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—Full Paper—

Follicle Size-Dependent Changes in Follicular Fluid Components and Oocyte Diameter in Antarctic Minke Whales (*Balaenoptera bonaerensis*)

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Abstract. The concentrations of various components of follicular fluid were compared among three groups of follicles (small, <5 mm; medium: 5–10 mm; large, >10 mm) with a control that consisted of the components of umbilical serum using seven pregnant Antarctic minke whales. Follicular oocytes recovered from the follicles were also used for measurement of oocyte diameter after removing the cumulus cells. The mean diameter of the ooplasm in the oocytes from the large follicles (143.2 μm) was significantly greater than those from the small (127.1 μm) and medium (131.7 μm) follicles, although there were no significant differences in diameter of the whole oocyte and thickness of the zona pellucida among the three follicular sizes. The osmolarity of the follicular fluid from the small follicles (363.3 mOsmol) was significantly lower than that of the medium follicles (388.9 mOsmol) and tended to be lower than that of large (381.9 mOsmol) follicles, respectively, both of which were similar to that of the umbilical serum (379.5 mOsmol). There was no significant difference in the concentrations of all components of the follicular fluid between the medium and large follicles. As compared with the values of the umbilical serum, the total-protein, glucose, albumin and chlorine concentrations of the follicular fluid from the medium and large follicles were significantly higher, and the total cholesterol and calcium concentrations were significantly lower. The concentrations of lactic acid (85.3–136.0 mg/dl) of the follicular fluid from the three groups of follicles were significantly lower than that of the umbilical serum (360.0 mg/dl). Among the follicles, the follicular fluid from the small follicles (136.0 mg/dl) contained a significantly higher concentration of lactic acid than that from the large follicles (85.3 mg/dl). The progesterone concentrations were not significantly different among the fluid from the three group of follicles and the umbilical serum: however, the estradiol 17-β concentrations of the follicular fluid increased with the size of the follicle (14.3 and 34.6 ng/ml for small and large follicles, respectively). These results offer new information concerning whale reproductive physiology, especially for improvement of *in vitro* oocyte maturation and related technologies in whales.

Key words: Follicular fluid, Follicle size, Minke whale (*Balaenoptera bonaerensis*), Oocyte diameter

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Studies of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of Antarctic minke whale (*Balaenopetera bonaerensis*) oocytes have been performed in our laboratory [1–3]. Iwayama *et al*. [4] reported a successful shortening of the IVM culture period for fresh minke whale oocytes from 120 to 30 h by adding pregnant whale

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follicular fluid (wFF) to an IVM medium in place of supplementation with calf or whale serum. Furthermore, by adjusting the osmolarity of the IVM medium to 390 mOsmol, Fujihira *et al*. [5] improved the IVM rates for freshly collected immature oocytes from adult and prepubertal Antarctic minke whales (60.9 and 53.1%, respectively). These results indicate the importance of adding follicular fluid (FF) collected from pregnant females to IVM medium for whale immature oocytes. It has recently been suggested that chemically defined culture media can be used for IVM and IVC in order to clarify the effectiveness of various additives in the media, such as hormones and growth factors [6–9]. However, FF has been widely demonstrated to have a positive effect on IVM culture of various mammalian oocytes, and it has been shown that addition of FF during IVM enhances the male pronuclei formation of oocytes after IVF and subsequent embryonic development [10–14]. Furthermore, FF collected from larger follicles contains some proteins and higher expression of mRNA that improve the progression of nuclear and cytoplasmic maturation and the developmental competence of bovine and porcine oocytes after IVF [13, 14]. The relationships between follicular size and oocyte developmental capacity have been established for many species, including cattle [15], goats [16], pigs [17] and buffaloes [18]. It has also been reported the developmental competence is affected by oocyte diameter and that the oocytes recovered from smaller follicles are generally smaller in diameter and less competent compared with those recovered from larger follicles [19–22].

