Ultrastructural Changes during Maturation and Cryopreservation of Follicular Oocytes of Antarctic Minke Whales (*Balaenoptera bonaerensis*)

Masatsugu ASADA ^{1,4)}, Masafumi TETSUKA ²⁾, Hajime ISHIKAWA ³⁾, Seiji OHSUMI ³⁾ and Yutaka FUKUI ¹⁾

1) Department of Food Production Science, Obihiro University of Agriculture and Veterinary Medicine,
West 2-line, Inada-cho, Obihiro 080-8555, Japan

2) Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine,
West 2-line, Inada-cho, Obihiro 080-8555, Japan

3) The Institute of Cetacean Research, 4-5, Toyomi-cho, Tokyo 104-0055, Japan

4) Present address: National Livestock Breeding Center, Tottori Station, 14, Kotoura-cho, Toh-haku-gun,
Tottori 689-2501, Japan

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南極海産クロミンクジラ卵母細胞の体外成熟および 凍結過程における微細構造の変化

浅田正嗣 ^{1, 4)}, 手塚雅文 ²⁾, 石川 創 ³⁾, 大隅清治 ³⁾, 福井 豊 ¹⁾

1) 帯広畜産大学食料生産科学講座 〒080-8555 北海道帯広市稲田町西 2 線

2) 帯広畜産大学家畜生命科学講座 〒080-8555 北海道帯広市稲田町西 2 線

3) 日本鯨類研究所 〒104-0055 東京都中央区豊海町 4-5

4) 現所属：家畜改良センター鳥取牧場 〒689-2501 鳥取県東伯郡琴浦町大字出上 14

ABSTRACT. Important information for cryopreservation and *in vitro* culture could be obtained through ultrastructural observation of minke whale oocytes. In the present study, transmission electron microscopy was used to examine the ultrastructure of immature and *in vitro* mature Antarctic minke whale oocytes before and after freezing and thawing. Close communication existed between ooplasm and cumulus cell projections, and between mitochondria and membranous organelles, including the smooth endoplasmic reticulum in the ooplasm of minke whale immature oocytes. Cytoplasmic organelles, such as mitochondria and lipid droplets, were clustered in the ooplasm cortex region, while vesicles were located centrally in the ooplasm. After *in vitro* maturation, cytoplasmic organelles were observed to have distributed throughout the ooplasm, and the morphology of the mitochondria was changed. Cytoplasmic degeneration was observed in the minke whale oocytes after freezing and thawing. The slow freezing procedures employed in this study appeared to generate extensive and severe ultrastructural damages in minke whale ooplasm.

Key words: *Balaenoptera bonaerensis*, minke whale, oocyte, transmission electron microscopy (TEM)

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INTRODUCTION

No information is available regarding details of the normal oocyte structure of large baleen whales. In a previous study [1], the ultrastructure of immature oocytes of minke whales after freezing and thawing was described. There are reports in cattle oocytes undergoing maturation that cytoplasmic organelles, such as mitochondria and cortical granules (CGs), exhibit specific patterns of reorganization and morphological changes [2-6]. Cytoplasmic organelle reorganization was a result of interrupted communication between oocytes and cumulus cells [5]. Cumulus cells form processes that traverse the zona-pellucida (ZP) and end in foot processes that penetrate into the oocyte cortex region, where they form gap junctions

with oolemma [7,8]. These heterogeneous contacts are also important during final maturation in cattle oocytes [9]. Gap junctions as metabolic couplings are vital for normal oocyte growth and for maintenance of meiotic arrest in mouse oocytes [10].

It has been demonstrated through ultrastructural observations that cytoplasmic organelles in cryopreserved mammalian oocytes were impaired during freezing and thawing procedures. These include plasma membrane disruption [11,12], release of CGs [13,14], extensive ooplasm disorganization, and loss of oocyte-cumulus cell attachment [15]. Viability of immature (31-44%) and *in vitro* mature (IVM; 49-69%) minke whale oocytes has been reported to be low after freezing and thawing [1,16]. Observations by

transmission electron microscopy suggested that low viability of minke whale oocytes after freezing and thawing might be due to rupture of the membranes of the ooplasm and vacuolation of mitochondria [1].

