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Fertilisability of ovine, bovine or minke whale (*Balaenoptera acutorostrata*) spermatozoa intracytoplasmically injected into bovine oocytes

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Summary

This study was conducted to investigate the possibility of using bovine oocytes for a heterologous fertility test by intracytoplasmic sperm injection (ICSI) and to compare the pronuclear formation of ram, bull and minke whale spermatozoa after injection into bovine oocytes. Bovine oocytes were cultured *in vitro* for 24 h and those with a polar body were selected for ICSI. Frozen-thawed semen from the three species were treated with 5 mM dithiothreitol for 1 h and spermatozoa were killed by storing them in a –20 °C refrigerator before use. ICSI was performed using a Piezo system. Three experiments were designed. In experiment 1, a higher ($p < 0.05$) male pronuclear formation rate was found in the oocytes injected with ram (52.6%) or bull (53.4%) spermatozoa than with minke whale spermatozoa (39.1%). In experiment 2, sperm head decondensation was detected at 2 h after ICSI in the oocytes injected with a spermatozoon of each species. Male pronuclei were first observed at 4 h in the oocytes injected with ram or bull spermatozoa and at 6 h in oocytes injected with minke whale spermatozoa. The mean diameters of male pronuclei derived from both whale and bull spermatozoa were larger than those from ram spermatozoa (30.4 μm and 28.3 μm vs 22.4 μm, $p < 0.005$). The mean diameter of female pronuclei in the oocytes injected with whale spermatozoa was also larger than with ram spermatozoa (29.3 μm vs 24.7 μm, $p < 0.05$). The development of male and female pronuclei was synchronous. In experiment 3, ethanol-activated oocytes injected with a spermatozoon from any of the three species achieved significantly higher ($p < 0.05$–0.001) cleavage rates than control oocytes. Blastocyst formation was only observed when bull spermatozoa were used. The results of this study indicate that dead foreign spermatozoa can participate in fertilisation activities in bovine oocytes after ICSI.

Keywords: Bull, ICSI, Minke whale, Oocyte, Ram, Spermatozoon

Introduction

Although sperm parameters such as motility, viability and acrosomal status are important in evaluating sperm fertilisability, it does not mean that a semen sample with good sperm parameters must be highly fertile. Sperm–oocyte interactions, such as sperm–zona pellucida binding, sperm penetration through the zona pellucida, sperm–egg fusion and subsequent male pronuclear formation, also influence fertilisability. The best way to study sperm–oocyte interaction *in vitro* is to conduct homologous *in vitro* fertilisation (IVF), which allows direct insight into the nature of fertilisation events. Homologous oocytes, however, are not easily available in some species, such as endangered wild animals and the human. Heterologous oocytes from some experimental animals such as the hamster (Naish *et al*., 1987; Goud *et al*., 1998), mouse (Rybouchkin *et al*., 1995) and cat (Donoghue *et al*., 1992; Roth *et al*., 1995), and from farm animals such as cattle (Slavik *et al*., 1990; Smith & Murray, 1996) and pigs (Kim *et al*., 1999), have therefore been employed as substitutes for homologous ones to analyse sperm fertilisability of various species.

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With conventional IVF techniques, a problem arises when heterologous oocytes are used for a sperm fertility test. Although an oocyte can easily be penetrated by sperm from certain species, it has generally been accepted that the mammalian oocyte is protected against foreign sperm penetration by a barrier located at the level of the zona pellucida, ooplasm or both (Yanagimachi, 1981). It is difficult to predict whether or not oocytes from a selected species can be penetrated by heterologous spermatozoa following cross-insemination. The technique of intracytoplasmic sperm injection (ICSI), which introduces a spermatozoon directly into the ooplasm, allows sperm to overcome the oocyte barrier, therefore making it possible to analyse and compare fertilisation events such as sperm head decondensation and pronuclear formation in different species using heterologous oocytes. Mouse oocytes have been used to investigate oocyte-activating capacity of human spermatozoa by ICSI (Rybouchkin *et al*., 1995). Kim *et al*. (1999) have compared oocyte activation, pronuclear formation and pronuclear apposition in porcine oocytes following injection of porcine, bovine, mouse or human spermatozoa. There has been no report to date, however, on using bovine oocytes, which have been frequently studied and successfully used for both homologous IVF (Lu *et al*., 1987) and ICSI (Goto *et al*., 1990) and heterologous IVF (Slavik *et al*., 1990; Smith & Murray, 1996; Roth *et al*., 1998, 1999), to test the fertilisability of foreign sperm via ICSI.