In regard to whales, Fukui *et al*. [23] and Suzuki *et al*. [24] have previously measured the plasma or serum concentrations of gonadotropins and steroid hormones in pregnant and non-pregnant Antarctic minke whales. However, the concentrations of the chemical components of wFF from follicles of different sizes have not been investigated in detail, except for some steroid hormones such as progesterone (P_4), testesterone (T) and estradiol 17-β (E_2) [25]. In our previous studies [4, 5], wFFs collected from various sizes of follicles from pregnant females were pooled and used for IVM culture of Antarctic minke whale oocytes. If only wFF collected from larger follicles in IVM culture positively affect IVM and subsequent embryonic development, as shown in several studies of domestic animals [13, 14], analyses of wFF components could contribute the design of a chemically defined IVM medium for whale oocytes.

Therefore, the present study was conducted to compare the concentrations of various components of wFF collected from follicles of different sizes (small, <5 mm; medium, 5–10 mm; large, >10 mm) with a control that consisted of the components of umbilical serum using seven pregnant Antarctic minke whales. Vitrified and warmed follicular oocytes derived from the three groups of follicles were also used for measurement of the diameters of the whole oocyte, ooplasm and zona pelllucida after removing the cumulus cells.

Materials and Methods

Whales

The Antarctic minke whales used in this study were obtained from November 2005 to March 2006 as part of the Japanese Whale Research Program with a Special Permit for the Antarctic (JARPA), which was organized by the Institute of Cetacean Research (Tokyo, Japan). The seven pregnant minke whales (body length: 9.04 ± 0.10 m) used were obtained in an area 60 degrees south to the ice edge and between 130 degrees east and to 145 degrees west. All the minke whales possessed a pregnant corpus luteum in one of their ovaries. The fetal length ranged from 60.4 to 149.5 cm (mean ± SEM: 118.1 ± 12.3 cm). The whales were killed with explosive harpoons, which have been recognized by the International Whaling Commission as the most humane method for killing whales, as provided for by Schedule III (Capture) of the International Convention for the Regulation of Whaling. Special efforts were made to reduce the time to death for all sampled whales. Explosive harpoons were used for all whales as the primary killing method. A large-caliber rifle was used as the secondary killing method as required. The ovaries were collected within 3 h after death, and were kept warm at 20–25 C until oocyte collection (within 8 h).

Oocyte collection and vitrification

Cumulus-oocyte complexes (COCs) were aspirated from three groups of follicles (<5 mm, small; 5 to 10 mm, medium; >10 mm, large in diameter) of the minke whales using a 10-ml syringe fitted with an 18-gauge needle. The oocytes surrounded by

more than two layers of cumulus cells and with homogeneous cytoplasm were separately selected and washed three times in Medium 199 (M199, Sigma-Aldrich Chemical, St. Louis, MO, USA) containing 0.1% (w/v) polyvinyl alcohol (PVA, Sigma), 2 mM $NaHCO_3$, (Wako Pure Chemicals, Osaka, Japan), 10 mM HEPES (Sigma) and 75 mg/l kanamycin (Sigma; M199-PVA).

The oocytes were essentially vitrified-warmed according to the method of Fujihira *et al*. [5]. In brief, the COCs were transferred to M199-20% (v/v) newborn calf serum (NBCS; Gibco-BRL, Grand Island, NY, USA; M199-NBCS, osmolarity, 270 mOsmol) containing 7.5 μg/ml cytochalasin B (Sigma) for 30 min. The COCs were then exposed to 7.5% cryoprotectant (3.75% ethylene glycol, EG, and 3.75% dimethyl sulfoxide, DMSO) in M199-NBCS for 8 min and then 15% cryoprotectant (7.5% EG and 7.5% DMSO) in M199-NBCS for 4 min at 37 C. Finally, the COCs were exposed to 30% cryoprotectant (15% EG and 15% DMSO) and 0.5 M sucrose (Wako) in M199-NBCS. Within 1 min, 3 to 5 COCs were placed on a sheet of cryotop (Kitazato Supply, Tokyo, Japan) [5, 26], which was then immersed in liquid nitrogen. For warming, the cryotop sheet holding the COCs was immersed in M199-NBCS containing 0.5 M sucrose at 37 C for 5 min and was further diluted by exposure to M199-NBCS containing 0.25 and 0.125 M sucrose for 2 and 2 min, respectively, at 37 C.

