Revised

For Theriogenology

Negative correlations of mitochondrial DNA copy number in commercial frozen bull spermatozoa with the motility parameters after thawing

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One figure and six tables

Running head: Mitochondrial DNA copy number in frozen bull spermatozoa

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Abstract

The purpose of the current study was to investigate the relationship between mitochondrial content of commercial frozen-thawed bull spermatozoa and motility. Firstly, mitochondrial DNA copy number per spermatozoon (MDCN), mitochondrial content (MC), the percentage of spermatozoa with high mitochondrial membrane potential (HMMP), intracellular reactive oxygen species (ROS) and motility parameters of frozen-thawed spermatozoa derived from five bulls were determined by using qPCR, flow cytometry and CASA, respectively, and analyzed the relationships. Results showed that all parameters examined, including MDCN, MC, HMMP, ROS and motility indicators, significantly differed among frozen spermatozoa from different bulls. Both MDCN and MC were negatively correlated with HMMP and motility indicators, but positively with ROS, of course, whereas there was a highly positive relationship between MDCN and MC. Secondly, when MDCN and MC were examined in frozen spermatozoa prepared at different points in the lives of four bulls, those did not correlate overall throughout their lives (1.3-14.3 years old), but did correlate significantly in two sires. From these results, we conclude that MDCN and MC of frozen spermatozoa differ among sires, and are negatively correlated with HMMP and sperm motility parameters, probably due to mitochondrial oxidative stress resulted in the presence of ROS, demonstrating that these appear to be useful markers to assess sires' spermatozoa. It should be noted that the MDCN and MC of bull spermatozoa ~~do~~ may not vary overall with the age of the sire, whereas those change with age in some individuals and may affect sperm motility.

Keywords: Spermatozoa, Bulls, Mitochondrial content, Motility, Frozen semen

1. Introduction

Mitochondria are an important organelle to have multiple functions, including synthesis of adenosine triphosphate (ATP), production of intracellular reactive oxygen species (ROS), calcium signaling, thermogenesis and apoptosis [1]. Although energy production in bull spermatozoa appears to be carried out not only through the oxidative phosphorylation in the midpiece (mitochondria) but also through the anaerobic glycolysis in the principle piece [2], sperm mitochondria still play an important role in energy production for progressive motility, and other functions, such as capacitation, hyper-activation and acrosome reaction [3]. In fact, mitochondrial membrane potential has been reported to be a potent indicator of sperm motility [4]. Mitochondrial membrane potential (MMP) and DNA integrity in buffalo [5] and human spermatozoa [6] seem to be significantly associated with the presence of intracellular ROS, since oxidative stress induced by an overproduction of ROS in mitochondria [7-9] could impair plasma membrane, mitochondrial homeostasis, and consequently penetrability of spermatozoa [10].

On the other hand, the mitochondrial DNA copy number (MDCN) and mitochondrial content (MC) per spermatozoon are known to drastically reduce through mammalian spermatogenesis, especially between the late spermatocyte and mature spermatozoa stages [11-13]. Recently, a study on the MDCN from two groups of bulls separated by the artificial insemination performance and the sperm parameters determined by using CASA has reported a higher copy number in the superior scoring bull group [14]. However, MDCN has been known to be negatively correlated with motility parameters in stallion [15, 16] and boar spermatozoa [17], as well as clinical studies in human [18-22]. If MC is, as reported in equine, swine and human, negatively correlated with sperm motility, then an incomplete reduction in MC during spermatogenesis would have a negative effect on the sperm motility. However, only bull spermatozoa may require a contrary interpretation, although the previously reported study with bovine sperm was conducted in two groups of cows [14] and did not reveal correlations for each individual. Furthermore, although it has been demonstrated that motility and fertility of bull spermatozoa could decrease with the age

of sires [23], it is also not clear whether the ability to regulate MDCN and MC to a constant level varies across the bulls' lives.