The purposes of the present study using Antarctic minke whales (*Balaenoptera bonaerensis*) were verification and comparison of fresh and frozen-thawed immature and IVM oocyte ultrastructures such as characteristics of microvilli, mitochondria, and cortical granules. If ultrastructural observation of Antarctic minke whale oocytes were possible, important information for cryopreservation [1] and *in vitro* culture [17,18] of the whale oocytes could be obtained approaching the level of *in vitro* embryo production for other mammals, such as cattle and sheep.

MATERIALS AND METHODS

Animals

This study was undertaken as part of the 1999/2000 Japanese Whale Research Program under Special Permit in the Antarctic. The present research area comprised Area IV (60-68° S and 70-130° E) and the eastern part of Area III (58° S to the ice edge and 35-70° E) in the Antarctic Ocean; it was conducted from 5 December 1999 to 10 March 2000 [19]. All the whales used in the present study were killed by explosive harpoons which have been recognized by the IWC/SC as the most humane method to kill whales, and is accepted by schedule III (Capture) in the International Convention for the Regulations of Whaling. Special attention to reduce the time to death was given to all sampled whales; explosive harpoons for the primary method and the large caliber rifle as the secondary method when required. Whales were killed with grenaded harpoons according to the regulation of whaling under the International Convention.

Oocyte Collection and *In vitro* Maturation

Oocytes were collected from 34 female whales. Follicles (1-10 mm in diameter) were aspirated with an 18-gauge needle connected with a 10-ml syringe containing a modified phosphate-buffered saline (mPBS; Embryo-Tech; Nihon-Zenyaku Industrial, Japan) supplemented with 0.5% (v/v) sodium citrate solution (Chitoramin; Fuso Pharmaceutical, Japan). Recovered follicular oocytes were selected as described by Fukui et al. [18]. Cumulus-oocyte complexes were washed three times with mPBS. The medium used for IVM was a Tissue Culture Medium 199 (TCM199; Gibco Life Technologies, USA) supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (all from Sigma Chemical, USA). The maturation medium contained 1 µg/ml estradiol-17β (Sigma), 0.01 mg/ml porcine pituitary follicle stimulating hormone (Antrin; Denka Pharmaceutical, Japan) and 20% (v/v) fetal whale serum [17]. Follicular oocytes (5-30) were co-cultured with 5×10^5 live granulosa cells/ml in a 4-well dish in 0.5 ml of maturation medium covered with mineral oil (E.R. Squibb and Sons, USA) for 120 h [18,20] at

37°C in 5% CO₂ in air and ≤ 95% humidity. Every 48 h during culture, 0.3 ml of maturation medium was replaced with fresh medium. Granulosa cells were collected at the time of aspiration of follicles and washed once (600 x g for 10 min) with the mPBS, and then washed twice with the TCM199 after being kept for 30 min at room temperature (22-24°C). The supernatant was discarded after centrifugation; the granulosa cell pellet was suspended with TCM199.

Freezing and Thawing

Dulbecco's PBS (D-PBS; Nissui Pharmaceutical, Japan) containing 1.5 M10% (v/v) ethylene glycol, 0.1 M sucrose, and 10% (v/v) heat-treated fetal calf serum (FCS) was used as a cryoprotective medium. Immature and IVM oocytes were transferred to the cryoprotective medium and equilibrated for 10 min at room temperature (20-22°C). After equilibration, a group of 10 oocytes was loaded into a 0.25-ml straw; the open end of the straw was plugged with polyvinyl alcohol powder, and the straw was then transferred into a pre-cooled methanol bath (0°C) within a programmable freezer (ET-UM; Fujiya Yano Science, Japan). Straws were cooled from 0°C to -6.5°C at 0.5°C/min, and then seeded at -6.5°C for 10 min. After seeding, straws were further cooled to -35°C at the rate of 0.3°C/min before they were plunged into liquid nitrogen (LN₂). Oocytes were kept in LN₂ for 3-5 months prior to being thawed and subsequent experimentation. Straws were warmed rapidly (10 sec at 25°C in air, followed by 20 sec at 37°C in a water bath), and cryoprotectants were removed by transferring oocytes directly into D-PBS supplemented with 10% FCS [21].