After mechanical removal of cumulus cells from the post-warm COCs, the diameter of the whole oocyte including the zona pellucida, diameter of the ooplasm and thickness of the zona pellucida were recorded by means of two perpendicular measurements. Ten oocytes derived from each group of follicles (small, medium and large) were used for the measurement.

Follicular fluid and umbilical serum

The FF was aspirated from the three groups of follicles (small, medium and large) for collection of oocytes from the seven pregnant whales and was individually stored in 1.8 ml cryo-tubes at –20 C. Umbilical blood was collected from the fetal cords of four out of the seven pregnant whales, and the samples were stored for 1 h at 4 C to allow clotting. The samples were centrifuged (600 g × 20 min) to separate the serum and were kept at –20 C until analyses.

Measurement of the concentrations of follicular fluid and umbilical serum components

Osmolarity: The osmolarity of the FF from the three groups of follicles (n=14 for small, n=22 for medium and n=24 for large) and the umbilical serum (n=4) was measured using an osmometer (OM801, Vogel, Giessen, Germany). Each sample was measured two times.

Chemical components: The concentrations of total-protein (TP, g/dl), total-cholesterol (T-CHO, mg/dl), glucose (GL, mg/dl), albumin (ALB, g/dl), sodium (Na, mg/dl), potassium (K, mg/dl), chlorine (Cl, mg/dl), calcium (Ca, mg/dl), phosphate (P, mg/dl) and magnesium (Mg, mg/dl) in the FF of the medium (n=20) and large (n=20) follicles and the umbilical serum (n=4) were measured at the Tokachi Clinic Center (Obihiro, Japan); TP was measured by a Biuret method, ALB was measured by a bromocresol green method, T-CHO and GL were measured by an enzyme method, Na, K and Cl were measured by an electric-rod method, Ca was measured by an o-cresolphthalein complexone method, Mg was measured by a xylidyl blue method and P was measured by an molybdenum-direct method.

Due to the limited sample volume, the chemical components in the FF collected from the small follicles could not be obtained for measurement with exception of lactic acid. The concentration of lactic acid was measured by an enzyme assay using a commercial kit (Determiner LA; Kyowa-Hakkou, Osaka, Japan). The intra- and interassay coefficients of variation were 3.0 and 1.9%, respectively. Each sample was measured two times.

Steroid hormones: The concentrations of P_4 and E_2 in the FF from the three groups of follicles (n=18 for small, n=25 for medium and n=25 for large) and the umbilical serum (n=4) were measured by enzyme immunoassay as reported in our previous studies [24, 25]. The intra- and interassay coefficients of variation were 5.2 and 9.3% and 4.8 and 8.6% for P_4 and E_2, respectively. Each sample was measured two times.

Statistical analysis

All data are presented as means ± SEM. Kruskal-Wallis tests and the Scheffe method using the StatView program (Abacus Concepts, Berkeley, CA, USA) were used to determine differences for each component of the wFF collected from the three groups of follicles (small, <5 mm; medium, 5–10

Table 1. Diameters (μm, mean ± SEM) of the whole oocyte and ooplasm of the Antarctic minke whales

	Follicular size (mm)		
	<5	5–10	>10
n	10*[1]	10*[1]	10*[1]
Whole oocyte	179.2 ± 3.1	184.4 ± 3.0	188.7 ± 2.5
Ooplasm	127.1 ± 3.1[a]	131.7 ± 3.7[a]	143.2 ± 3.1[b]
Zona pellucida	26.1 ± 1.6	26.3 ± 1.4	22.7 ± 0.8

*[1] Numbers of follicles examined. [a, b] The different superscripts indicate significant differences (P<0.05).