Fukui *et al*. (1997) collected follicular oocytes and deferent ductal spermatozoa from the southern minke whale (*Balaenoptera acutorostrata*) in the Antarctic Ocean for further research. Although IVF has been conducted on the minke whale (Fukui *et al*., 1997), the outcome was limited due to the inadequate supply of oocytes, the difficulty of oocyte maturation and the low motility of spermatozoa collected from vasa deferentia. Little is known about the characteristics of fertilisation events, such as pronuclear formation ability and the time course of pronuclear formation, of minke whale sperm during IVF.

For the reasons given above, we used bovine oocytes for the investigation on fertilisability of minke whale spermatozoa (WS) by ICSI. Ram spermatozoa (RS) were also used in this study as the fertility of ram sperm has been successfully tested using bovine oocytes through IVF (Slavik *et al*., 1990; Smith & Murray, 1996). Bovine spermatozoa (BS) were used for homologous fertilisation (control).

Materials and methods

Experimental design

Three experiments were designed in this study. In each experiment, BS, RS and WS were injected into bovine oocytes respectively. In experiment 1, oocytes were fixed at 18 h after sperm injection for examination of pronuclear formation. In experiment 2, oocytes were fixed at 2, 4, 6, 8, 12 or 18 h after sperm injection to determine the time course of pronuclear formation. To investigate the synchronisation of male and female pronuclei, the diameters of both male and female pronuclei (MPN and FPN) were measured in the oocytes fixed at 4, 6, 12 and 18 h after sperm injection. In experiment 3, sperm-injected bovine oocytes were cultured for 10 days to observe cleavage and embryonic development. Since the sample of WS used for this study was limited and the sperm motility was very low (less than 10%), it was not possible to use motile sperm for all experiments. To create the same conditions for ICSI with spermatozoa from different species in this study, all the spermatozoa used for ICSI were killed by storing them in a –20 °C refrigerator (Goto *et al*., 1990).

Oocyte preparation

Ovaries were collected from Holstein and Japanese Black Cattle at a local slaughterhouse and brought to the laboratory in 0.9% NaCl (w/v) at 30–35 °C within 3 h. Cumulus–oocyte complexes (COCs) were collected by aspirating follicles 2–8 mm in diameter with an 18 gauge needle. The COCs were then matured in Tissue Culture Medium 199 (TCM-199 with Earle's salts and glutamine; ICN Biomedicals, Costa Mesa, CA) 100 µl drops (10–15 oocytes/drop) supplemented with 10% heat-inactivated fetal calf serum (FCS; Mitsubishi Kasei, Tokyo, Japan), 1 µg/ml oestradiol-17β (Sigma Chemical Co., St Louis, MO) and 0.02 AU/ml porcine FSH (Antrin; Denka Chemical Co., Japan), at 39 °C in 5% CO_2 and 95% humidified air. After 24 h of *in vitro* maturation, oocytes were denuded with 0.1% (w/v) hyaluronidase (Sigma) in TCM-199 and centrifuged in Hepes-buffered TCM-199 for 5 min at 6000 *g* (Rho *et al*., 1998) to clarify the ooplasm and facilitate the operation of sperm injection.