Therefore, the objective of the present study was to determine (1) what correlation exists between MDCN or MC and the percentage of spermatozoa with high mitochondrial membrane potential (HMMP), ROS or sperm motility indicators in commercial frozen-thawed spermatozoa from multiple sires, and (2) if MDCN, MC, HMMP and motility parameters vary among spermatozoa collected at various times during the lives of sire bulls.

2. Materials and methods

2.1. Chemicals, media and frozen semen samples

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The basic media used for washing and manipulating the frozen-thawed bull spermatozoa was modified TL-HEPES-PVA (composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 NaH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 12 mM sorbitol, 10 mM HEPES, 0.2 mM Na-pyruvate, 0.1% (w/v) polyvinyl alcohol (PVA), 25 μg/ml gentamicin sulfate, and 65 μg/ml potassium penicillin G) or phosphate-buffered saline (PBS, Sigma) solution (PBS only used for washing and pelleting sperm samples again in DNA extraction protocol for mtDNA copy number analysis).

Commercial frozen semen derived from totally nine Japanese Black bulls (prepared at 1.3-14.3 years old, bulls A-I) with proven excellent fertility quality and prepared in 0.5 mL straws through the national standard protocols [24] was obtained from a local public AI center, the Okayama Prefectural Center for Animal Husbandry and Research. No ethics approvals were required because commercial frozen semen was used in the current study.

Straws of frozen semen were thawed individually in water bath at 70°C for 8 seconds and then stabilized at 39°C for 52 seconds. Immediately, the semen was washed three times with modified TL-HEPES-PVA by centrifugation at 700 ×g for 3 min at 39°C. Thawed spermatozoa

were re-suspended in pre-warmed TL-HEPES-PVA at a concentration of 1 x 10^8 cells/mL and used in each experiment.

2.2. Relative quantification of mtDNA copy number

Any somatic cells contained in the washed sperm sample were removed by using a cell strainer (PluriSelect, San Diego, CA, USA) according to a protocol described previously [15]. Total genomic DNA of spermatozoa was extracted with the High Pure PCR Template Preparation Kit (11796828001, Roche, Nippon Genetic Co., Ltd, Tokyo, Japan) according to the manufacturer's protocols with modifications. In brief, sperm samples were lysed with 200 μL tissue lysis buffer, 5 μL of 100 mM dithiothreitol and 40 μL of proteinase K at 55°C for 1 h. Then, after incubation at 70°C for 10 min following adding 200 μL of binding buffer, the lysed samples were cultured on ice for 10 min with 100 μL of isopropanol (100%) to adjust DNA binding conditions. Subsequently, sperm mixture was loaded in the filter column tube and centrifuged at 8,000 ×g for 1 min. After the flow-through liquid was discarded, inhibitor removal buffer (500 μL) was added into the column and centrifuged at 8,000 ×g for 1 min, followed by washing twice with silica membrane (500 μL wash buffer and centrifugation at 8,000 ×g for 1 min each time). The column was again centrifuged for 30 seconds at 13,000 ×g to dry the silica membrane before DNA elution step. The pre-warmed elution buffer (50 μL) was added into the column, incubated at 37°C for 2 min and centrifuged at 8,000 ×g for 1 min to collect all DNA into a 1.5-mL microcentrifuge tube. DNA concentration was quantified by using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the total DNA was stored at -20°C until PCR analysis.

The relative quantification of mitochondrial DNA copy number per spermatozoon (MDCN) was determined by using a quantitative real-time PCR (qPCR). Primers (Fasmac Co., Ltd. Kanagawa, Japan) specific for mtDNA (F: 5'-ATATGCACGTAGGACGAGGC-3', R: 5'-TGCCGATGTATGGGATTGCT-3') and a reference (F: 5'-TTATGGTCGACAACGGGCTC-3', R: 5'-CCGTGCTCAATGGGGTACTT-3') were synthesized from the reference genomic