Table 2. Osmolarity (mOsmol, mean ± SEM) of the follicular fluid derived from the different sizes of the follicles of the Antarctic minke whales

	Follicular size (mm)			Umbilical serum
	<5	5–10	<10	
n	14*[1]	22*[1]	24*[1]	4*[2]
Osmolarity	363.3 ± 4.2[a]	388.9 ± 6.6[b]	381.9 ± 4.4[a,b]	379.5 ± 5.8[a,b]

*[1] Numbers of follicles examined. *[2] Numbers of fetuses examined. [a, b] The different superscripts indicate significant differences (P<0.05).

mm; large, >10 mm) and the umbilical serum. Groups producing P values <0.05 were considered significantly different.

Results

Diameters of the whole oocyte and ooplasm

As shown in Table 1, the diameters of the ooplasm of the oocytes recovered from the small (127.1 ± 3.1 μm) and medium (131.7 ± 3.7 μm) follicles were significantly smaller (P<0.05) than that of the ooplasm of the oocytes recovered from the large follicles (143.2 ± 3.1 μm), although there was no significant difference in diameter of the whole oocyte and thickness of zona pellucida among the three follicular size groups.

Osmolarity of follicular fluid and umbilical serum

As shown in Table 2, the osmolarity of the FF of the small follicles (363.3 ± 4.2 mOsmol) was significantly lower than that of the FF collected from the medium follicles (388.9 ± 6.6 mOsmol; P<0.05) and tended to be low compared with the large follicles (381.9 ± 4.4 mOsmol), which had a value similar to that seen in the umbilical serum (379.5 ± 5.8 mOsmol).

Concentrations of chemical components in follicular fluid and umbilical serum

The concentrations of various components of the FF collected from the medium and large follicles and umbilical serum are shown in Table 3. There were no significant differences in the concentrations of any of the components of the wFF collected from the medium and large follicles. However, the TP, GL, ALB and Cl concentrations of the FF from the medium and large follicles were significantly higher (P<0.05) and T-CHO and Ca concentrations were significantly lower (P<0.05) than those of the umbilical serum. The concentrations of the other components of the FF collected from these two groups of follicles and the umbilical serum were not significantly different.

Lactic acid and steroid hormone concentrations

As shown in Table 4, the concentrations of lactic acid in the FF collected from the three groups of follicles were significantly (P<0.05) lower than that in the umbilical serum. Among the follicles, the FF collected from the small follicles (136.0 ± 16.1 mg/dl) had a significantly higher concentration of lactic acid than that collected from the large follicles (85.3 ± 10.0 mg/dl: P<0.05).

The P_4 concentrations were not significantly different among the three groups of follicles and the umbilical serum, while the E_2 concentrations of the wFF increased as the size of the follicles increased

Table 3. Concentrations of various components of follicular fluid and umbilical serum (mean ± SEM) of the Antarctic minke whales

Components	Follicular size (mm)		Umbilical Serum
	5–10	>10	
n	20*1	20*1	4*2
Total-protein (g/dl)	5.2 ± 0.1[a]	5.3 ± 0.1[a]	3.7 ± 0.2[b]
Total-cholesterol (mg/dl)	101.2 ± 6.0[a]	102.7 ± 6.7[a]	159.0 ± 4.1[b]
Glucose (mg/dl)	82.6 ± 4.8[a]	87.9 ± 5.7[a]	3.8 ± 0.9[b]
Albumin (g/dl)	3.8 ± 0.1[a]	3.8 ± 0.1[a]	2.7 ± 0.1[b]
Na (mg/dl)	387.8 ± 7.4	387.0 ± 6.8	354.2 ± 2.8
K (mg/dl)	48.9 ± 2.7	45.1 ± 2.9	54.1 ± 3.3
Cl (mg/dl)	439.7 ± 15.7[a]	435.9 ± 13.0[a]	330.2 ± 3.8[b]
Ca (mg/dl)	8.5 ± 0.3[a]	9.7 ± 0.5[a]	15.1 ± 0.4[b]
P (mg/dl)	9.6 ± 0.2	10.6 ± 0.5	11.3 ± 0.4
Mg (mg/dl)	3.9 ± 0.3	3.9 ± 0.3	4.5 ± 0.2

