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—Original—

Validation of the Sperm Quality Analyzer and the Hypo-osmotic Swelling Test for Frozen-thawed Ram and Minke Whale (*Balaenoptera bonarensis*) Spermatozoa

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Abstract. The object of the present study was to investigate the validation of the sperm quality analyzer (SQA) and the hypo-osmotic swelling (HOS) test with standard sperm analysis methods in frozen-thawed ram and minke whale spermatozoa. In rams, highly significant correlations were observed in the percentage of motile spermatozoa ($P<0.01$) and sperm concentration ($P<0.01$) between the standard and SQA methods. But, the percentage of morphologically normal spermatozoa did not significantly correlate between the standard and SQA methods. The percentages of swollen spermatozoa at 15 minutes by the HOS test were significantly correlated with the motility by the standard ($P<0.05$) and by the SQA ($P<0.05$) methods. For minke whale spermatozoa, the SVI (sperm viability index) values by the standard method were significantly ($P<0.001$) correlated with the sperm motility index (SMI) values by SQA. The percentage of motile spermatozoa was also significantly correlated ($P<0.01$) with the motility measured by SQA. Using different hypo-osmotic solutions and incubation times, the HOS test with 25, 100 and 150 mOsM did not show significant variations. Motility observed by the standard method and the percentage of swollen spermatozoa were significantly correlated ($P<0.05$). These results indicate that the SQA and HOS test can be utilized to assess the post-thawing motility of ram and minke whale spermatozoa, and that the SQA and HOS test values are significantly correlated in ram spermatozoa. However, sperm concentration and morphologically normal spermatozoa are not assessed accurately by SQA in minke whales.

Key words: Sperm quality analyzer, Hypo-osmotic swelling test, Frozen sperm, Sheep, Minke whale

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Standard semen analysis has involved a number of parameters such as sperm concentration, motility, morphology, and live or dead sperm proportion for the assessment of male fertility. However, these parameters have a limited value for predicting sperm fertilizability and pregnancy after artificial insemination (AI). Several assays have been developed to evaluate functional parameters of spermatozoa more objectively, such as zona-free hamster egg penetration (ZHEP), triple staining, sperm acrosome activity, the *in vitro* fertilization (IVF) test, and various protein assays [3]. However, for routine semen evaluation, a test that is inexpensive, technically simple, and accurate, needs to be established.

The hypo-osmotic swelling (HOS) test has been applied to various species such as dogs [5, 13],

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horses [17, 18], cattle [4, 21], pigs [19], and humans [9, 25, 26]. The HOS test is a simple and useful method, and it has been reported that a significant correlation was obtained with *in vivo* fertility using boar spermatozoa [19]. The HOS test is a stable method with good reliability and repeatability for stallion spermatozoa [18]. There was a good correlation between the percentage of swollen spermatozoa and the outcome of the ZFHP assay as well as IVF outcomes in humans [11, 12]. On the other hand, Rota *et al.* [21] did not find a correlation between *in vitro* fertilization and the HOS test in five frozen bull semen samples.

As a simple objective device for human semen analysis, the sperm quality analyzer (SQA) has been developed. This SQA system initially was proposed for bull spermatozoa in 1981 [2]. A new version, SQA-IIB supplying numerical readouts of the three main indices, sperm concentration, motility and morphology, has been used for human sperm analysis [1, 14, 15]. Iguer-Ouada and Verstegen [10] reported that data of dog semen analyzed by SQA were as reliable as those obtained by a computerized sperm motility analyzer (HTM-IVOS: Hamilton-Thorn Research, Danvers, U.S.A.). However, the repeatability and accuracy of the SQA method will depend on the number and quality of motile spermatozoa in the ejaculate [10, 14].