Sperm preparation

Frozen-thawed semen from a Holstein bull (collected via an artificial vagina and frozen in straws), a Merino × Dorset ram (collected via an artificial vagina and frozen in pellets) and a male minke whale was used in this study. The WS were recovered from vasa deferentia, diluted and frozen in cryomicrotubes as described previously (Fukui *et al*., 1997; Mogoe *et al*., 1998), during November 1995 to March 1996 in the Japanese Whale Research Programme with Special Permit in the Antarctic (JARPA), organised by the Institute of Cetacean Research in Tokyo, Japan. Spermatozoa were suspended in bovine serum albumin (BSA)-free TALP

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containing 5 mM dithiothreitol (Rho *et al*., 1998) (DTT; Wako Pure Chemical Industries, Japan) for 1 h before being washed twice by centrifugation in TALP supplemented with 0.6% (w/v) BSA (fatty acid free, fraction V; Sigma). The sperm were then added to 0.9% NaCl solution containing 8% (w/v) polyvinylpyrrolidone (PVP-360; Sigma). The resultant sperm-containing solutions (sperm-PVP) were distributed into microtubes and stored in a –20 °C refrigerator for up to 1 month.

Pipette preparation and sperm microinjection

Preparation of capillaries, oocyte-holding pipettes and injection pipettes was conducted as described in a previous study (Wei & Fukui, 1999) with some modifications. Neither a bevel nor a spike was made on the tip of injection pipettes. A small volume (about 1 µl) of mercury was added in the proximal end of the pipette before use.

The cover of a plastic dish (100 mm × 15 mm, cat. no. 1001; Falcon Plastics, Oxnard, CA) was used as a microinjection chamber. For sperm from each species, a row consisting of two drops (15 µl/drop) of 8% PVP in 0.9% NaCl medium, one drop (15 µl) of frozen-thawed sperm-PVP and three drops (20 µl/drop) of low $NaHCO_3$ (2 mM)-containing Hepes (10 mM)-buffered TCM-199 was placed in sequence in a vertical line. These drops were covered with culture-grade paraffin oil (Sigma). Twenty to 30 oocytes were placed into the second drop of TCM-199 in the line. Spermatozoa used for injection were tail-cut at the midpiece with a sharpened glass needle (10–20 µm in diameter) via micromanipulation. Tail-cutting reduces the volume of vehicle medium injected into oocytes by shortening the length of spermatozoa. A piezo-micropipette-driving unit (System PMM-150FU; Prime Tech, Japan) was used for sperm injection. An injection pipette was fixed to the holder of the Piezo impact unit which was connected to a water-filled syringe system. Before ICSI, the injection pipette was lowered into the first drop of PVP medium to be washed by repeatedly aspirating and expelling the medium, and was then moved to the second drop for aspiration of fresh PVP medium before transfer to the sperm-PVP drop to pick up a tail-cut sperm. The sperm-containing pipette was then washed in the first drop of TCM-199 to remove the PVP medium attached to the outside wall. One oocyte, with a first polar body, was chosen and positioned with the polar body at 6 or 12 o'clock and held by a holding pipette. Sperm injection was carried out as described by Kimura & Yanagimachi (1995). The sperm-injected oocytes were transferred to the third TCM-199 drop.

Activation and *in vitro* culture of oocytes

Oocytes were activated with 7% ethanol in Hepes-buffered synthetic oviductal fluid (Hepes-SOF) twice (for 5 min each time): 30 min before sperm injection and 1 h after sperm injection. The activated sperm-injected oocytes were then cultured in SOF supplemented with 0.8% (w/v) BSA, 2% (v/v) minimal essential medium (MEM) essential amino acids (EAA, 50-strength; Life Technologies, Grand Island, NY) and 1% (v/v) MEM non-essential amino acids (NEAA, 100-strength; Life Technologies) (Jung *et al*., 1998).

Fixation and examination of oocytes

Oocytes were fixed overnight with acetic acid:ethanol (1:3) in 35 × 10 mm dishes (Falcon). The oocytes were then placed in the scale area of a haemocytometer, covered with a cover slip and stained with 1% acetic acid:orcein by adding the stain solution into the space between the cover slip and the haemocytometer. Five minutes after staining, the oocytes were examined through a phase contrast microscope. The status of sperm and oocyte nuclei was recorded. MPN and FPN were identified by their positions relative to the remaining sperm mid-piece and the second polar body of the oocyte. For example, if a pronucleus was obviously nearer the remaining sperm mid-piece and further away from the second polar body, it was considered to be a MPN (Fig. 3*c*). The diameters of MPN and FPN were measured using a micro-ruler installed in the eyepiece of the microscope.