*1, *2 See the footnotes of Table 2. [a, b] The different superscripts indicate significant differences (P<0.05).

Table 4. Concentrations of lactic acid and steroid hormones in the umbilical serum and follicular fluid from different sizes of follicles (mean ± SEM) of the Antarctic minke whales

Components	Follicular size (mm)			Umbilical serum
	<5	5–10	>10	
n	18*1	25*1	25*1	4*2
Lactic acid (mg/dl)	136.0 ± 16.1[a]	96.8 ± 9.0[a,b]	85.3 ± 10.0[b]	360.0 ± 46.3[c]
Progesterone (ng/ml)	13.3 ± 3.1	16.7 ± 2.5	19.6 ± 3.9	12.4 ± 4.8
Estradiol-17β (ng/ml)	14.3 ± 2.6[a]	22.9 ± 4.0[a,b]	34.6 ± 7.0 [b]	1.2 ± 0.2 [a,b]

*1, *2 See the footnotes of Table 2. [a, b, c] The different superscripts indicate significant differences (P<0.05).

with a significant difference between the small and large follicles (14.3 ± 2.6 and 34.6 ± 7.0 ng/ml, respectively: P<0.05), and tended to be higher than that of the umbilical serum.

Discussion

The present study shows that the osmolarity of the wFF of the small follicles was significantly low (363 mOsmol) compared with that in the medium follicles, and that the osmolarity of the wFF of the medium and large follicles were similar to that of the umbilical serum (380 to 390 mOsmol). Our previous reports [1, 3] showed that the nuclear maturation rate of Antarctic minke whale oocytes was approximately 30% after 96–120 h of IVM culture. In these studies, 15–20% fetal whale serum was incorporated into IVM media with an osmolarity of approximately 310 mOsmol. Iwayama *et al*. [4, 27] added 10 or 50% wFF into IVM media for fresh or vitrified Antarctic minke whale oocytes, and found that the IVM duration could be greatly shortened to 30–40 h. The osmolarity of wFF (387.9 ± 3.1 mOsmol; n=23, [4]) is higher than those of bovine and porcine FF (approximately 300 mOsmol: unpublished data, Iwayama H.). Addition of 10% wFF to IVM medium adjusting the osmolarity to 390 mOsmol provides a better and more physiological environment for maturation of Antarctic minke whale oocytes *in vitro* [4, 5, 27].

Although no significant difference was found in the diameters of the zona pellucida intact oocytes recovered from the three sizes of follicles, the diameters of the ooplasm of the oocytes recovered from the small and medium follicles were significantly smaller than that recovered from the large follicles. Formation of the vitelline space and shrinkage of the cytoplasm were not observed in any of the oocytes categorized into the three groups. This was because there were significant differences in diameter of the ooplasm of the oocytes, but no difference

in diameter of the whole oocyte or thickness of the zona-pellucida; this was not clarified in the present study. However, it should be kept in mind that the whale oocytes used for diameter measurement were derived from COCs vitrified immediately after release from the wFF and had been placed in vitrification solution with an unsuitable osmolarity (around 270 mOsmol) that did not contain wFF; however, the present vitrification procedures may not have only affected the ooplasm diameter. An increase in osmolarity (up to 390 mOsmol) by adding wFF to a vitrification solution could be an alternative means of maintaining oocyte quality leading to subsequent IVM competence.