It may be desirable to analyze raw semen rather than frozen-thawed semen, because the physical and functional characteristics of frozen-thawed spermatozoa are changed during or after freezing and thawing procedures. However, to assess the fertilizability before AI or IVF and to predict the fertility of frozen-thawed spermatozoa after AI, frozen-thawed semen is a more usable resource than fresh raw semen. Minke whale spermatozoa can not be obtained as raw semen. Only spermatozoa frozen on the research vessel, collected from vasa deferentia of minke whales captured in the Antarctic Ocean are available at present [8, 16]. A simple, practical and inexpensive sperm analysis method is required for sheep AI and minke whale IVF studies.

The objective of the present study was to investigate the validation of the SQA and the HOS test in frozen-thawed ram and minke whale spermatozoa with standard sperm analysis methods.

Materials and Methods

Preparation of ram semen specimens

The present study was approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine, in accordance with Guiding Principles for the Care and Use of Research Animals.

Fresh raw semen was collected from five mature South Down and Merino × Dorset rams. Only semen specimens with more than 80% forward movement and 3×10^9 sperm concentration were diluted 5-fold with a diluent consisting of Tris (300 mM), glucose (27.75 mM), citric acid (94.7 mM), glycerol (5%, v/v) and egg yolk (15%, v/v) [7, 22]. The diluted semen was cooled and frozen in pellet form (0.2 ml in volume). After storage of the frozen semen in liquid nitrogen for 1–2 months, the specimens were thawed at 37 C and used for analyzing sperm quality .

Minke whale sperm specimens used in this study were collected from vasa deferentia of two (A, B) mature minke whales (body length: 8.19 and 8.71 meters, body weight: 6.3 and 7.3 tons, right and left testicular weight: 1,825 and 1,929 g and 1,065 and 1,067 g for males A and B, respectively) captured in December, 1999 for the Japanese Whale Research Program with Special Permit in the Antarctic (JARPA) in the area of 64 – 66°S, 136–153°E. On the research ship, the sperm specimen was diluted 5-fold with the same diluent used for ram semen and cooled to 5 C for 3 hours. Thereafter, an aliquot of 0.2–0.3 ml in a 0.6 ml micro-centrifuge tube (Quality Scientific Plastics; 502-PLNS, Porex Bio Products Inc., U.S.A.) was frozen in a –80 C freezer and placed into liquid nitrogen. After storage for 2–3 months, the specimens were thawed at 37 C, and used for analyses.

Sperm evaluation: Standard method

After thawing, the ram and minke whale sperm specimens were immediately placed on a pre-warmed glass slide and covered with a 18 × 18 mm coverslip. The percentage of motile spermatozoa were determined subjectively by one observer using phase-contrast optics (× 100 and × 400). Classification of live and dead spermatozoa was performed with eosin (1.67%, w/v)-nigrosin (10%, w/v) staining [6]. Two hundred spermatozoa from different fields on each slide were examined with a microscope (× 400). Viability was expressed as the

proportions of non-stained (live) spermatozoa out of the total counted. The percentages of morphologically normal and abnormal spermatozoa were also assessed. Finally, sperm concentration in the specimens diluted with 3% (w/v) saline solution in various ratios were determined with a hemocytometer. For comparison with the SQA method, values for estimating sperm motility and viability (Sperm Viability Index, SVI) were determined by the standard method. Briefly, the sperm motility pattern was classified into four categories (+++, ++, +, ±) and gave 100, 75, 50, 25, points were given to each category, respectively. The sum of four categories multiplied by each point was divided by 100 to yield the final SVI value.

Evaluation with the Sperm Quality Analyzer (SQA)

Ram and minke whale sperm specimens were analyzed by the SQA II-B (Medical Electric Systems, Migdal Haemek, Israel). Following the User's Guide, each sample aspirated into the accessory disposable capillary, was inserted into the machine. The total functional sperm concentration (TFSC), the total sperm concentration, the percentage of motile spermatozoa, the percentage of morphologically normal spermatozoa, and the sperm motility index (SMI) which is a measurement of optical density fluctuations caused by motile spermatozoa [1], were displayed sequentially within 1 minute. TFSC value ($\times 10^5$/mL) is the morphologically normal and motile sperm concentration. The average of two readouts at 10-minute intervals was used as the final result.