DNA sequence of mitochondrial cytochrome b gene and nuclear β-actin gene, respectively, using the online tool Primer-BLAST (NIH, National Center for Biotechnology Information, USA). The specificity of each primer pair was confirmed by using UCSC In-Silico PCR online tool (UCSC Genome Browser). DNA samples were diluted to a final concentration of 10 ng/μL for qPCR. The qPCR reactions contained 1 μL of DNA solution, 0.5 μM of each primer, and FastStart Essential DNA Green Master (Roche, Nippon Genetic Co., Ltd, Tokyo, Japan) in 10 μL total volume (final DNA concentration of 1 ng/μL for qPCR reactions). Reactions were performed in LightCycler 96 (Roche) with the heat profile as followed and every reaction was repeated twice, including the pre-incubation (95°C for 5 min), 2-step amplification of 40 cycles (95°C for 10 seconds, 58°C for 30 sec), melting analysis at ramping temperature from 65 to 95°C with continuous acquisition and cooling (37°C for 5 seconds). The amplification efficiency of each primer pair was checked by performing a standard dilution series of six points of 10-fold dilutions from 10 ng/μL of a sample DNA. MDCN was calculated with the $2^{-\Delta\Delta Ct}$ method [17].

2.3. Evaluation of mitochondrial content, the activity and ROS of spermatozoa

Sperm suspension samples were analyzed for mitochondrial content, the activity and ROS by using a Gallios flow cytometer (Beckman Coulter Inc., Brea, CA, USA) after repeatedly washing before and after staining with specific dyes to remove non-sperm particles which can affect the results. Green fluorescence emission was detected in 525 nm long pass detector, and orange fluorescence wavelength in 575 nm band pass filter. For each sperm sample, one test tube containing 0.5 mL of diluted sperm suspension (5 x 10^6 sperm/mL) was acquired result of 10,000 events per replicate at the forward and side scatter channels.

Mitochondrial content (MC) of spermatozoa was determined by using MitoTracker Green FM (MTG), according to the manufacturer's protocols (Molecular Probes, Eugene, OR, USA) and a previous report [14] with small modifications. In brief, sperm samples were fixed in 2% paraformaldehyde in PBS for 15 min, washed once by centrifugation at 700 × *g* for 3 min with fresh TL-HEPES-PVA and then stained with a final concentration of 20 nM MTG in TL-HEPES-

PVA for 60 min at 39°C. After washing once by centrifugation (700 ×g, 3 min), MC of frozen-thawed bull spermatozoa was analyzed by using flow cytometer and presented as mean fluorescence intensity (MFI) from 10,000 events by using Kaluza software installed with the flow cytometer.

Mitochondrial activity of spermatozoa determined as mitochondrial membrane potential was assessed by using JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine iodide; Molecular Probes/Thermo Fisher Scientific, Waltham, MA, USA) by which forms monomers making a green fluorescence at 527 nm (low mitochondrial membrane potential) and aggregates giving an orange wavelength at 590 nm (high mitochondrial membrane potential) [25]. The samples of sperm suspension in pre-warmed TL-HEPES-PVA were incubated with JC-1 dye at a final concentration of 0.76 μmol/L at 39°C for 8 min in dark [26, 27]. The percentage of spermatozoa with high mitochondrial membrane potential (HMMP) was determined by the proportion of cells in the orange fluorescence gate in flow cytometry of 10,000 events by using Kaluza software installed with the flow cytometer.

Reactive oxygen species in spermatozoa were detected by using a ROS Assay kit with highly sensitive DCFH-DA (Dojindo Laboratories, Kumamoto, Japan) [28, 29]. The fluorescence dye, DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) can permeate cell membrane, is de-esterified in the cell to release DCFH and could be transformed in the presence of ROS into a DCFD form that emits green fluorescence [9, 28]. Sperm suspension samples were incubated with a final concentration of 10 μmol/L PI and highly sensitive DCFH-DA reagent diluted at 1:1,000 with the loading buffer for 30 min at 39 °C in the dark. The percentage of live spermatozoa with ROS detected as green fluorescence was observed in the green fluorescence gate in flow cytometry of 10,000 events by using Kaluza using a flow cytometer.