Preparation of Oocytes for Transmission Electron Microscopy

Fresh immature (Fig. 1A; n=10) and IVM (Fig. 1C; n=5) oocytes were pre-fixed by Karnovsky's solution (1% glutaraldehyde and 4% paraformaldehyde containing 0.1 M phosphate buffer [PB; pH 7.4]) on board the vessel. Karnovsky's solution was changed to a fresh solution every 1 month for 5-6 months. In the laboratory, fresh and frozen-thawed [immature (Fig. 1B) : n=8; IVM (Fig. 1D) : n=4] oocytes were re-fixed by 2.5% glutaraldehyde in 0.1 M PB for 2 h at 4°C, followed by 1% osmium tetroxide in 0.1 M PB for 2 h at 4°C according to the procedures described previously for bovine oocytes [22,23]. Briefly, after routine dehydration with a graded series of ethanol, oocytes were infiltrated successively with 3:1, 1:1, and 1:3 mixtures of absolute propylene-oxide and Spurr's epoxy resin, and then with absolute Spurr's epoxy resin. Oocytes were embedded in beam capsules with fresh Spurr's epoxy resin. Ultrathin sections (80 nm) were cut with a diamond knife and double-stained with uranyl acetate and lead citrate; they were then examined under an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Fresh Immature Oocytes

Aggregation of membrane-bound vesicles and mitochondria

Ultrastructure of Minke Whale Oocytes

Fig.1 A: A COC with homogeneous cytoplasm and surrounded with numerous cumulus layers. B: A follicular oocyte after freezing and thawing. C: An *in vitro* matured oocyte with an extruded first polar body (arrowhead). D: An *in vitro* matured oocyte after freezing and thawing, which was partially surrounded with expanded cumulus cells (bar: 50 μ m).

was observed in peripheral ooplasm (Figs. 2A, 3 and 4A). Central ooplasm was largely free from cytoplasmic organelles, and there were confluent vesicles (Fig. 2A). The cortical region of ooplasm was occupied by some coated vesicles (Fig. 3). Fresh oocytes and cumulus cells were tightly attached to the zona-pellucida (ZP; Fig. 2A). Cumulus cell processes into the oocyte were located in a perivitelline space with microvilli (Fig. 4A). Also, 2-3 μ m diameter mitochondria (Fig. 6A), about 0.5 μ m diameter cortical granules (CGs), and Golgi apparatus were located in the ooplasm cortex region (Fig. 3). The most abundant form of endoplasmic reticulum appeared as smooth single sacs closely associated with outer surfaces of mitochondria, vesicles, Golgi apparatus, and lipid droplets in the ooplasm (Fig. 3). The smooth endoplasmic reticulum (SER) was closely associated with the mitochondria; these mitochondria occasionally possessed a "hood-like" process or appendage forming an extra mitochondrial cavity that often appeared to be intra-mitochondrial when the plane of the section passed through the hood (Fig. 6B). Fresh follicular oocytes had a multi-layered cumulus cell investment in which cells had well-developed cisternae of ER and Golgi apparatus (Fig. 7). Cumulus cells adjacent to the ZP showed numerous

projections which penetrated through and finally attached to the oolemma (Fig. 4A).

Fresh Oocytes after *In vitro* Maturation

The oocytes after maturation had no deviations in specific organelle arrangements (Fig. 2B). A few cumulus projections were observed in the perivitelline space of an oocyte after maturation (Fig. 4C). Cytoplasmic organelles, homogeneous mass, lipid droplets, and vesicles were evenly intermixed and distributed throughout the ooplasm. Mitochondria of 3-7 μ m in diameter after maturation culture were located in an even distribution in the ooplasm, and (Fig. 4C). After maturation, Golgi apparatus, SER, and lipid droplets became small in size, and vesicles tended toward more homogenous distribution (Fig. 4C vs A). CGs were dispersed in a lining beneath the cytoplasmic membrane (Fig. 4C). Mitochondria were intermixed with the more developed SER and Golgi apparatus (Fig. 4C), and showed numerous transverse criste (Fig. 6E). Figure 4C shows heterogeneous density of the ZP layer outer and inner surfaces. The outer ZP was rough compared to the inner surface.

