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## Summary

Prior to attempting the *in vitro* production of embryos in the Bryde's whale (*Balaenoptera edeni*), we investigated whether spermatozoa can retain the capacity for oocyte activation and pronucleus formation as well as chromosomal integrity under cryopreservation by using intracytoplasmic sperm injection (ICSI) into mouse oocytes. Regardless of motility and viability, whale spermatozoa efficiently led to the activation of mouse oocytes (90.3–97.4%), and sperm nuclei successfully transformed into male pronucleus within activated ooplasm (87.2–93.6%). Chromosome analysis at the first cleavage metaphase (M) of the hybrid zygotes revealed that a majority (95.2%) of motile spermatozoa had the normal chromosome complement, while the percentage of chromosomal normality was significantly reduced to 63.5% in immotile spermatozoa and 50.0% in dead spermatozoa due to the increase in structural chromosome aberrations. This is the first report showing that motile Bryde's whale spermatozoa are competent to support embryonic development.

Keywords: Assisted reproductive technology, Bryde's whale, Chromosome, Fertilization, Intracytoplasmic sperm injection

## Introduction

*In vitro* fertilization, *in vitro* culture of embryos, and cryopreservation of gametes and embryos have been intensively and extensively studied in land mammals including laboratory animals, livestock and humans, thus leading to great development in their assisted reproductive technology. Conversely, the study of such reproductive technologies in marine mammals has been delayed because of limited availability of

their gametes. To ameliorate this situation, we have participated in the Japanese Whale Research Program with Special Permit in the Antarctic and tackled the cryopreservation of gametes, *in vitro* maturation of oocytes, and *in vitro* fertilization and *in vitro* production of embryos in minke whales (Fukui *et al.*, 1997a, b; Mogoe *et al.*, 1998; Asada *et al.*, 2000, 2001a, b; Iwayama *et al.*, 2004). Although spermatozoa could penetrate into 55–63% of oocytes following *in vitro* insemination, a considerable number of penetrated sperm nuclei failed to transform into male pronuclei, and none of the fertilized ova developed to blastocysts (Fukui *et al.*, 1997b; Asada *et al.*, 2001a). The failure in male pronuclear formation and the arrest of embryo cleavage were observed when oocytes were microsurgically injected with frozen-thawed spermatozoa (Asada *et al.*, 2001b). The unsuccessful results may not be completely unrelated to functional defects and DNA damage in spermatozoa.

A recent study revealed that the productivity of spermatozoa within testes during the feeding

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(non-breeding) season was very low in common minke whales and Bryde's whales. Testicular spermatozoa of Bryde's whales showed low viability, and approximately 40% of them had abnormal morphology (Watanabe *et al.*, 2004). Spermatozoa collected during the feeding season may be physiologically immature, and their chromatin may be unstable despite their appearance. As sexually mature males are not always captured during the breeding season, it should be evaluated in advance whether collected spermatozoa have normal fertilizability representing oocyte activation (resumption of the second meiosis), nuclear decondensation and male pronuclear formation, and whether they maintain chromosomal integrity.

Wei & Fukui (2000) microsurgically injected minke whale spermatozoa into bovine oocytes to evaluate the fertilizability and detected male pronuclear formation in 39.1% of oocytes. The transformation of sperm nuclei into male pronuclei in Bryde's whale spermatozoa was observed when spermatozoa were injected into porcine oocytes (H. Watanabe, unpublished data). However, whale sperm chromosomes have never been analysed due to some technical difficulties in the chromosome preparation of bovine and porcine eggs. Recently, Amemiya *et al.* (2004) reported that 76.5% of mouse oocytes underwent successful activation following ICSI of Antarctic minke whale spermatozoa, as shown in hamster, rabbit, pig and human spermatozoa (Kimura *et al.*, 1998). In addition, ICSI into mouse oocytes has already enabled us to observe paternal chromosomes at the first cleavage metaphase of hybrid zygotes derived from human spermatozoa (Rybouchkin *et al.*, 1995, 1996a; Lee *et al.*, 1996; Tateno *et al.*, 2000; Watanabe, 2003, 2004; Fedorova *et al.*, 2005) and minke whale spermatozoa (H. Tateno, unpublished data).