Evaluation with the hypo-osmotic swelling (HOS) test

For ram semen, a HOS solution consisting of fructose (1.35%, w/v) and sodium citrate (0.735%, w/v) at 157 mOsM was used [11, 12]. Aliquots of the mixture (semen : solution = 50 μL : 500 μL) were incubated at 35 C at 15 minutes intervals until 60 minutes, to determine the time for the maximum number of swollen spermatozoa. After incubation, 1 or 2 drops of the treatment mixture were examined on a slide coverslip preparation by phase contrast microscopy at × 400. Two hundred spermatozoa per slide were counted, and the proportion of swollen spermatozoa was determined (number of spermatozoa with swollen tails divided by the total number of spermatozoa counted, multiplied by 100).

For minke whale sperm, the response to the HOS test was assessed with fructose and sodium citrate solutions at different osmolalities (10, 25, 50, 100, 150, 300 mOsM) and incubation times (0, 15, 30, 45, and 60 min). To avoid confusion with the number of abnormal spermatozoa with coiled or bent tails before the HOS-test, 200 frozen-thawed minke whale spermatozoa were examined for morphology of the sperm tail. The number of pre-curled spermatozoa before HOS test was expressed as 'pseudo'-swollen spermatozoa (X). After mixture with HOS solutions, the number (Y) of all swollen spermatozoa was determined in each treatment, and the proportion (%) of 'true'-swollen spermatozoa was calculated by the formula: (Y–X)/ 200 × 100.

Statistical analysis

The proportions (mean ± SEM) of swollen spermatozoa in both ram and minke whales, were analyzed by Duncan's multiple range test using Statistical Analysis System (SAS). Pearson's coefficient of correlation (r=values) was calculated [23], and relationships between the standard method, the SQA method, and the HOS test were analyzed by SAS. Values were considered significant when $P < 0.05$.

Results

Ram

Highly positive correlations were observed in the percentage of motile spermatozoa ($r=0.78$; $P<0.01$) and sperm concentration ($r=0.86$; $P<0.01$) between the standard and SQA methods (Fig. 1 and 2). The SVI values by the standard method were also significantly ($r=0.60$; $P<0.01$) correlated with the SMI values by the SQA methods. However, the percentage of morphologically normal spermatozoa did not significantly correlate between the standard and SQA method. Also, the SVI values by the standard method were not significantly correlated with the TFSC values by the SQA method.

The response of sperm tails examined in the HOS solution at 4 incubation times is shown in Table 1. An increase of the incubation period was consistent with an increase of the percentage of swollen

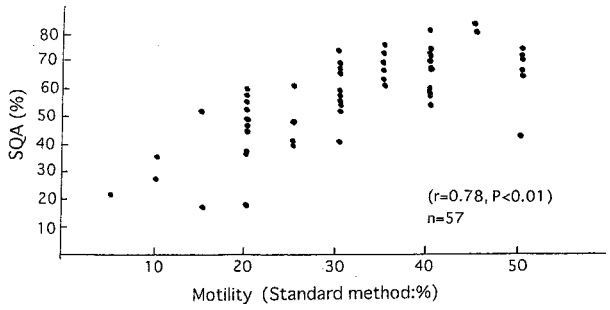

Fig. 1. Relationship of the motility of frozen-thawed ram semen between the standard and the SQA methods.

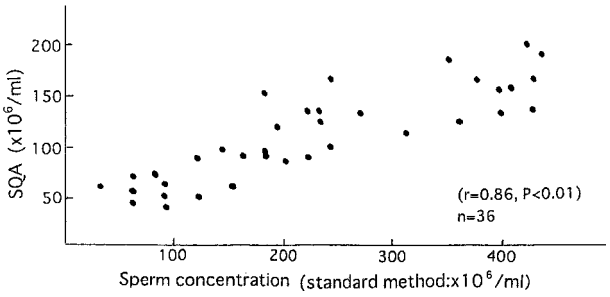

Fig. 2. Relationship of the sperm concentration of frozen-thawed ram semen between the standard and the SQA methods.