Fig.2 A: A whole structure of a fresh immature oocyte surrounded with cumulus cells (CC), zona-pellucida (ZP), and ooplasm (O). Many vesicles were located in the center of the ooplasm, and the clusters of organelles under the oolemma were observed. Numerous microvilli (arrow) were well developed. B: A fresh oocyte cultured for *in vitro* maturation. Cumulus cells surrounding an oocyte were not shown. (bar: 10 μ m)

Cryopreserved Oocytes

Electron microscopy revealed widespread damage in cryopreserved immature and IVM oocytes, such as ooplasm membrane rupture (Fig. 4B), different structure microvilli vacuolation (Fig. 4B), disappeared CGs (Fig. 5) and presence of swollen ER (Fig. 5) and vacuolated mitochondria (Fig. 6D). After freezing and thawing, vacuoles were found to be present at the periphery of the oocytes (Figs. 4B and D). Microvilli in frozen-thawed immature oocytes exhibited degeneration and detachment of foot processes; they were also distorted or even absent (Fig. 4B). Numerous vesicles in abnormal oocytes appeared to migrate peripherally in the ooplasm,

became confluent, and occasionally resulted in rupture of the oolemma (Figs. 4B and D). It was shown that the mitochondrial matrix becomes hollow in both frozen-thawed immature and IVM oocytes (Figs. 6C and D). Cumulus cells surrounding an immature oocyte after freezing and thawing displayed more vesicles than those of a fresh oocyte. (Fig. 7B vs A).

DISCUSSION

The present study, using immature and IVM oocytes of minke whales, attempted to clarify ultrastructural aspects. In addition, cytoplasmic organelle changes were shown after *in vitro* oocyte maturation, and freezing and thawing procedures.

Ultrastructure of Minke Whale Oocytes

Fig. 3 The various organelles were shown in cortical region of a minke whale immature oocyte. The cortical granule (CG) and transport coated vesicles (arrowhead) in an endocytic pathway were located beneath oolemma. The dilated sacs of endoplasmic reticulum (ER) were surrounded with Golgi apparatus (G) and mitochondria (M) (ZP: zona-pellucida, LD: lipid droplet). (bar: 5 μ m)

It has been reported that various cytoplasmic organelles appear in cumulus-oocyte complexes of human and domestic animals [4,24,25]. Also, the close association among mitochondria, vesicles and lipid droplets through SER in the oocyte has confirmed descriptions reported for cattle [3,5].

It is well known that cumulus cells play a very important metabolic role during follicular growth or IVM, supplying nutrients or other substances for oocyte development [4,26,27]. It was shown that numerous microvilli in the fresh immature oocytes intermediated with connection between cumulus cell projections (Figs. 2A, 3 and 4A). In contrast, cumulus cell projections disappeared in the ZP and

perivitelline space after maturation (Fig. 4C). Therefore, close communication of ooplasm and cumulus cells would no longer be essential after oocyte maturation. In immature oocytes, vesicles appeared under lipid droplets and a mitochondria mass (Fig. 3). However, after IVM, vesicles were dispersed and smaller than in a follicular oocyte (Fig. 4C). It is reasonable to infer that vesicle contents were consumed during oocyte maturation because it is likely that the coated vesicles were caused by endocytosis or exocytosis occurring on the surface or inside oocytes (Fig. 3). Also, some mitochondria had a curved appendage consisting of both inner and outer membranes forming a "hood-like" structure (Fig. 6B).

