# Studies on Vitrification Methods of Immature Oocytes in Three Baleen Whales

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# 三種のヒゲクジラにおける未成熟卵子のガラス化保存法に関する研究

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# General Introduction

## 1. Application of reproductive technologies in whales

In domestic animals, the reproductive technologies such as *in vitro* embryo production (IVP), oocytes/embryos cryopreservation and embryo transfer (ET) have been developed. These technologies can be applied for human assisted reproduction and endangered wild animals for protection. Whales are the largest wild mammalian species and some of their species are endangered such as blue (*Balaenoptera musculus*) or bowhead (*Balaena mysticetus*) whales. However, studies of the IVP and oocytes/embryos cryopreservation for cetaceans were limited and the blastocyst stage embryos have not yet produced. Therefore, the technology of the IVP, ET and oocytes/embryos cryopreservation for whales should be established for the conservation, artificial reproduction and gene banking.

## 2. Study of whale *in vitro* production until now

Since first Fukui *et al.* [1] reported that whale semen was successfully cryopreserved, several studies were performed regarding whale IVP in common minke (*B. acutorostrata*), Antarctic minke (*B. bonaerensis*), sei (*B. borealis*) and Bryde's (*B. edeni*) whales [2-18]. It was reported that immature oocytes were matured approximately 30% after 96-120 h of *in vitro* maturation (IVM) culture [2]. Iwayama *et al.* [10] reported a successful shortening of the IVM culture period by adding whale follicular fluid (WFF) to an IVM medium. Furthermore, Fujihira *et al.* [11] improved the IVM rates of Antarctic minke whale immature oocytes (60.9%) by adjusting the

osmolarity of the IVM medium to 390 mOsmol. Asada *et al.* [6] succeeded in improvement of IVM and IVF culture condition and also produced morula stage embryos. Furthermore, attempts at intracytoplasmic sperm injection (ICSI) and inter-species somatic cell nuclear transfer were reported [7, 8, 11, 15, 18]. However, it was still the low cleavage rate and not yet completely succeeded to produce the blastocyst stage embryos.

### 3. Cryopreservation of whale immature oocytes

Cryopreservation of mammalian oocytes is one of the most important techniques for gene preservation. In the case of whales, it is difficult to carry out the culture devices such as $CO_2$ incubator and cylinder onto a research base ship. Therefore, a practical cryopreservation technique for immature whale oocytes has been required. Asada *et al.* [5, 12] reported that conventional two step freezing of Antarctic minke whale immature oocytes and ultrastructural observation during maturation and cryopreservation. Additionally, Iwayama *et al.* [9] reported that increasing the IVM rates of vitrified and warmed Antarctic minke whale immature oocytes approximately 30%. However, the results of cryopreservation whale immature oocytes were not sufficient level for subsequent IVP experiment.

### 4. Objectives of this study

The purpose of present study was optimization of vitrification condition to improve the IVM rates for whale immature oocytes. The equilibration

method of cryoprotectants and components of vitrification solution were examined. Furthermore, cytoplasmic organelles injury was observed by electron microscope. Additionally, until now, studies of cryopreservation of sei, Bryde's and common minke whale captured at the western-north pacific immature oocytes have been limited. Therefore, attempt at vitrification of immature oocytes derived from three baleen whales (sei, Bryde's and minke whale) was also performed in this study.

# Chapter I

## Effects of equilibration steps and addition of whale follicular fluid to vitrification solution on *in vitro* maturation of vitrified baleen whale oocytes

# 1. Introduction

*In vitro* production of baleen whale embryos including IVM, IVF and IVC has been studied [2, 3, 5-7, 9-11, 15, 16]. Antarctic minke whale oocytes developed to the morula stage after IVM and IVF, but have not reached to the blastocyst stage. Recently, Bhuiyan *et al.* [16] also attempted IVM and IVF in sei and Bryde's whales and achieved some cleaved embryos developing to the morula stage. However, there was no report with regard to vitrification of immature oocytes collected from sei, Bryde's and common minke whales captured at the western-north pacific.