2.4. Assessment of sperm motility

A computer-assisted sperm analysis (CASA) system (with the Sperm Motility Analysis System software, Digital Image Technology, Tokyo, Japan) with 60 frames per second was used

according to the manufacturer's protocols and previously described in more specifications for bull semen [9, 30] to identify the conventional motion characteristics such as the total motility (TM), progressive motility (PM), rapid progressive motility (RPM) and kinematic parameters [31, 32]. The kinematic parameters assessed in the present study were the velocity straight line (VSL, μm/s), velocity curved line (VCL, μm/s), velocity average path (VAP, μm/s), linearity (LIN, %), straightness of trajectory (STR, %), wobble coefficient (WOB, %), amplitude of lateral head displacement (ALH, μm/s), and beat cross frequency (BCF, Hz). Briefly, sperm samples in pre-warmed TL-HEPES-PVA were diluted to a concentration of 1 x 10^7 cells/mL and then analyzed in a Makler counting chamber (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) at 39°C with CASA system. A minimum of 300 spermatozoa per suspension were analyzed by the system at 3 different microscopic fields.

2.5. Experimental design

2.5.1. Relationships between MDCN, MC, HMMP, ROS and motility parameters of frozen-thawed spermatozoa from five bulls (experiment 1)

The frozen semen straws, which had been prepared using the same ejaculate from each of five sire bulls (A-E), were used in this experiment. After thawing and washing, spermatozoa were evaluated for MDCN, MC, HMMP, ROS and conventional motility parameters. Correlations among those were also examined. Experiments were replicated 6 times with different frozen straws from each bull semen in all experimental groups.

2.5.2. MDCN, MC, HMMP and motility parameters of frozen-thawed semen prepared at various points in the lives of four bulls (experiment 2)

Frozen semen straws prepared at three different times during the lifetime of each of four bulls (F-I) were used in this experiment. Thawed and washed spermatozoa were evaluated for the parameters described above to determine if MDCN and MC in spermatozoon varied across the

lifetime of the bulls and affected the motility. Experiments were replicated 5 times using different frozen straws at each time point during the lives of the bulls.

2.6. Statistical analysis

Data from five or six replicated trials were evaluated using one-way analysis of variance (ANOVA) in GraphPad Prism 8.3 statistical software (GraphPad Software Inc., San Diego, CA, USA). Pearson correlation coefficients were applied to characterize relationships among MDCN, MC, HMMP, ROS and conventional motility parameters in sperm samples. Since all percentage data in the current study consequently distributed within 10-90%, those data were not subjected to the arc-sin transformation for fitting to a normal distribution before analyses. All data are expressed as the mean $\pm$ SEM. Findings were considered significantly different at $P < 0.05$ and, when there was a significant effect, values were compared with a Turkey's multiple range post hoc test.

3. Results

3.1. Relationships between MDCN, MC, HMMP, ROS and motility parameters of frozen-thawed spermatozoa from five bulls (experiment 1)

As shown in Tables 1 and 2, not only MDCN, MC, HMMP and ROS, but also a majority of motility parameters, such as TM, PM, RPM, VCL, WOB, ALH and BCF, significantly differed ($P < 0.01$) among commercial frozen-thawed spermatozoa from five bulls, whereas no significant differences were found in VSL, VAP, LIN and STR (Table 2). When correlation matrix analyses were performed using these data (Table 3), MDCN had a significant positive correlation with MC ($r = 0.45$, $P < 0.05$), but HMMP had a negative correlation with MDCN ($r = -0.59$, $P < 0.01$) and MC ($r = -0.66$, $P < 0.01$). Both MDCN and MC were found to have a significant negative correlations ($P < 0.05$) with a majority of motility parameters, except for VSL ($r = -0.35$, $P = 0.06$), VAP ($r = -0.28$, $P = 0.13$), LIN ($r = -0.01$, $P = 0.95$), STR ($r = -0.26$, $P = 0.16$) and WOB ($r = 0.33$, $P = 0.08$) in MC, whereas MDCN and MC had positive correlations with BCF ($r = 0.37$,