Table 1. Percentage of swollen spermatozoa of frozen-thawed ram semen at different incubation times in hypo-osmotic solution

Incubation times (minutes)	Percentage of swollen spermatozoa (Mean ± SEM: %)
0	41.5 ± 4.1[a]
15	47.2 ± 7.0[b]
30	46.9 ± 5.5[b]
45	46.6 ± 5.5[b]
60	44.8 ± 6.0[a,b]

Experimental conditions are shown in Materials and Methods.

[a,b] Values with different superscripts are significantly different (P<0.05)

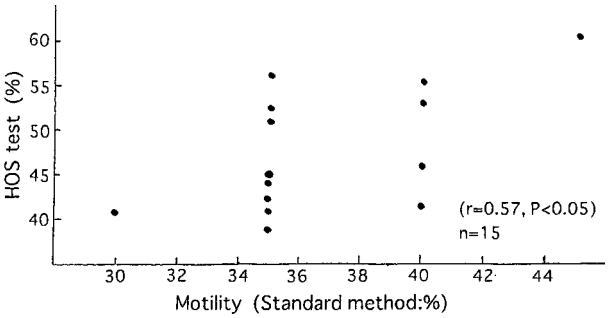

Fig. 3. Relationship of the motility of frozen-thawed ram semen assessed by the standard method and the HOS test.

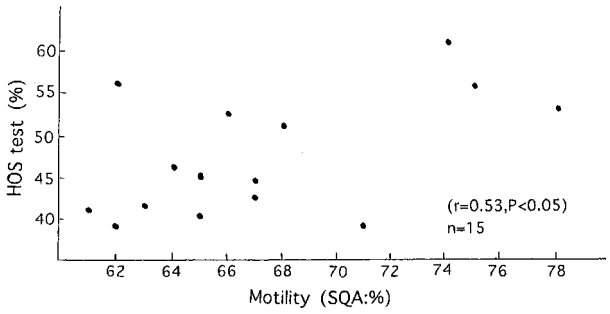

Fig. 4. Relationship of the motility of frozen-thawed ram semen assessed by the SQA method and the HOS test.

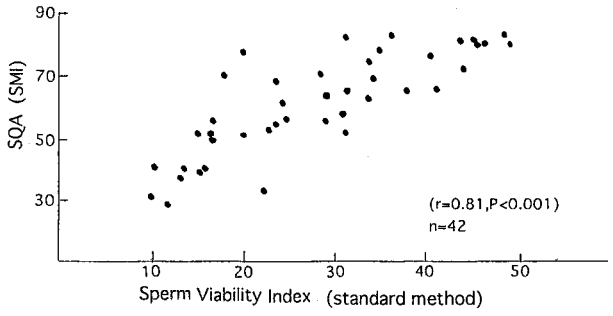

Fig. 5. Relationship between the sperm viability index (SVI) assessed by the standard method and the sperm motility index (SMI) assessed by the SQA method in frozen-thawed minke whale spermatozoa.

spermatozoa except for the 60 min observation, and no significant difference was found in the percentage of swollen spermatozoa between the four incubation periods. The percentages of swollen spermatozoa observed at the 15 minute observation were significantly correlated with the motility obtained by the standard (r=0.57; P<0.05, Fig. 3) and SQA (r=0.53; P<0.05, Fig. 4) methods.