Because the conduction of whale IVP techniques has limited on a research ship, vitrification of immature whale oocytes is an alternative way to expect producing whale embryos *in vitro*. Iwayama *et al.* [9] reported Cryotop was more effectively than open-pulled straw in Antarctic minke whale oocytes. Fujihira *et al.* [11] also reported high IVM rates using freshly collected immature oocytes from Antarctic minke whales (60.9%) by adjusting the osmolarity of the IVM medium to 390 mOsmol. However, IVM rates of vitrified-warmed whale oocytes is still low (approximately 30%) compared with those in other mammals [19-22]. Therefore, improvement of a vitrification method for immature whale oocytes has been strongly desired.

Kuwayama *et al.* [23, 24] reported a high survival rate of bovine blastocysts after vitrification using a 16-step method to equilibrate cryoprotectants. They showed that the stepwise manner to equilibrate cryoprotectants protected membrane microstructure change in bovine blastocysts. Other researcher also showed effectiveness of additive step

numbers on vitrification of immature oocytes [19, 20]. For whale immature oocytes, it was reported that cytoplasmic organelles were damaged by freezing and thawing process [12]. Therefore, it was able to hypothesize that an increased step number during equilibration of cryoprotectants may protect membrane integrity, leading to improve viability and IVM rate.

Recently, Iwayama *et al.* [10] found successful shortening of the IVM culture period for fresh minke whale oocytes from 120 to 30 h by addition of WFF into IVM medium instead of supplementation with calf or whale serum. Vitrification solution is usually containing biogenic substrate such as fetal calf serum (FCS). WFF is also *in vivo* derived substrate and it probably the most suitable maturation culture medium for whale oocytes. Nagai *et al.* [13] showed that concentration of various components in WFF. The compositions of WFF differ from FCS (Table1). Therefore, adding WFF to vitrification solution instead of FCS may be suitable for maintain oocyte quality and subsequent IVM capability.

In the present study, effects of equilibration step (3 vs 5 step) and addition of WFF to vitrification solution were investigated on viability and IVM ability of vitrified oocytes derived from three different baleen whales (sei, Bryde's and minke whales).

## 2. 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

*Whales*

Sei, Bryde's and common minke whales used in this study were captured at the western-north pacific from May to September 2007 in the second phase of the Japanese Whale Research Program under Special Permit in the Western North Pacific (JARPN II), which was organized by the Institute of Cetacean Research in Tokyo, Japan. All whales were killed by 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 of death for all whales. The ovaries were collected within 3 h after death and were kept at 20-25 °C until oocyte collection.

*Oocyte and whale follicular fluid collection*

Cumulus–oocyte complexes (COCs) and WFF were aspirated from follicles using a 20 ml disposable syringe and 18-gauge needle. Oocytes surrounded by more than one layer of cumulus cells and with homogeneous cytoplasm were selected by stereomicroscope and washed three times with

Medium 199 (Sigma Aldrich Chemicals, St. Louis, MO, USA) containing 0.1% (w/v) polyvinyl alcohol (Sigma), 2 mM $NaHCO_3$ (Wako Pure Chemical Industries, Osaka, Japan), 10 mM HEPES (Sigma) and 75 μg/ml kanamycin (Sigma). The WFF was prepared by centrifugation of follicular fluid at 1000 x g for 20 min followed by filter of supernatant using 0.45 μm sterile filters and was stored at -20 °C until used.

*Vitrification and warming*

The vitrification and warming methods were basically followed by the report of Fujihira *et al.* [11]. The basic medium for the equilibration or dilution of cryoprotectant was HEPES-buffered Medium 199 supplemented with 20% (v/v) FCS (Gibco-BRL, Grand Island, NY, USA, HM-FCS) or WFF (HM-WFF). Whale follicular fluid was used as the same oocyte species. The COCs were transferred to HM-FCS or HM-WFF with 7.5 μg/ml cytochalasin B (Sigma) for 15min. Then the COCs were equilibrated cryoprotectant by 3 or 5 step manner. In the 3 step, COCs were first exposed to 7.5% cryoprotectant (3.75% ethylene glycol, EG, and 3.75% dimethylsulfoxide, DMSO) in HM-FCS or HM-WFF for 8 min and then 15% cryoprotectant (7.5% EG and 7.5% DMSO) in HM-FCS or HM-WFF for 4 min at 37 °C. In the 5 step, COCs were sequentially exposed to 5, 10, 15 and 20% cryoprotectant (2.5, 5, 7.5, 10% EG and 2.5, 5, 7.5, 10% DMSO) in HM-FCS or HM-WFF for 5, 3, 2, 2 min, respectively at 37 °C. In both steps, the COCs were finally exposed to 30% cryoprotectant (15% EG and 15% DMSO) and 0.5 M sucrose (Wako) in HM-FCS or HM-WFF and then five to six COCs were placed on a sheet of Cryotop (Kitazatio Supplies Co., Shizuoka, Japan)