P < 0.05 and r = 0.50, P < 0.01, respectively). Contrary to the characteristics of MDCN and MC, HMMP was found to have significantly positive correlations (P < 0.01) with a majority of motility parameters except for VSL (r = 0.31, P = 0.10) and VAP (r = 0.30, P = 0.10), whereas it was a negative correlation with BCF (r = -0,73, P < 0.01). Whereas ROS had positive correlations with MDCN (r = 0.41, P < 0.05) and MC (r = 0.58, P < 0.01), it was significantly negative correlations with HMMP (r - -0.84, P < 0.01) and a majority of motility parameters, except for VSL (r = -0.30, P = 0.11), VAP (r = -0.31, P = 0.10), LIN (r = 007, P = 0.70), STR (r = -0.30, P = 0.11) and WOB (r = 0.34, P = 0.06). Within motility parameters, only VSL was not found any correlations with many parameters, except for with VCL (r = 0.55, P < 0.01), VAP (r = 0.43, P < 0.05), LIN (r = 0.75, P < 0.01) and STR (r = 0.62, P < 0.01).

3.2. MDCN, MC, HMMP and motility parameters of frozen-thawed semen prepared at various points in the lives of four bulls (experiment 2)

Interestingly, in only two of the four bulls examined, significant differences were observed (P < 0.05) in MDCN, MC, HMMP (one bull increased MDCN and MC with age and decreased HMMP, while another did the opposite; Table 4), and even a majority of motility parameters (P < 0.05), except for VSL, VAP, STR and WOB (from these two bulls), or LIN and ALH (from only one bull), among frozen-thawed sperm samples prepared at three different points in bull's lives (Table 5). When correlation matrix analyses were performed using these data (Table 6), BAC in the present study (from 1.3 to 14.3 years old) did not affect any with MDCN, MC, HMMP and all motility parameters examined. Results of correlations among MDCN, MC, HMMP, and motility parameters in this experiment (Table 6) were similar with those in Table 3. The scattering plots for MDCN and MC in frozen-thawed spermatozoa prepared at three different points during four bulls' lives again showed no clear trend between the age of the bulls and MDCN or MC at the time of semen collection (Fig. 1). Furthermore, even when the age of bulls at semen collection was classified into three groups (< 5 years old, 5-10 years old and ≥ 10 years old), there were no

significant differences in both MDCN (< 5 years old, 1.10 ± 0.05; 5-10 years old, 1.22 ± 0.05; ≥ 10 years old, 1.12 ± 0.04; P = 0.15) and MC (< 5 years old, 90.4 ± 1.7; 5-10 years old, 93.6 ± 1.4; ≥ 10 years old, 89.3 ± 1.6; P = 0.11).

4. Discussion

In the present study, we assessed MC by staining sperm mitochondria with MTG and then measuring their fluorescence by flow cytometry, according to previous reports [14, 17, 33]. However, there have been few reports on how accurately this method can assess MC, including comparative assessments with MDCN and other methods. Our current results demonstrated that MC had a significant positive correlations with MDCN determined by qPCR in frozen-thawed bull spermatozoa, indicating that this simple method of measuring sperm MC by fluorescence intensity under flow cytometry made clear to be a sufficiently reliable sperm evaluation method. On the other hand, HMMP had significant negative correlations with both MDCN and MC. This result was consistent with a previous report demonstrating a negative relation between MDCN and HMMP in stallion spermatozoa [16]. In the current study, the percentage of spermatozoa detected intracellular ROS not only had significant positive correlations with both MDCN and MC but also had negative correlations with HMMP. Since the intracellular ROS seems to be strongly associated with HMMP and DNA integrity in buffalo [5] and human spermatozoa [6], therefore, in spermatozoa with higher MDCN and MC, it is quite possible that oxidative stress due to excessive mitochondrial ROS production can significantly reduce sperm HMMP and motility. This interpretation is consistent with a previous report in human spermatozoa of patients with severe asthenozoospermia showing the relation between increased MDCN and elevated ROS level [34].