In this study, we applied ICSI into mouse oocytes to evaluate the fertilizability and chromosomal integrity of spermatozoa of the Bryde's whale, in which the *in vitro* fertilization and *in vitro* production of embryos is now being pursued. Since sperm samples of the whale usually contain a large number of immotile or dead spermatozoa, we have used these types of spermatozoa in addition to motile spermatozoa to estimate irremediable damage to their fertilizability and chromosomes.

## Materials and methods

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

## Reagents and media

All chemicals were purchased from Nacalai Tesque unless specifically stated. The culture medium of mouse oocytes after ICSI was Chatot–Ziomek–Bavister (CZB) (Chatot *et al.*, 1989) supplemented with 5.56 mM D-glucose. Mouse oocyte collection and microinjection were performed in a modified CZB supplemented with 20 mM HEPES–Na, 5 mM NaHCO<sub>3</sub>, and 0.1 mg/ml polyvinyl alcohol (cold water soluble; Sigma-Aldrich) in place of bovine serum albumin (HEPES–CZB). The medium for cryopreservation of whale spermatozoa consisted of 297.58 mM Tris–aminomethane (Merck), 96.32 mM citric acid (Wako Pure Chemical Industries), 82.66 mM fructose (Sigma-Aldrich), 15% (v/v) egg yolk and 5% (v/v) glycerol (Wako) (Mogoe *et al.*, 1998). Whale spermatozoa after thawing were handled in a modified Toyoda–Yokoyama–Hoshi (TYH) medium (Toyoda *et al.*, 1971) supplemented with 20 mM HEPES, 5 mM NaHCO<sub>3</sub>, and 0.1 mg/ml polyvinyl alcohol in place of bovine serum albumin (HEPES–TYH). The pH value of both HEPES–CZB and HEPES–TYH was adjusted to approximately 7.4 by the addition of 1 N HCl solution.

## Preparation of mouse oocytes

B6D2F1 female mice, 7–11 weeks of age, were super-ovulated by i.p. injection of 10 IU PMSG followed by injection of 10 IU HCG 48 h later. The oocytes recovered from oviducts between 14 and 16 h after HCG injection were denuded of their cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma-Aldrich) in HEPES–CZB. The denuded oocytes were repeatedly rinsed in CZB medium and kept at 37°C under 5% CO<sub>2</sub> in the same medium until ICSI.

## Collection and cryopreservation of whale spermatozoa

Sperm samples were obtained from a Bryde's whale (*Balaenoptera edeni*) captured under the Japanese Whale Research Program with Special Permit in the Western North Pacific between May 2003 and August 2003 (presumptive feeding season). The whale was killed by an explosive harpoon, which has been recognized as the best humane method of killing whales by the International Whaling Commission (IWC) and stipulated by Schedule III (Capture) of the International Convention for the Regulation of Whaling. Special attention to reduce the time to death was given to the whale by use of a large caliber rifle for the secondary method when required.

Immediately after death, *vasa deferentia* were carefully retrieved for fear of contamination by seawater. Spermatozoa collected from *vasa deferentia* were cryopreserved in liquid nitrogen as described previously

(Mogoe *et al.*, 1998). Briefly, spermatozoa were diluted five-fold with cryopreservation medium, and aliquots (0.5 ml) of the sperm suspension were put in microcryotubes. The tubes were exposed to liquid nitrogen vapour for 10 min and then plunged into liquid nitrogen.

### Preparation of whale spermatozoa for ICSI

Frozen Bryde's whale spermatozoa were thawed in a water bath at 37 °C. They were then washed with HEPES-TYH by centrifugation at 500 *g* for 5 min to remove cryopreservation medium. Motile and immotile spermatozoa were obtained at this step. Some spermatozoa in HEPES-TYH were refrozen without cryoprotectant at -20 °C to be completely killed. Within 24 h, they were thawed at 37 °C and prepared for ICSI.