and immersed in liquid nitrogen within 1 min. For warming, the Cryotop sheet holding the COCs was immersed in HM-FCS or HM-WFF containing 1 M sucrose at 37 °C for 1 min and was further diluted by exposure to HM-FCS or HM-WFF containing 0.5, 0.25, and 0.125 M sucrose for 4, 2, 2 min, respectively at 37 °C. After diluting cryoprotectant, COCs were kept in HM-FCS or HM-WFF at least 15 min.

*In vitro maturation (IVM)*

The IVM medium used was Medium 199 adjusted to 390 mosmol by changing the concentration of NaCl, KCl, $MgSO_4$ (anhydrous) and $CaCl_2 \cdot 2H_2O$ at a constant ratio with Medium 199 and supplemented with 10% (v/v) WFF, 0.33 mM sodium pyruvate (Wako), 1 mM glutamine (Wako), 100 μM cysteamine (Sigma), 25 mM HEPES, 75 mg/l kanamycin, and 10 ng/ml epidermal growth factor (Sigma). The COCs were washed three times in an IVM medium. Ten to thirty COCs were transferred to a 500 μl IVM medium in a four-well dish and cultured for 46-50 h at 37 °C in 5% $CO_2$ in air. During the first 24h culture period, 0.02 AU/ml pFSH (Antrin; Kawasaki Pharmaceutical Co., Kanagawa, Japan) and 1 μg/ml estradiol-17β (Sigma) were added into the IVM medium, and the last 22-26 h was without hormonal additive.

*Viability and maturation rate*

After IVM culture, the cumulus cells were removed by pipetting the COCs for 3-5 min in the presence of 0.1% (w/v) hyaluronidase (Sigma). The denuded oocytes were observed extruding the first polar body (PB). In the

present study, the oocyte with the extruded PB was considered to be viable and matured. The oocytes without PB were evaluated viability by fluorescein diacetate staining using fluorescence microscope (excitation 495 nm, emission 520 nm; Nikon, Tokyo, Japan). After viability assessment, the status of oocyte nucleus was examined by whole-mount preparation.

*Statistical analysis*

The rates of viability and maturation (number of oocytes matured with PB per number of oocytes warmed and cultured for IVM) were subjected to analysis of logistic regression, following a binominal distribution. All percentage data were analyzed using the models: $\ln (\alpha/ 1-\alpha) = \beta$ + main factors (equilibration steps and additives to basic medium) and their interaction, where $\alpha$ = frequency of positive outcome and $\beta$ = the intercept. Differences were considered significant when the P value was less than 0.05.

## 3. Results

In this study, enough numbers of common minke whale oocytes were not available to compare the effect of FCS and WFF. The significant interactions between the equilibration steps and the additives to vitrification solution were not revealed in both viability and IVM rates in sei and Bryde's whale oocytes.

In sei whales, the viability was not significantly different between the 3 and 5 step groups, but the IVM rate in the 3 step group (32.0%) was significantly ($P<0.05$) higher than that of the 5 step group (17.8%, Table 2). On the other hand, the viability of the WFF group was significantly ($P<0.05$) higher than the FCS addition group (90.6 and 75.7% respectively, Table 2), but there was no significant difference in the IVM rates between the WFF and FCS groups.

In Bryde's whales, the viability of the 5 step group (89.1%) was significantly ($P<0.05$) higher than that of the 3 step group (68.3%), but the IVM rates were not significantly different between the 3 and 5 step groups (Table 3). There were no significant differences in both viability and IVM rates between FCS and WFF groups.

In common minke whales, there was no significant difference in the viability between the 3 and 5 step groups. In contrast, the 5 step group resulted in the highest maturation rate (37.5%) obtained during this study, although the IVM rate was not significantly different from that of the 3 step group (31.6%).

Figure 1 shows immature and *in vitro* matured sei whale oocytes

following vitrification and warming, and the difference in viable and dead oocytes was also observed.