Fig. 4 A: A fresh immature oocyte. Clusters of mitochondria (M), and sacs of smooth endoplasmic reticulum (arrowhead) were observed between the mitochondria. Microvilli (Mv) were attached at the end point of cumulus cell projection (ECP) (LD: lipid droplet). B: Vesicles (*) were swollen in the oolemma in an immature oocyte after freezing and thawing. C: A fresh *in vitro* maturing oocyte. A few microvilli (white arrow) was shown on the smooth membrane. The cortical granules (arrowhead) were placed in parallel to an ooplasm membrane, and Golgi apparatus (G) were also shown. D: The circumstance of oolemma in a frozen-thawed oocyte after *in vitro* maturation. Vesicles (*) were swollen. (bar: 5 μ m)

"Hood-like" mitochondria structures were observed as reported in bovine and human oocytes [24,28]. In mature human oocytes, mitochondria are often observed to locate on the endoplasmic reticulum side with a mass of numerous transverse cristae, and are either elongated or transformed into "dumb-bell" shapes [25]. Rieger et al. [29] suggested that the relative effects of some cellular growth factors on the oocyte pyruvate metabolism generally correlated with cleavage and subsequent development. It was shown clearly that mitochondria were elongated and had multiple-layered cristae in minke whale oocytes after IVM (Fig. 6E). Thus, morphological change of mitochondria is considered to

show functional characteristics during oocyte maturation. Furthermore, CGs after oocyte maturation culture migrate to individual positions forming a line along the oolemma. CGs were also observed under the oolemma in mature bovine oocytes [4,28]. The present observations revealed that CGs also appeared beneath the cytoplasm membrane after oocyte maturation (Fig. 4C). Probably, CGs release their contents into the perivitelline space when minke whale oocytes have been stimulated by fertilization or activation as fertilization events in other mammals. On the other hand, cytoplasmic organelles including mitochondrial clusters and vesicles were reorganized in bovine oocytes more than 19 h after LH surge [5]. It seems

Ultrastructure of Minke Whale Oocytes

Fig. 5 The periplasm of a frozen-thawed oocyte cultured after *in vitro* maturation was shown. A part of cortical granules was indistinctness (arrowhead). The characteristic morphology of mitochondria (M) and swollen endoplasmic reticulum (ER) attached with them were shown. (ZP: zona-pellucida) (bar: 5 μ m)

that cytoplasmic organelles in minke whale oocytes become rearranged and ultrastructurally change during maturation. For example, mitochondrial elongation was shown as described in reports of bovine [4] and human [24] oocytes after IVM. These observations suggested that metabolic function occurred in cytoplasmic organelles during maturation in minke whale oocytes.

During freezing and thawing, mammalian oocytes have been damaged by various factors, including cryoprotectant toxicity, chilling injury, osmotic swelling, and shrinkage [13,14,30-33]. Electron microscopy of frozen-thawed minke whale oocytes showed the various damage to cytoplasmic organelles that is normally associated with freezing and thawing procedures. The survival rate (40%) [16] in post-thawed minke whale oocytes was lower than those reported in other studies [34-37]. Observations of cryopreserved bovine [3] and mouse [14] oocytes showed that gathering vesicles often occupied the ooplasm and that the ooplasm was then ruptured. This vesicle confluence and plasma membrane destruction (Fig. 4B) in minke whale oocytes were also observed in the present study. This phenomenon was recognized during oocyte shrinkage at the time of freezing; increased vacuolation resulted from shrinking and changes in cellular volume. Osmotic change of cytoplasm volume probably caused membrane rupture and broken organelles. Also, it can be speculated that breakdown in contact of cumulus cell projections to the ooplasm and loss of mitochondrial integrity were consequences of osmotic shock; an oocyte seemed to appear first in change of these membranous structures by freezing and thawing.