Minke whale

The values of SVI examined by the standard method were significantly (r=0.81, P<0.001) correlated with the SMI values obtained by SQA (Fig. 5). The percentage of motile spermatozoa by the standard method was also significantly correlated (r=0.80, P<0.01) with the motility values measured by SQA (Fig. 6). However, a significant

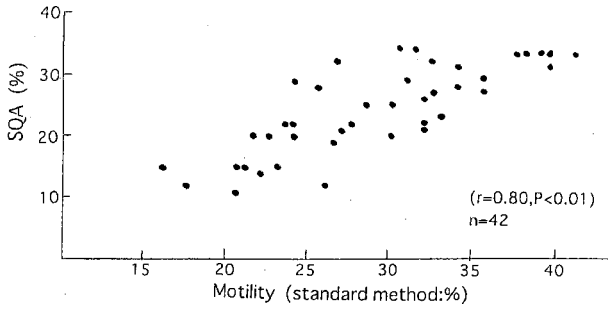

Fig. 6. Relationship of the motility of frozen-thawed minke whale spermatozoa between the standard and the SQA methods.

correlation was not found in either sperm concentration (r=–0.17) or the percentage of morphologically normal spermatozoa (r=–0.24) between the standard and SQA methods. Also, the SVI values were not significantly correlated with TFSC by the SQA method.

The results of HOS tests on frozen-thawed spermatozoa derived from two minke whales (A and B) are shown in Tables 2 and 3, respectively. The post-thawing motility was greatly different between males A (019: 52.3 ± 3.3%) and B (003: 9.0 ± 1.7%). In male A (Table 2), 300 mOsM resulted in significantly (P<0.05) lower numbers of swollen spermatozoa between the incubation times (except at 60 minutes), and tended to increase the percentage of swollen spermatozoa with the longer incubation period. Treatment with 25, 100 and 150 mOsM did not show a significant variation at any incubation period, but the percentage of swollen spermatozoa tended to be higher at 100 and 150 mOsM than at other osmolalities. At 15, 30 and 60 minutes of the incubation, the 150 mOsM treatment showed a significantly higher number of swollen spermatozoa as compared with the 10 or 25 mOsM treatments. In male B (Table 3), the percentage of swollen spermatozoa treated with 10 mOsM showed the lowest value at the 60 minutes observation, which was significantly (P<0.05) lower than treatments with higher osmolality. Also, the 60 minutes observation showed lower (P<0.05) percentages of swollen spermatozoa in 10, 50, and 300 mOsM treatments. The relationship between motility and the percentage of swollen spermatozoa was only examined in male A. The percentage of swollen spermatozoa were always higher than the percentage of motile spermatozoa

Table 2. Effects of different osmolality hypo-osmotic solutions and incubation times on the swelling of frozen-thawed minke whale spermatozoa (Male A: No. 019)

Incubation times (minutes)	Osmolality (mOsM)					
	10	25	50	100	150	300
0	$52.8^{Aa} \pm 3.0$	$57.2^{a} \pm 6.7$	$60.2^{Aa} \pm 2.7$	$56.5^{a} \pm 1.5$	$57.3^{a} \pm 5.9$	$21.5^{Ab} \pm 4.3$
15	$35.7^{Bab} \pm 5.8$	$41.5^{ab} \pm 7.0$	$57.3^{Ac} \pm 5.1$	$54.7^{c} \pm 2.2$	$55.5^{c} \pm 2.6$	$25.7^{Ab} \pm 5.3$
30	$37.5^{ab} \pm 3.3$	$38.8^{abc} \pm 9.1$	$51.2^{ad} \pm 2.2$	$53.8^{cd} \pm 0.7$	$57.5^{d} \pm 3.0$	$33.7^{b} \pm 5.2$
45	38.0 ± 3.8	38.8 ± 11.3	50.5 ± 3.3	56.2 ± 0.7	$57.8^{a} \pm 0.4$	$37.2^{b} \pm 7.4$
60	$35.8^{Ba} \pm 6.8$	$34.0^{a} \pm 10.0$	$44.5^{B} \pm 5.5$	52.7 ± 3.8	$57.5^{b} \pm 2.9$	$43.7^{B} \pm 1.9$

The values are shown as Mean ± SEM: %.
The different superscripts in columns (A, B) and rows (a, b, c, d) are significantly different (P<0.05).