## 4. Discussion

Last decade, some studies on cryopreservation of whale immature oocytes have been performed [5, 7, 9, 11, 12], but these studies were limited to Antarctic minke whale oocytes. The present study is the first attempt to vitrification of western-north pacific sei, Bryde's and common minke whale immature oocytes, and investigated effects of cryoprotectant equilibration step and WFF addition to vitrification solution on the subsequent viability and IVM ability.

Generally, vitrification needs high concentration of cryoprotectants to prevent ice crystal formation, therefore the high osmolarity has caused detrimental effects in post-warmed oocytes, and impaired cytoskeletal organization, viability and developmental competence [25, 26]. Kuwayama *et al* [23, 24] suggested that cryoprotectant equilibration performed by a stepwise manner protected membrane integrity from osmotic pressure in bovine blastocysts. Other researchers also showed that a stepwise strategy had a beneficial effect on post-warmed viability and developmental competence by preventing from osmotic stress [27-29]. Although, in the present study, the difference in the viability between the 3 and 5 step methods was not observed in both sei and minke whale oocytes, the viability of the 5 step group was significantly improved compared with the 3 step group in Bryde's whales. This result has agreed with above studies [23, 24, 27-29], and indicates that the step number might affect viability of vitrified Bryde's whale immature oocytes. However, the step number did not enhance IVM rate in Bryde's whale (3 vs 5 step, 20.6 and 23.4% respectively). On the

contrary, the 5 step method (17.8%) resulted in a lower IVM rate than that of the 3 step group (32.0%) in sei whales. This absurdity reason is unclear, but it is likely that the present 5 step method was not optimal for an appropriate equilibrates time. It was observed using porcine oocytes that the 3 step method resulted in more recovered cell volume than the 5 step method at the end of equilibration (data is not shown). Insufficient permeation of cryoprotectant into oocyte cytoplasm is one factor limiting survivability of vitrified oocytes using a shorter expose and low concentration of cryoprotectant [28]. Some studies recommended that select method that included kind of cryoprotectant, equilibration step and time should be based on cell permeability and volume change [30, 31].

Previous studies [10, 11] showed that WFF addition to IVM medium lead to successful shortening of IVM period and improved IVM rate of fresh Antarctic minke whale oocytes. Furthermore, the concentration of various components in WFF was examined, and WFF may be more suitable additive to vitrification solution instead of FCS [13]. In general, vitrification solution contains macromolecule such as FCS, bovine serum albumin, polyvinylpyrrolidone, Ficoll and polyvinyl alcohol to reduce the amount of intracellular cryoprotectant and the toxicity in the solution and to protect against zona pellucida cracking [32]. Whale follicular fluid contains several components such as protein, cholesterol, albumin and steroid hormones similarly the FCS [13]. The study of vitrification using a vitrification solution containing follicular fluid has not been conducted, at least for whale oocytes. In this study, although there was no significant difference on viability in Bryde's whales between the WFF and FCS group, the WFF group (90.6%)

resulted in higher viability, in sei whales, than the FCS group (75.7%). In the present study, the WFF efficiency was not determined, but it was shown that WFF might be able to replace FCS and protect oocytes from cryoinjury, as shown in sei whales that there was no significant difference in the IVM rates between WFF and FCS groups.

The IVM rate of vitrified immature whale oocytes has been around 30% in previous studies [9, 11]. For whale IVP, improvement of IVM rate of vitrified whale oocytes is necessary. In the present study, it was expected that improvement of viability resulted in beneficial change to IVM rate. However throughout the present study, advanced viability has not lead to improvement of the IVM rate. Possible explanation is that assessment of viability was not a sufficient manner for detailed information on vitrified-warmed oocyte quality. Fluorescein diacetate has been used for an evaluation method of viability of post-warmed oocytes and embryos [22, 33, 34]. But, this agent could not observe the detailed cytoskeletal organization changes including spindle or tubular damage. During the present study, some vitrified whale oocyte nucleus had arrested at metaphase I stage. This suggested that some oocytes have been impaired by cryoinjury, which disturbs meiosis function such as microtubule or spindle organization [21, 26, 33]. Additionally, Asada *et al* [5, 12] reported that cytoplasmic organelles of the cryopreserved oocytes were impaired during the freezing and warming procedures in Antarctic minke whales oocytes.