Before injection, spermatozoa were transferred to a droplet (10 µl) of HEPES-TYH supplemented with 10% polyvinyl pyrrolidone (molecular weight: 360 000), which had been prepared in the ICSI chamber on the stage of an inverted microscope with a piezo-micromanipulator and covered with paraffin oil (Merck). Concurrently, a batch of five to 10 oocytes was transferred into a droplet (10 µl) of HEPES-CZB in the same chamber. A single spermatozoon was aspirated into the injection pipette tail first, and the tail was cut at the mid-piece by applying a few piezopulses. The tail-cut spermatozoon was individually injected into a mouse oocyte according to the method of Kimura & Yanagimachi (1995). The injected oocytes were returned to CZB for cultivation. This cycle was repeated several times. A series of experiments for ICSI was finished within 1.5 h of sperm preparation.

### Chromosome preparation

After 6–8 h of ICSI, surviving eggs were transferred to CZB containing 0.04 µg/ml colcemid to inhibit the first cleavage division. At 19–20 h after ICSI, they were treated with 0.5% protease (Kaken Pharmaceuticals) in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline to digest zona pellucida. Then, they were kept in hypotonic solution (1% sodium citrate:30% fetal

**Table 1** Activation of oocytes and transformation of sperm nucleus into male pronucleus (PN) following microinjection with frozen-thawed Bryde's whale spermatozoa into mouse oocytes

Sperm type (no. of experiments)	No. of oocytes examined	Non-activated <sup>a</sup>	Activated without male PN <sup>a</sup>	Activated with male PN <sup>a</sup>
Motile (5)	78	2 (2.6)	8 (10.3)	68 (87.2) <sup>b</sup>
Immotile (3)	72	7 (9.7)	2 (2.8)	63 (87.5)
Dead (4)	78	4 (5.1)	1 (1.3)	73 (93.6) <sup>c</sup>

<sup>a</sup> No. (%) of oocytes.

<sup>b, c</sup> Values with different superscripts were significantly different ( $p < 0.05$ ).

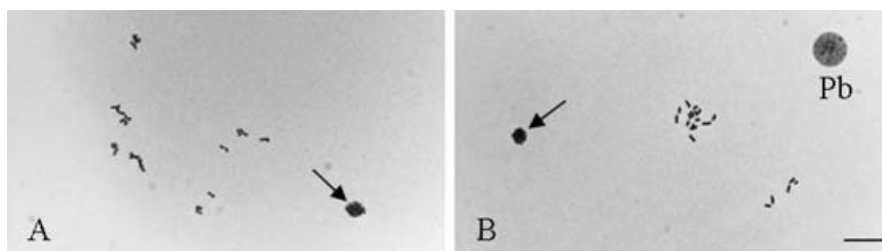
calf serum, 1:1) for 10 min at room temperature. Chromosome slides were made by the gradual-fixation/air drying method (Mikamo & Kamiguchi, 1983). The slides were conventionally stained with 2% Giemsa (Merck) in buffered saline (pH 6.8) for 10 min.

### Statistical analysis

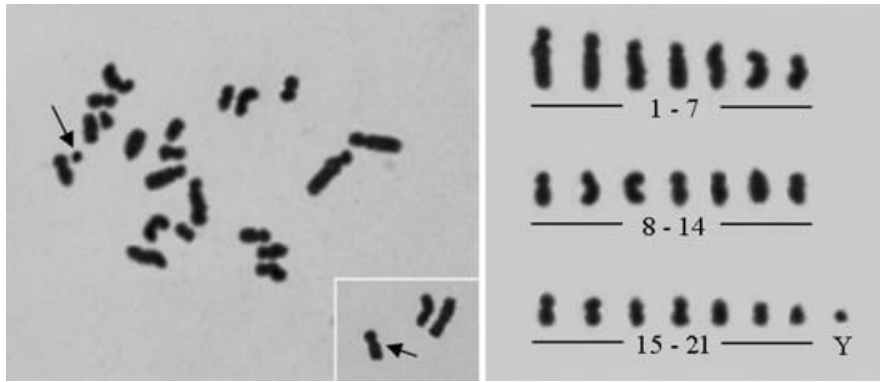
Comparison of group values was performed by either Fisher's exact probability test or chi-square test where necessary. Differences at  $p < 0.05$  were considered significant.