Table 3. Effects of different osmolality hypo-osmotic solutions and incubation times on the swelling of frozen-thawed minke whale spermatozoa (Male B: No.003)

Incubation times (minutes)	Osmolality (mOsM)					
	10	25	50	100	150	300
0	$17.8^{A} \pm 1.7$	15.2 ± 6.0	$12.0^{A} \pm 1.7$	$14.3^{A} \pm 3.6$	15.5 ± 1.7	$10.8^{A} \pm 2.4$
15	$9.7^{B} \pm 1.7$	15.8 ± 4.7	9.5 ± 4.2	15.3 ± 2.1	14.7 ± 1.6	11.5 ± 1.8
30	$7.2^{B} \pm 2.7$	10.2 ± 5.4	17.5 ± 6.1	$9.7^{B} \pm 1.9$	9.7 ± 1.9	8.0 ± 2.5
45	$7.7^{B} \pm 0.9$	10.2 ± 3.2	10.5 ± 3.3	11.5 ± 1.0	11.5 ± 1.0	8.8 ± 0.2
60	$4.7^{Ba} \pm 1.8$	$8.5^{b} \pm 2.1$	$10.8^{Bb} \pm 0.7$	$12.8^{b} \pm 1.4$	$12.8^{b} \pm 1.4$	$7.3^{Bb} \pm 1.7$

The values are shown as Mean ± SEM:%.
The different superscripts in columns (A, B) and rows (a, b) are significantly different (P<0.05).

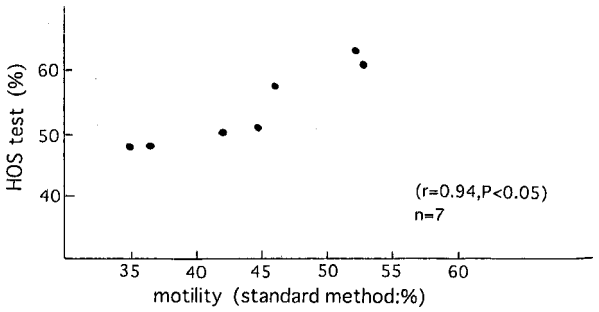

Fig. 7. Relationship of the motility of frozen-thawed minke whale spermatozoa assessed by the standard method and the HOS test.

during the 60 minutes of observation. The motility by the standard method and the percentage of swollen spermatozoa by the HOS-test were significantly correlated (r=0.94; P<0.05, Fig. 7).

Discussion

In the present study, relationships between standard sperm analysis method (motility, sperm concentration, and normal morphology), the SQA and the HOS test in frozen-thawed ram and minke whale spermatozoa were investigated. The reason for using rams and minke whales in this study is that the two animals are seasonal breeders with some similarities in reproductive morphology and physiology, and it is considered that a sheep model could possibly be used for understanding of whale reproduction, although the two species were not directly compared in this study. Generally, sperm evaluation has been performed on raw semen, but it does not always correlate with the outcome of fertilization or fertility in animals following AI. Sperm analysis using frozen-thawed semen may more accurately predict the fertilizablity of spermatozoa before AI or IVF as some workers have reported in cattle [4, 21] and horses [17].