In the present study, some abnormal oocytes, which did not reach to metaphase II stage, were observed. They were likely formed pseudo-pronucleus. Cryoprotectant such as DMSO and EG containing in

vitrification solution may introduce the meiotic resumption in sheep oocytes [35]. In previous study [3] also reported that some Antarctic minke whale oocytes collected from ovaries had already resumed the meiosis. Furthermore, oocytes with the first PB were fertilized by intracytoplasmic sperm injection, but no cleaved embryos were observed during the two days of IVC. This also indicated that oocytes vitrified and matured *in vitro* have been impaired cytoskeletal function and developmental competence by cryoinjury.

In conclusions, the present study was firstly showed that western-north pacific sei, Bryde's and common minke whales immature oocytes could be vitrified and produced a similar IVM rate (over 30%) obtaining in our previous studies. The present study also showed that the present step number (3 or 5 step) of cryoprotectant equilibration and WFF addition did not improve IVM rate of vitrified baleen whale oocytes. However, the three step was better than the five step because there were significant difference in sei whale IVM rate. Further studies are needed to improve the vitrification procedure and IVM and IVC culture systems of presumptive zygotes for establishing whale IVP technologies.

Table 1. Components of Antarctic mink whale follicular fluid (WFF) and fetal calf serum (FCS)

| Components | | WFF | FCS |
|---|---|---|---|
| Total protein | g/dl | 5.2 | 4.2 |
| Total cholesterol | mg/dl | 101.2 | 34.2 |
| Glucose | mg/dl | 82.6 | 130.0 |
| Albumin | g/dl | 3.8 | 2.6 |
| Na | mg/dl | 387.8 | 308.2 |
| K | mg/dl | 48.9 | 59.0 |
| Cl | mg/dl | 439.7 | 372.4 |
| Ca | mg/dl | 8.5 | 14.6 |
| P | mg/dl | 9.6 | 11.2 |
| Progesterone | ng/ml | 16.7 | 0.03 |
| Estradiol | ng/ml | 22.9 | 13.8 |

5

Table 2. Effect of equilibration steps and additives (fetal calf serum: FCS, whale follicular fluid: WFF) to vitrification solution on viability and *in vitro* maturation rate of vitrified-warmed sei whale oocytes

| Step* | Additives | No. (%) of oocytes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IVM | Survived | GV | GVBD | MI | AII/TII | MII | DG[1] | Others[2] |
| 3 | FCS | 76 | 59 (77.6) | 1 | 1 | 16 | 7 | 27 (35.5) | 16 | 8 |
| | WFF | 24 | 22 (91.7) | 0 | 0 | 8 | 4 | 5 (20.8) | 4 | 3 |
| | total | 100 | 81 (81.0) | 1 | 1 | 24 | 11 | 32 (32.0) [a] | 20 | 11 |
| 5 | FCS | 72 | 53 (73.6) | 0 | 2 | 25 | 6 | 15 (20.8) | 20 | 4 |
| | WFF | 29 | 26 (89.7) | 0 | 6 | 9 | 2 | 3 (10.3) | 3 | 6 |
| | total | 101 | 79 (78.2) | 0 | 8 | 34 | 8 | 18 (17.8) [b] | 23 | 10 |
| Total | FCS | 148 | 112 (75.7) [a] | 1 | 3 | 41 | 13 | 42 (28.4) | 36 | 12 |
| | WFF | 53 | 48 (90.6) [b] | 0 | 6 | 17 | 6 | 8 (15.1) | 7 | 9 |

[a,b]Values with different superscripts within the same column are significantly different ($P<0.05$).

[1]DG: degenerate. [2]Others: pseudo-pronuclear formation

*3 step: Oocytes sequentially exposed to 7.5, 15, and 30% cryoprotectant for 8, 4, 1 min, respectively

5 step: Oocytes sequentially exposed to 5, 10, 15, 20, and 30% cryoprotectant for 5, 3, 2, 2, 1 min, respectively