Statistical analysis

The percentages of male pronuclear formation in experiment 1 and the rates of cleaved oocytes in experiment 3 were examined by a modified chi-square analysis. Student's *t*-test was used to compare the means of pronuclear diameters in experiment 2.

Results

Experiment 1. Nuclear decondensation and male pronuclear formation

The results of experiment 1 are shown in Table 1. Male pronuclear formation rate was significantly higher ($p < 0.05$) in the oocytes injected with BS (53.4%) or RS (52.6%) than with WS (39.1%). The total rate of sperm transformation, including sperm transformed to the stages of decondensed sperm head and MPN, was also higher ($p < 0.001$) with BS (84.5%) and RS (82.2%) than with WS (61.7%).

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Table 1 Nuclear decondensation and pronuclear formation of bovine, ovine and minke whale spermatozoa injected into bovine oocytes

Sperm source	No. of oocytes examined	No. (%) of oocytes with		
		1DSH	1MPN	1DSH or 1MPN
Bull	103	32 (31.1)	55 (53.4)[a]	87 (84.5)[c]
Ram	135	40 (29.6)	71 (52.6)[a]	111 (82.2)[c]
Whale	133	30 (22.6)	52 (39.1)[b]	82 (61.7)[d]

DSH, decondensed sperm head; MPN, male pronucleus.
[a–d] Values with different superscripts within a column differ significantly ([a, b] $p < 0.05$; [c, d] $p < 0.001$).

Experiment 2. Time course of pronuclear formation

Bull, ram and minke whale sperm heads were decondensed 2 h after injection (Fig. 1*A*, Fig. 3*A*). The formation of MPN was observed in the oocytes injected with BS and RS from 4 h after sperm injection, and from 6 h with WS (Figs. 1*B*, 3*B*). When oocytes were fixed at 18 h after ICSI, the mean sizes (diameters) of MPN from both BS and WS were larger than that from RS (28.3 μm and 30.4 μm vs 22.4 μm, $p < 0.005$). The mean size of FPN in the oocytes injected with WS was also larger than that with RS (29.3 μm vs 24.7 μm $p < 0.05$). No significant difference was found between the mean diameters of MPN and FPN in an oocyte injected with spermatozoa from any of the three species (Fig. 2).