### Results

Regardless of their motility and viability, Bryde's whale spermatozoa led to the activation of more than 90% of mouse oocytes (Table 1). Non-activated oocytes remained as MII regardless of the presence of a decondensed sperm nucleus (Fig. 1*a*). The rate of transformation of sperm nucleus into male pronucleus within activated ooplasm was 87.2% in motile spermatozoa, 87.5% in immotile spermatozoa and 93.6% in dead spermatozoa. Interestingly, the rate was significantly higher ( $p < 0.05$ ) in dead spermatozoa than in motile



**Figure 1** Air-dried preparations of mouse eggs showing uncompleted fertilization with Bryde's whale spermatozoa. (a) A non-activated oocyte. A spread of mouse MII chromosomes (left) and a decondensed whale sperm head (arrow) are seen. (b) An activated egg. Female pronucleus enters the first cleavage metaphase with 20 chromosomes (middle mass), while sperm nucleus remains decondensed. Pb, nucleus of the second polar body. (Bar = 20.0 µm).



**Figure 2** Normal chromosome spread (left) and karyotype (right) derived from motile Bryde's whale spermatozoa. An arrow indicates the Y chromosome. An arrow in the inset represents the X chromosome from a different metaphase.

**Table 2** Chromosomal analysis of frozen-thawed Bryde's whale spermatozoa

Sperm type	No. of sperm examined	Normal (%)	Structural anomaly <sup>a</sup>	Aneuploidy <sup>a</sup>	Diploidy <sup>a</sup>
Motile	62	59 (95.2) <sup>b</sup>	2 (3.2) <sup>b</sup>	0 (0)	1 (1.6)
Immotile	52	33 (63.5) <sup>c</sup>	19 (36.5) <sup>c</sup>	0 (0)	0 (0)
Dead	60	30 (50.0) <sup>c</sup>	30 (50.0) <sup>c</sup>	1 (1.7)	0 (0)

<sup>a</sup> Chromosomal aberrations (%).

<sup>b, c</sup> Values with different superscripts were significantly different ( $p < 0.001$ ).

spermatozoa. In eggs without formation of a male pronucleus, a decondensed sperm nucleus and a female pronucleus or its chromosomes were observed (Fig. 1b).

The rate at which hybrid zygotes were able to reach the first cleavage metaphase was 92.6% (63/68) in the motile sperm group, 82.5% (52/63) in the immotile sperm group and 87.7% (64/73) in the dead sperm group. All the metaphases in the immotile sperm groups were karyoanalysed, while one and four metaphases in the motile and dead sperm groups, respectively, were unsuitable for chromosome analysis owing to the underspread of chromosomes. As shown in Fig. 2, whale sperm chromosomes duplicated and well condensed within mouse ooplasm. The haploid chromosome number of the Bryde's whale was 22. They consisted of 18 metacentric, submetacentric or subtelocentric chromosomes and three acrocentric or telocentric chromosomes. The Y chromosome was the smallest one, and the X chromosome was regarded as a medium-sized metacentric chromosome. Table 2 shows the results of the chromosome analysis. Out of 62 hybrid zygotes in the motile sperm group, 59 (95.2%) had a normal chromosome complement, and two (3.2%) had a chromosome break and a chromatid exchange, respectively. On the other hand, the incidence of spermatozoa with a normal chromosome complement was significantly reduced to 63.5% in the immotile group and 50.0% in the dead group due to the remarkable increase of



**Figure 3** Multiple chromosome fragments (arrows) in the metaphase plate derived from dead Bryde's whale spermatozoa.

structural chromosome aberrations (Fig. 3). Although the incidence of structural chromosome aberrations was higher in the dead sperm group than in the immotile sperm group, there was no significant difference between the two groups. Neither aneuploidy nor diploidy increased in any sperm groups. The overall ratio of Y-bearing spermatozoa was 56%.