Table 3. Effect of equilibration steps and additives (fetal calf serum: FCS, whale follicular fluid: WFF) to vitrification solution on viability and *in vitro* maturation rate of vitrified-warmed Bryde's whale oocytes

| Step* | Additives | No. (%) of oocytes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IVM | Survived | GV | GVBD | MI | AII/TII | MII | DG[1] | Others[2] |
| 3 | FCS | 39 | 27 (69.2) | 0 | 9 | 12 | 0 | 7 (17.9) | 7 | 4 |
| | WFF | 24 | 16 (66.7) | 0 | 0 | 8 | 1 | 6 (25.0) | 6 | 3 |
| | total | 63 | 43 (68.3) [a] | 0 | 9 | 20 | 1 | 13 (20.6) | 13 | 7 |
| 5 | FCS | 40 | 37 (92.5) | 0 | 4 | 15 | 7 | 9 (22.5) | 3 | 2 |
| | WFF | 24 | 20 (83.3) | 0 | 3 | 5 | 0 | 6 (25.0) | 5 | 5 |
| | total | 64 | 57 (89.1) [b] | 0 | 7 | 20 | 7 | 15 (23.4) | 8 | 7 |
| Total | FCS | 79 | 64 (81.0) | 0 | 13 | 27 | 7 | 16 (20.3) | 10 | 6 |
| | WFF | 48 | 36 (75.0) | 0 | 3 | 13 | 1 | 12 (25.0) | 11 | 8 |

[a,b]Values with different superscripts within the same column are significantly different ($P<0.05$).

[1]DG: degenerate. [2]Others: pseudo-pronuclear formation

*3 step: Oocytes sequentially exposed to 7.5, 15, and 30% cryoprotectant for 8, 4, 1 min, respectively

5 step: Oocytes sequentially exposed to 5, 10, 15, 20, and 30% cryoprotectant for 5, 3, 2, 2, 1 min, respectively

Table 4. Effect of equilibration steps on viability and *in vitro* maturation rate of vitrified-warmed common minke whale oocytes

| Step* | No. (%) of oocytes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | IVM | Survived | GV | GVBD | MI | AII/TII | MII | DG[1] | Others[2] |
| 3 | 19 | 11 (57.9) | 0 | 1 | 2 | 0 | 6 (31.6) | 8 | 2 |
| 5 | 24 | 17 (70.8) | 0 | 0 | 3 | 0 | 9 (37.5) | 7 | 5 |

[1]DG: degenerate. [2]Others: pseudo-pronuclear formation

*3 step: Oocytes sequentially exposed to 7.5, 15, and 30% cryoprotectant for 8, 4, 1 min, respectively

5 step: Oocytes sequentially exposed to 5, 10, 15, 20, and 30% cryoprotectant for 5, 3, 2, 2, 1 min, respectively