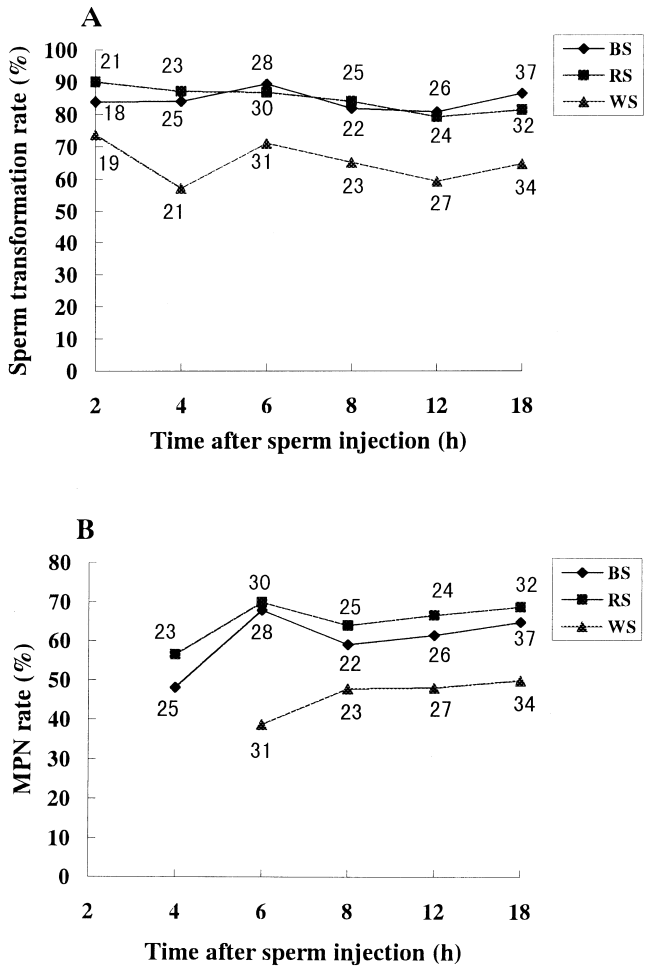

Figure 1 Status of bull (BS), ram (RS) and minke whale (WS) spermatozoa at different time intervals after injection into bovine oocytes. (*A*) Total rates of spermatozoa transformed to and beyond the sperm head decondensation stage. (*B*) Rates of spermatozoa developed to the male pronucleus (MPN) stage. Numbers are oocytes examined.

Experiment 3. Cleavage and embryonic development

When activated with ethanol, the oocytes injected with sperm from any of the three species achieved significantly higher ($p < 0.05$–0.001) cleavage rates than those oocytes treated by sham injection, or the activation and non-treated controls (Table 2). Blastocyst formation (5.7%), however, was only observed when BS were used. Oocytes injected with RS or WS did not develop

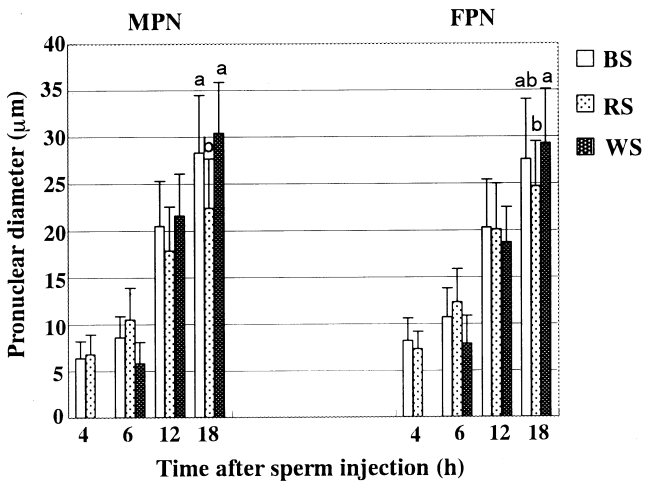

Figure 2 Change in male (MPN) and female (FPN) pronuclear diameters (means ± SD) at different time intervals in bovine oocytes injected with bull (BS), ram (RS) or minke whale (WS) spermatozoa. Within each time interval, columns with different superscripts differ significantly ($p < 0.05$).