## Discussion

Successful activation of mouse oocytes following microinjection with Bryde's whale spermatozoa in the present study shows that sperm-borne oocyte activating factor (SOAF) of the Bryde's whale is not highly species specific, and the activity of SOAF is not necessarily dependent on motility and viability. The results support a previous report that frozen-thawed minke whale spermatozoa could activate mouse oocytes (Amemiya *et al.*, 2004). Furthermore, the high rate of transformation of sperm nuclei into male pronuclei within activated mouse ooplasm indicates that whale spermatozoa well retain the fertilizability under cryopreservation and *in vitro* operation. Wei & Fukui (2000) reported that the rate of male pronuclear formation after microinjection of minke whale spermatozoa into bovine oocytes was 39.1%. The value is obviously lower than that (87.2%) obtained in the present study. A possible explanation is that cell cycle-regulated proteins including maturation promoting factor and cytostatic factor may be stable in bovine oocytes compared to mouse oocytes. The stability of these proteins in whale oocytes remains to be investigated. Interestingly, the rate of male pronuclear formation was significantly higher in dead spermatozoa than in motile spermatozoa. Because unprotected freezing of spermatozoa causes severe damage to the cell membrane, ooplasmic factors implicated in sperm chromatin remodeling (McLay & Clarke, 2003) can easily access to the nuclei of dead spermatozoa.

There may be risks in generating structural chromosome aberrations in assisted reproductive technology. In interspecific *in vitro* fertilization between Chinese hamster spermatozoa and golden hamster oocytes, the lag of sperm nuclear decondensation behind the second meiotic division of oocytes causes structural chromosome aberrations in male genomes (Tateno & Kamiguchi, 1999a, b). This seems to be due to the rich content of disulfide bonds in protamine molecules in Chinese hamster spermatozoa compared with golden hamster spermatozoa. In ICSI, the plasma membrane of spermatozoa enters oocytes. If the sperm plasma membrane is relatively stable, delayed decondensation of sperm nucleus will occur. In the present chromosome analysis, the incidence of structural chromosome aberrations in motile Bryde's whale spermatozoa was 3.2%. This value was similar to that (3.8%) of motile mouse spermatozoa estimated by ICSI into homologous oocytes (Tateno & Kamiguchi, 2005), suggesting that there is no critical delay in the decondensation of Bryde's whale sperm nuclei. Thus the findings indicate that ICSI into mouse oocytes is useful for analyzing the chromosomes of Bryde's whale spermatozoa.

Although immotile and dead spermatozoa retained the fertilizability well, 36.5% of the former and 50% of the latter suffered structural chromosome damage. A significant increase of structural chromosome aberrations was found in mouse and human spermatozoa that had been killed by sonication (Tateno *et al.*, 2000). A similar detrimental effect on chromosomes was found in human immotile spermatozoa obtained from the ejaculated semen (Rybouchkin *et al.*, 1997; Watanabe, 2004) and human dead spermatozoa following unprotected freezing (Rybouchkin *et al.*, 1996b). Usually the plasma membrane of immotile and dead spermatozoa is disintegrated, which may be why structural chromosome aberrations frequently occur in immotile and dead spermatozoa. Tateno *et al.* (2000) reported that when dead spermatozoa were stored in a nucleus isolation medium with a low concentration of Na<sup>+</sup> and a high concentration of K<sup>+</sup> instead of common culture medium, the occurrence of structural chromosome aberrations was mostly suppressed in mouse spermatozoa and was completely suppressed in human spermatozoa. Therefore, it is conceivable that the disturbance of ion balance within the nucleus through plasma membrane disintegration causes structural chromosome aberrations, though the molecular mechanism remains obscure. Because a majority of embryos with structural chromosome aberrations abort early or late in the development process, the *in vitro* production of embryos from immotile and dead spermatozoa is undesirable.

Although the data obtained in the present study are limited to a sperm sample from only a Bryde's whale, we conclude that frozen-thawed motile spermatozoa are competent to support embryonic development. When an *in vitro* culture environment of oocytes and embryos can be adequately improved in a future study, successful *in vitro* production of whale embryos should be possible. Furthermore, the present results provide a new perspective on our freezing protocol of whale spermatozoa as a method for preservation of a genetic source of whales. Additionally, we have shown that chromosomal analysis of whale spermatozoa is a useful technique for measuring the influences of marine pollution on reproduction in cetacean species that occupy the top niche in the marine ecosystem.

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