It has been well demonstrated that oocytes recovered from smaller follicles have less competency to fertilize and develop after maturation [13, 14, 19–22]. In the present study, the E_2 concentration of wFF collected from the small follicles was significantly or tended to be lower than those of wFF collected from the two larger follicles. Iga *et al*. [25] previously reported that the E_2 levels were detectable (≥0.2 ng/ml) in most follicles of Antarctic minke whales and that large follicles (>8 mm in diameter) could be classified into growing and atretic follicles based on their E_2 levels. In the bovine, there is an increase in the concentration of E_2, as follicular size increases and there is no relationship between follicular size and the concentration of P_4 in the FF [28]. The concentrations of E_2 and P_4 in the wFF were similar to those seen in bovine follicles. Therefore, the present results suggest that the oocytes and wFF derived from large follicles with >10 mm in diameter should be used for IVM culture of whale oocytes.

All the Antarctic minke whales used in the present study were in the second trimester of gestation period, and the sample size (n=7 animals) was very limited. Therefore, we could not directly compare the metabolic components and electrolytes in the FF with other species of whales and dolphins. However, to the best of our knowledge, this is the first study of the concentrations of various components of FF, including steroid hormones, in relation to follicular size in Antarctic minke whales. The concentrations of various components of the wFF collected from the medium and large follicles were similar, without any significant differences, although some of the characteristics of the wFF were notable when compared with the umbilical serum (Table 3). The concentrations of several components (TP, GL, ALB, Na and Cl) were significantly higher than those in the umbilical serum, whereas the T-CHO and Ca concentrations were lower than those in the umbilical serum. However, no significant differences were found in the concentrations of the chemical components among the three groups of follicles, except for the E_2 and lactic acid concentrations. The concentration of lactic acid of the small follicles was significantly higher than those of the wFF collected from the two larger follicle size groups. In the bovine, Iwata *et al*. [29] found that the concentration of lactic acid was negatively correlated with the GL ($P<0.05$) and Mg (–0.23, not significantly) concentrations of the FF. However, in the present study, the GL concentrations of the small follicles (<5 mm) were not measured due to the limited volume of wFF. However, the concentrations of GL and T-CHO in Antarctic minke whale FF were greater compared with those of bovine FF [29–31], and the concentrations of ALB, Ca and Mg were similar to those of bovine FF. It may be that the characteristics of FF change greatly during different developmental and estrous stages [32, 33]. These previous studies of the characteristics in FF of domestic animals indicate that the concentrations of the FF components are different depending on follicular size and suggested that the FF collected from larger follicles positively affects oocyte quality and developmental competence of oocytes after IVM culture [13, 14, 19–22]. The present wFF collected from the medium and large follicles had higher concentrations of TP, GL, ALB, Na, Cl and E_2 and lower concentrations of T-CHO, Ca and lactic acid than those of the umbilical serum. These data indicate that wFF would be a more suitable additive for IVM medium of Antarctic minke whale oocytes rather than umbilical serum.

To produce whale embryos *in vitro*, we have been attempted IVM, IVF and intracytoplasmic sperm injection (ICSI) and IVC [1–5] and have obtained early morula stage minke whale embryos; however, no blastocysts have been obtained to date. The present results suggest that it is better to use the follicular fluid recovered from larger follicles (>10 mm in diameter) for IVM culture and that the diameter of the ooplasm, osmolarity and concentrations of various components, including steroid hormones of wFF should be considered. This approach would be applicable to development of a new IVM medium for Antarctic minke oocytes and

could contribute to improvement of *in vitro* production of whale embryos in the near future. In conclusion, the present results offer new information concerning the follicular components of Antarctic minke whales and contribute whale reproductive physiology, especially for improvement of IVM and related technologies in whales.

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