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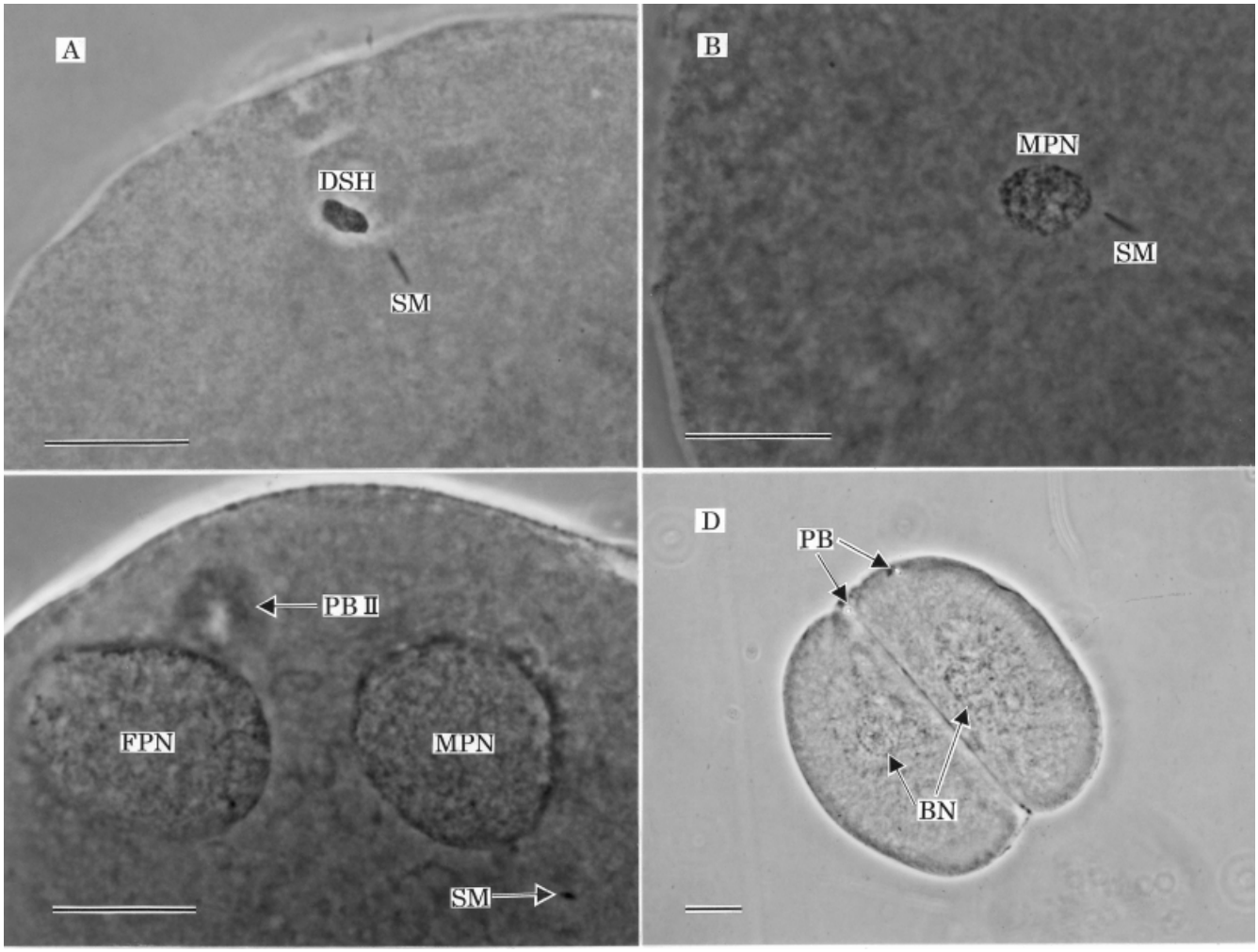

Figure 3 Fate of ram or minke whale spermatozoa after injection into bovine oocytes. (*A*) An oocyte fixed 2 h after intracytoplasmic sperm injection (ICSI). A ram sperm head is starting decondensation. (*B*) An early male pronucleus derived from a ram spermatozoon observed 4 h after ICSI. (*C*) A male pronucleus derived from a minke whale spermatozoon and a female pronucleus observed 18 h after ICSI. (*D*) The 2-cell stage of a minke whale × cow embryo observed 24 h after ICSI. DSH, decondensed sperm head; SM, sperm midpiece; MPN, male pronucleus; FPN, female pronucleus; PB, polar body; PB II, second polar body; BN, blastomere nucleus. Scale bar represents 20 μm.

beyond the 4-cell stage. When oocytes were not activated, BS-injected oocytes cleaved at a rate of 28.1%, but none developed beyond the 16-cell stage. No cleavage was observed in the oocytes injected with RS or WS for ICSI without oocyte activation.

