaculated spermatozoa with relatively intermediate PKA-mediated signaling activities primarily exhibited round movement in the medium without $CaCl_2$ by the anti-symmetrization of flagellar bends (Figs. 1, 2 and 5). These results suggest that asymmetrical bends induced by partial reduction of PKA-meditated signaling activities in non-hyperactivated spermatozoa differ in the regulatory molecular mechanisms (in the dependency on millimolar concentrations of extracellular Ca^{2+}) from asymmetrical bends induced by the increase of the intracellular Ca^{2+} in hyperactivated spermatozoa.

Recently, Nagata *et al*. [36] sorted bull cryopreserved spermatozoa into two subpopulations by using the microfluidic system. One subpopulation was predominately composed of rapidly linear progressive nonsinuous spermatozoa and the other was predominately composed of relatively slowly transitional sinuous spermatozoa. Interestingly, suitable AI timing was different between these subpopulations; the latter achieved pregnancy when AI was performed at a later time (closer to the timing of ovulation). From these results, they proposed that relatively slowly transitional sinuous spermatozoa are associated with fertility and may be truly functional cells, rather than the rapid and more linear spermatozoa. However, it is necessary to note that microfluidic-sorted spermatozoa are in the process of capacitation, as mentioned in this paper [36]. According to our previous paper [7], when the cAMP-PKA-mediated signaling activity is enhanced to promote the capacitation-related events in bull spermatozoa, sperm motility temporally becomes slower and less linear (like the transitional sinuous type) at the early stage of the capacitation and finally becomes hyperactivated after the completion of capacitation. The same capacitation-related changes in motility were originally observed in hamster spermatozoa [8]. Thus, it is likely that the above-mentioned new proposal is valid for spermatozoa that are undergoing the process of capacitation. On the other hand, in the AI station, bull ejaculated spermatozoa that rarely undergo the capacitation-related events are routinely used for motility evaluation. Therefore, we believe our criteria are more suitable for the motility evaluation of bull ejaculated (unfrozen) spermatozoa (before entering the capacitation process) in the AI station.

Based on the results obtained in this study, we indicate that bull ejaculated spermatozoa with high PKA-mediated signaling activities exhibit progressive/planar movement as well as fast movement. Reduction of PKA-mediated signaling activities is accompanied by the appearance of helical movement (with rotation) in place of planar movement (without rotation), as well as a decrease in movement velocities and linearity. Thus, it is beneficial to add a new parameter of "rotation" to the evaluation criteria of sperm motility that are currently based on the degrees of progressive movement (linearity and straightness) and movement velocities. Recently,

computer-assisted sperm analysis (CASA) systems are often used for objective evaluation of bull frozen-thawed spermatozoa, though it is still difficult to predict the ability of bull frozen-thawed spermatozoa to impregnate cows solely from the results of CASA [3, 4]. When CASA parameters were compared between cells with helical movement (with rotation) and cells with planar movement (without rotation) in bull frozen-thawed spermatozoa, the amplitude of lateral head displacement (ALH) values were significantly higher in the former (5.69 μm) than in the latter (1.71 μm) [6]. This suggests that the ALH value is a valid scale for grading the exhibition of planar movement (without rotation). However, it should be noted that bull frozen-thawed semen includes many spermatozoa exhibiting planar and slow movement [6]. Therefore, we suggest that it is necessary to evaluate the movement patterns of spermatozoa individually and objectively in order to determine the exact percentages of spermatozoa exhibiting both progressive/planar movement (without rotation) and fast movement.

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