Furthermore, the current results in both experiments 1 and 2 demonstrated that both MDCN and MC had significant negative correlations with a majority of motility parameters in commercial frozen-thawed spermatozoa from a total of nine sires. Recently, an analysis of frozen

bull semen, divided into two groups based on artificial insemination performance, reported that larger MDCN were observed in spermatozoa with higher motility [14]. Our current results were in contrast to their report [14], but were consistent with previous reports in stallion [15, 16] and boar spermatozoa [17] and clinical studies in human [18-22]. Therefore, it seems reasonable to conclude that both MDCN and MC are negatively correlated with sperm motility, similar to what has been reported in other species, rather than that only bovine sperm require a reverse special interpretation. Since MDCN and MC drastically decrease through the mammalian spermatogenesis, especially between the late spermatocyte and mature spermatozoa stages [11-13], spermatozoa with relatively larger copy numbers and contents may be immature during the process of spermatogenesis. It has also been demonstrated that less mitochondrial and mtDNA contents did not affect the mitochondrial activity and conventional sperm motility [15, 17, 19]~~,~~. Again, in the present study, we found that the incidence of spermatozoa with detected ROS significantly correlated with both MDCN and MC positively and with HMMP negatively. Therefore, MDCN- and MC-rich spermatozoa may have further reduced mitochondrial membrane potential due to severe oxidative stress from relatively more ROS production, consequently resulting in lower sperm motility.

In the second experiment, when we observed whether MDCN, MC or HMMP in frozen-thawed bull spermatozoa varied among the time points when the semen was collected during bulls' lives (1.3-14.3 years old), there were significant variations in two of the four bulls examined (one bull increased MDCN and MC with age and decreased HMMP, while another did the opposite), but no significant variations overall. There were no correlation between bull's age at time of semen collection and MDCN, MC or HMMP, whereas both MDCN and MC differ with age at semen collection in a few individuals and may also affect sperm motility. To our knowledge, no study has examined whether MDCN, MC and HMMP in frozen bull spermatozoa from the same sires vary with the age of the sire at the time when the semen is collected. Our results are consistent with the results on sperm MDCN when collected from a large number of males of various ages in human [18] and stallion [15, 16]. Although it has been demonstrated that motility and fertility of

bull spermatozoa could decrease with the age of sires [23, 35], our current results showed that bull's age at semen collection did not correlate with all sperm motility parameters examined. Therefore, within the range of semen collection ages of the four Japanese Black bulls examined in the present study, the sperm MDCN and MC, as well as the HMMP and motility, do not appear to be affected by aging. Although some samples showed significant differences in sperm motility parameters when the semen was collected at three different time points in the lives of sire bulls in the second experiment. However, in the current study, which was designed using commercial frozen semen provided from a local AI center, we had limitations in systematically matching the ages of the sire bulls. Further strict research may be required to clarify the relationship between the age of the bulls at the time of semen collection and MDCN/MC in more details.

In conclusion, MDCN and MC of commercial frozen-thawed spermatozoa differ among sire bulls and have significantly negative correlation with HMMP and a majority of sperm motility parameters, probably due to significant positive correlations with ROS. It should be noted that the MDCN and MC of commercially available frozen-thawed bull spermatozoa do not vary overall with the age of the sire from which the semen was collected, whereas they change with age in some individuals and may also affect sperm motility. Furthermore, the current simple method of measuring sperm MC by fluorescence intensity under flow cytometry is a sufficiently reliable sperm evaluation method, since there is a significant correlation with MDCN.

Author contributions

HTN and HF were responsible for the experimental concept, design, formal analyses, manuscript preparation and edition. SQD and TW contributed to the experimental design adjustment and MDCN analyses. HK contributed to the preparation of frozen semen samples and analyses. All authors have read and agreed to the completely final version of this manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

The authors acknowledge the Japanese Government Scholarship (MEXT) to HTN for his Ph.D. research. This research was supported in part by a research grant for Ph.D. student from Graduate School of Environmental and Life Science, Okayama University.

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Legends of figure

Fig. 1. The scattering plots for the relation between (A) the mtDNA copy number ~~(A) and~~ or (B) mitochondrial content ~~(B)~~ in frozen-thawed spermatozoa from four bulls (F-I) and the age of bulls when their semen were collected and cryopreserved. The dot points represent the one value in the replicated data.