Discussion

Several studies have investigated the use of bovine oocytes for the heterologous fertility test of spermatozoa from rams (Slavik *et al*., 1990; Smith & Murray, 1996) and an endangered African antelope, the scimitar-horned oryx (*Oryx dammah*) (Roth *et al*., 1998, 1999), by IVF. The rates of sperm penetration into oocytes and fertilisation (male pronuclear formation) were obtained at levels comparable to those with BS. In the oryx × cow IVF, 58% of the inseminated bovine oocytes developed to the 2- to 16-cell stage. It was explained that these species share significant physiological similarities. No highly effective species-specific fertilisation barrier exists among the Bovidae, which includes cattle, sheep and oryx, or between non-domestic wildlife species and their domesticated relatives (Roth *et al*., 1998). In the present study, the male pronuclear formation rate was similar in bovine oocytes injected with RS and BS, but lower with WS. This may be because the ram is a member of the Bovidae family but the minke whale is not. Another possible reason is that the whale semen used in this study was collected from vasa deferentia, which was different from the ejaculated ram and bull semen. Nevertheless, 61.7% of

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Table 2 Cleavage and embryonic development of the oocytes injected with spermatozoa from bull, ram or minke whale

Treatment of oocytes		No. (%) of oocytes		
Sperm injection	Activation	Cultured	Cleaved	Developed to blastocysts
Treatments				
BS	+	53	22 (41.5)[a]	3 (5.7)
	–	32	9 (28.1)[ab]	0
RS	+	52	21 (40.4)[a]	0
	–	29	0	
WS	+	56	22 (39.3)[a]	0
	–	34	0	
Controls				
Sham injection	+	86	19 (22.1)[b]	0
Activated control	+	124	22 (17.7)[b]	0
Non-treated control	–	158	3 (1.9)[c]	0

BS, bull sperm; RS, ram sperm; WS, minke whale sperm.
[a–c] Values with different superscripts within a column differ significantly ([a, b] $p < 0.05$; [b, c] $p < 0.001$).

injected WS decondensed and 39.1% developed to MPN. These rates are comparable to those in a pioneer attempt (Fukui *et al*., 1997) at minke whale IVF, which achieved a sperm penetration rate of 55.1% and a male pronuclear formation rate of 40.4%. The present results indicate that foreign spermatozoa from either bovid or non-bovid species can participate in the fertilisation activities in bovine oocytes after ICSI. This fact demonstrates that bovine oocytes can be applied, through ICSI, as a model to investigate the fertilisation events of heterologous spermatozoa. By using the technique of ICSI, Rybouchkin *et al*. (1995) found that mouse oocytes can be used not only to study the ability of human spermatozoa to activate oocytes but also to investigate the chromosomal complement of human spermatozoa. Kim *et al*. (1999) reported the successful use of porcine oocytes to study male pronuclear formation and pronuclear apposition after ICSI with bovine, mouse, human or porcine spermatozoa. It seems that the evolutionary relation is not of consequence in the heterologous fertility test by ICSI.

In the present study, spermatozoa from all three species were found decondensed 2 h after injection into bovine oocytes (Fig. 3*A*). The earliest male pronuclear formation was observed at 4 h after ICSI with BS or RS (Fig. 3*B*). Similar results were reported in ovine ICSI by Gomez *et al*. (1998), who found the earliest decondensation and pronuclear formation of RS were 1 and 3 h after sperm injection – 1 h earlier than the results of our study. The difference may be due to the different time interval between observations in the two studies: 1 h in theirs and 2 h in ours. WS decondensed at the same time as RS and BS but required 2 h longer to form MPN. Before ICSI, we measured the head sizes of BS, RS and WS in order to make injection pipettes of appropriate diameters. It was found that the head length × head width was approximately 5–6 × 8–10 (μm) for BS and RS, and 3 × 5 (μm) for WS. The same size for WS was also reported in a previous study (Mogoe *et al*., 1998). The smaller size of WS might explain the delayed male pronuclear formation. The minke whale sperm head is more compact when compared with those of RS and BS and therefore the chromatin in WS needs more time to decondense fully before pronuclear formation.