Microvilli loss indicates close coupling between the oocyte and its investment of cumulus cells [38]. Disappearance of cumulus cell projections hinders nutrient transportation

to minke whale oocytes. Therefore, it was considered that the oocytes, after freezing and thawing, would demonstrate lower proportions of survivability and maturation ability. For cryopreserved horse oocytes [15], the freezing method induces some ultrastructural changes such as mitochondria vacuolation and, peripheral ooplasm destruction and communication. In the present study, the mitochondrial matrix in ooplasm of the fresh minke whale oocytes was quite highly density (Figs. 6A and E), whereas mitochondria swelling with reduced matrix density was observed in the frozen-thawed oocytes (Figs. 6C and D). This observation implies that the vacuolated mitochondrial matrix was affected by the cryopreservation procedures. Changes in the ZP area associated with decreased CG number did not occur in isolated ZP from ooplasm [39]; also, it was suggested that effects of cooling and cryoprotective agents on the ZP lead to premature CG exocytosis and zona hardening [40]. In the present study using minke whale IVM oocytes after freezing and thawing, indistinct CGs (Fig. 5) might have been induced by low temperature and cryoprotectants during freezing and thawing as reported in cryopreserved bovine oocytes [32,41]. Cumulus cells surrounding oocytes (Fig. 7) were morphologically similar to bovine cumulus cells as reported by Zhao et al. [23]. However, vesicles in cumulus cells of minke whales were swollen after freezing and thawing, suggesting that cumulus cells were contracted by the cryoprotective medium.

Salient points of the present results are that minke whale immature oocytes had close communication between the ooplasm and cumulus cell projections, and between mitochondria and SER in the ooplasm. A cytoplasmic organelle mass including mitochondria and lipid droplets was located in the ooplasm cortex region. In addition, vesicles were evident

Fig. 6 A: The high matrix density in the ellipse shape of mitochondria (M) was attached with smooth endoplasmic reticulum (SER) and a lipid droplet (LD) in a fresh immature oocyte. B: The cavity of a "hood-like" mitochondrion (arrow) which was passed to the SER membrane (arrowhead) in a fresh immature oocyte. C: The matrix density of mitochondria was lowered in a frozen-thawed immature oocyte. D: The vacuolation of mitochondrial matrix was observed in a frozen-thawed oocyte after *in vitro* maturation. E: Numerous transverse cristae (arrow) into mitochondria was observed in a fresh IVM oocyte. (bar: 3 μ m)

within the central ooplasm in immature oocytes. Therefore, the ultrastructure of immature and *in vitro* maturing minke whale oocytes was quite similar to that reported in bovine oocytes. On the other hand, cytoplasmic organelles in whale oocytes after IVM were distributed homogeneously throughout the ooplasm; then mitochondria and SER changed morphologically. It is suggested that the cytoplasmic organelles of whale oocytes

undergo functional changes during maturation. Regarding ultrastructural damages to cryopreserved minke whale oocytes, these observations were caused by the freezing and thawing procedures used in this study. Physical damage to oocytes was also present in both immature and IVM oocytes after cryopreservation.

Ultrastructure of Minke Whale Oocytes

Fig. 7 A: Cumulus cells surrounding a fresh immature oocyte had a large decondensation of nuclei (N) and lipid droplets (white arrow), and endoplasmic reticulum (ER) were extended to the many cytoplasmic projections (CP). B: Cumulus cells surrounding a frozen-thawed immature oocyte had the similar components with fresh cumulus cells. The swollen vesicles (arrowhead) were seen. (ZP: zona-pellucida) (bar: 5 μ m)

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要 約

超微細構造の観察を行うことによって、卵母細胞の凍結保存や体外培養のための重要な知見が得られる。本研究では、透過型電子顕微鏡を使用して、クロミンククジラ卵母細胞の体外成熟の培養前後において、凍結前および凍結・融解後の超微細構造を観察した。成熟前の卵母細胞では、卵細胞質と卵丘細胞突起間に、また卵細胞質内のミトコンドリアや滑面小胞体を含む細胞内小器官の間に緊密な接着部が存在していた。ミトコンドリアや脂肪滴のような卵細胞質内小器官は細胞質の辺縁で塊状に分布していたが、小胞は細胞質の中心部に位置していた。体外成熟後の新鮮卵母細胞では、細胞質内小器官は細胞質内全体に分布し、ミトコンドリアの形態に変化が見られた。凍結・融解後の卵母細胞では、卵細胞質の変性が観察され、凍結・融解過程において卵細胞質の微細構造に大きな障害が生じたことが伺われた。

キーワード：卵母細胞，凍結保存，体外成熟，細胞質，クロミンククジラ

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