In frozen-thawed ram semen, sperm concentration and post-thawing motility examined by the standard methods were significantly correlated with the values measured by the SQA. The motility by the SQA method was higher than the values by the standard method (Fig. 1), and the SQA method appears to have underestimated sperm concentration as compared with the standard method (Fig. 2). However, the differences in the motility and sperm concentration between the SQA and standard methods were consistent. In minke whales, the post-thawing motility by the standard method was significantly correlated with the SQA value, but sperm concentration was not correlated. The minke whale spermatozoa were collected from vasa deferentia after death from animals captured in the Antarctic Ocean during the feeding season (December in the southern hemisphere). To obtain ejaculated semen from live minke whales is technically impossible at present. Baleen whales including minke whales migrate through the northern or southern oceans throughout the year and they are believed to be seasonal breeders. During the feeding season in the Antarctic, minke whales are out of the breeding season (August to October). In the present study, the relationship between motility and the percentage of swollen spermatozoa was only examined in Male A, because of the low post-thawing motility and high proportion of abnormal spermatozoa in Male B. Mogoe *et al.* [16] reported that more than 80% of the southern minke whale spermatozoa during the feeding season were morphologically abnormal. Therefore, the SQA might not have counted all sperm heads of minke whale spermatozoa, and this may have led to the lack of a significant correlation of sperm concentration between the standard and SQA methods. In the present study, motility was significantly correlated between the SQA method and the HOS test for ram spermatozoa. For minke whale spermatozoa, motility by the standard method was significantly correlated with the values by the SQA method and the HOS test. However, the relationship on sperm motility between the SQA method and HOS test was not examined in the present study, because the HOS test conditions were not determined due to the use of only 2 males with different motility. The HOS test has been reported as a simple, inexpensive, and reliable method for several mammalian species, such as cattle [4, 20, 24], horses [17, 18], pigs [19], dogs [5, 13], and humans [9, 25]. However, Rota *et al.* [21] using five samples of frozen-thawed bull semen reported that there was no significant correlation between *in vitro* fertilization and the HOS test. The sperm motility of raw and frozen-thawed semen was similar in the five rams used in the present study, but the post-thawing motility of the two male mike whales were quite different and only small samples from a minke whale (Male A) were

available for the HOS test. HOS solutions containing fructose and sodium citrate have been employed for the HOS test in different mammalian species: 60 mOsM for dogs [13] and 100 mOsM for cattle [21] and horses [18]. The present HOS solution for ram spermatozoa was adapted to 157 mOsM following the report of Jeyendran *et al.* [11, 12]. An incubation period of 60 minutes using frozen-thawed ram semen did not show any difference in the percentage of swollen spermatozoa. Nie and Wenzel [18] incubated stallion spermatozoa for 15 to 180 minutes, and reported that the proportion of swollen spermatozoa did not differ in samples incubated at 37 C for between 15 and 180 minutes. Other workers incubated horse [17], dog [13] and cattle spermatozoa for 30, 45 and 60 minutes, respectively. A shorter incubation time (5 minutes) has also produced tail swelling as most swelling occurs within 1 minute [19, 21]. Therefore, it should be possible to shorten the incubation time used in the present study (15 minutes).

For minke whale spermatozoa, an HOS solution at 100 or 150 mOSM resulted in constantly high numbers of identifiable swollen spermatozoa. An HOS solution at 10, 50 or 300 mOsM with 60 minutes incubation time resulted in a lower percentage of swollen spermatozoa. The reason for the lower percentage of swollen spermatozoa in an HOS solution with the lower osmolality (10 or 25 mOsM) in minke whales is unknown. Differences observed in the percentages of swollen spermatozoa could have resulted from different HOS solutions containing various sugars, types of spermatozoa (fresh or frozen-thawed) and animals used [4, 11, 13]. Especially, spermatozoa with membrane damaged or inactivated during the freezing-thawing process are unable to support osmotic swelling [17]. Although the present HOS test results show that the optimal osmolality of a HOS solution for frozen-thawed minke whale spermatozoa is in the range between 100 to 150 mOsM similar to those of other mammalian species [4, 11, 18, 21], the most appropriate conditions for osmolality of the HOS solution and incubation time for the HOST test in post-thaw minke whale spermatozoa remains to be determined in a future study.

From the results of the present study, it was concluded that: 1) the SQA and the HOS test can be utilized to assess the post-thawing motility of ram and minke whale frozen-thawed spermatozoa; 2) the values by the SQA and the HOS-test were significantly correlated in ram spermatozoa; 3) the values of SVI and TFSC by the standard and SQA methods respectively, were not significantly correlated in either ram or minke whale spermatozoa; and 4) sperm concentration and morphologically normal minke whale spermatozoa were not assessed accurately by the SQA method.

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