Delayed male pronuclear formation and asynchronous pronuclear development have been reported in cattle (Li *et al*., 1993; Iwasaki & Li, 1994; Wei & Fukui, 1999), sheep (Gomez *et al*., 1998) and rabbit (Hosoi & Iritani, 1993) ICSI. In the present study, however, it was noticed that the formation of MPN was synchronous with that of FPN. No significant difference was found between the mean diameters of MPN and FPN. We infer the reason to be that the volume of the vehicle medium injected into oocytes during ICSI could affect the formation of MPN. In this study, the sperm tail was cut away before ICSI to shorten the sperm length and thereby reduce the volume of vehicle medium (PVP medium) injected into oocytes. Less vehicle medium around the injected spermatozoon would be less harmful in its contact with the ooplasm and therefore benefit sperm–oocyte interaction after ICSI. This may lead to the synchronous development of MPN and FPN.

The most significant finding in the present study is that the development of MPN can regulate the development of FPN. The results show that, after 18 h of *in vitro* culture, both MPN and FPN in WS-injected bovine oocytes developed to sizes significantly larger than their equivalents in RS-injected bovine oocytes. It is obvious that the MPN derived from WS developed at a faster rate than did RS-derived MPN (Fig. 2), and the developmental speed of MPN might regulate that of FPN in order to achieve synchronous development. Therefore, as a result, the FPN in the minke whale × cow embryos were also larger than those of ram × cow embryos. It is inexplicable why a later-formed WS-derived MPN developed to a larger size than the earlier-formed MPN from RS, and how MPN regulated the development of FPN. Nevertheless, in this study it is clear that foreign spermatozoa can participate in the fertilisation activities in bovine oocytes after ICSI with their own characteristics, and the oocytes recognise and respond to the signal from foreign spermatozoa, which influences the development of FPN. This signaling, if it exists, should provide a new understanding of sperm–oocyte interaction. It was also noticed that

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decondensed sperm heads were present only in the oocytes that resumed the second reductional division, in other words, those that had been activated. Oocytes with MPN alone were rare. These results suggest that, in the ICSI of the present study, the decondensation of spermatozoa and the formation of MPN are dependent on the resumption of the second reductional division of oocytes and the formation of FPN. Similar findings were reported in ovine ICSI (Gomez *et al*., 1998), although swollen heads of spermatozoa have been found in equal proportions in non-activated and activated human oocytes after injection of human spermatozoa (Dozortsev *et al*., 1994; Flaherty *et al*., 1995).

The results of the present study demonstrate that sperm-injected bovine oocytes cleaved at significantly higher rates than the controls, after activation with ethanol. Fig. 3*D* shows a cleaved WS-injected oocyte fixed 24 h after ICSI which contains two polar bodies (a first polar body and a second polar body) and blastomere nuclei, demonstrating that the cleavage was a result of fertilisation rather than parthenogenesis or fragmentation. This fact indicates that, after heterologous ICSI, syngamy can take place between bovine FPN and foreign MPN, and the zygote can undertake cell division. The hybrid embryos, however, could not develop beyond the 4-cell stage, whereas homologous bovine embryos developed to blastocysts. It has been reported that oryx × cow hybrid embryos, produced by IVF, developed to the 5- to 8-cell stage (Roth *et al*., 1998). None of the porcine oocytes following injection of bovine, mouse or human spermatozoa developed to the mitotic metaphase or divided at the 2-cell stage (Kim *et al*., 1999). The reason for the difference between these results is still unknown. It was also found that when oocyte activation had not been conducted, the oocytes injected with WS or RS did not cleave, while 28.1% of BS-injected oocytes developed to the 2- to 16-cell stage. This indicates that the interaction between foreign spermatozoa and bovine oocytes is weaker than that between bovine spermatozoa and their homologous oocytes. Therefore, oocyte activation is necessary for foreign spermatozoa to participate in further fertilisation activities in bovine oocytes.

From this study we conclude that bovine oocytes can be used as a model for the fertility test of foreign spermatozoa by ICSI. Our results reveal, to our knowledge for the first time, that MPN play a role in synchronising the development of FPN. Further research is required on the interaction between MPN and FPN.

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