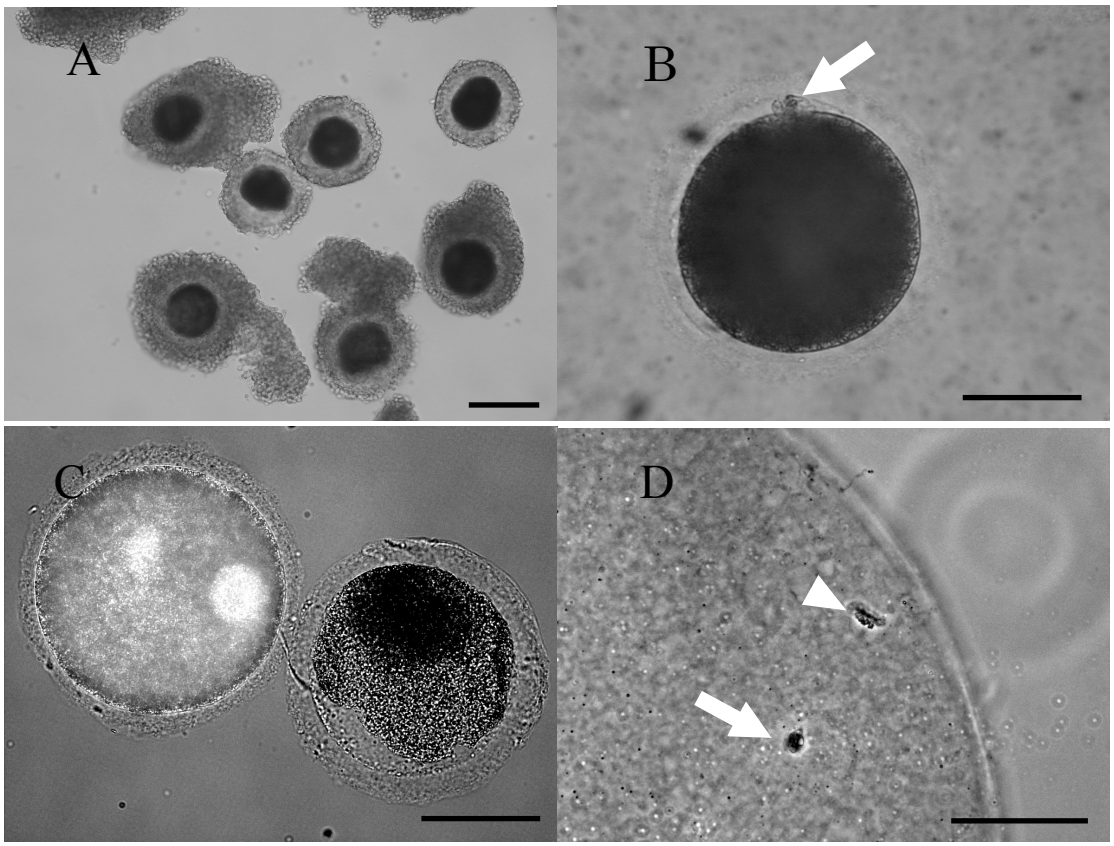


Fig. 1 (A) Immature sei whale oocytes after vitrification and warming (scale bar represents 100 μm). (B) A sei whale oocyte extruded the first polar body (arrow) after vitrification, warming and IVM (scale bar represents 50 μm). (C) Viable (left) and dead (right) sei whale oocytes stained with FDA after vitrification, warming and IVM (scale bar represents 50 μm). (D) Whole mount preparation of *in vitro* maturing sei whale oocyte (arrow, first polar body; arrowhead, second metaphase plate; scale bar represents 50 μm)

# Chapter II

## Ultrastructural observation of vitrified sei (*Balaenoptera borealis*) and Bryde's (*B. edeni*) whales immature oocytes

# 1. Introduction

Many factors affect the successful cryopreservation of oocytes, including ultrastructural changes and their functional alteration. It has been demonstrated that cytoplasmic organelles in cryopreserved mammalian oocytes were impaired during freezing and thawing procedures [25, 26, 36-38]. These include plasma membrane disruption, release of cortical granules, extensive ooplasm disorganization and loss of oocyte-cumulus cell attachment [25]. In previous studies [5, 12], the ultrastructure of Antarctic minke whale oocytes after freezing and thawing was described. However, there is no information that the ultrastructural observation of vitrified-warmed sei and Bryde's whale oocytes.

Vitrification needs a high concentrated cryoprotectant solution which has toxic effects and produce irreversible damage to cytoskeleton and cytoplasmic organelles [25]. The supplementation of nontoxic cryoprotectant, such as sugars, in vitrification solution was considered that beneficial counteracting the osmotic effect and reduce the toxicity by decreased the extracellular concentration of the cryoprotectant [37, 39]. It was reported that beneficial effect of microinjection [40] and co-incubation [41] of trehalose on cryosurvival. Additionally, trehalose was less harmful than sucrose [42]. Therefore, there is a possibility that vitrification solution was able to optimize by altering the sugar, sucrose to trehalose.

In Chapter 1, the effect of WFF addition to vitrification solution instead of FCS was examined. Although WFF addition has a significant effect on vitrified-warmed sei whale immature oocytes viability, the effect of WFF

addition was not detectable on IVM rate and also in other whales. Therefore, additional study was needed about whether or not the WFF addition to vitrification solution has beneficial effects on *in vitro* maturity and subsequent developmental capacity.

In the present study, the effect of extracellular sugar and WFF addition to vitrification solution on post-warmed IVM ability and cytoplasmic organelle changes were examined using sei and Bryde's whale oocytes.

## 2. 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.

*Whales*

Sei and Bryde's whales used in this study were captured at the western-north pacific from June to August 2008 in the second phase of the Japanese Whale Research Program under Special Permit in the Western North Pacific (JARPN II), which was organized by the Institute of Cetacean Research in Tokyo, Japan. All whales were killed by 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 of death for all whales. The ovaries were collected within 3 h after death and were kept at 20-25 °C until oocyte collection.

*Oocyte collection*

Cumulus–oocyte complexes (COCs) were aspirated from follicles using a 20 ml disposable syringe and 18-gauge needle. Oocytes surrounded by more than one layer of cumulus cells and with homogeneous cytoplasm were selected by stereomicroscope and washed three times with Medium 199

(Sigma Aldrich Chemicals, St. Louis, MO, USA) containing 0.1% (w/v) polyvinyl alcohol (Sigma), 2 mM $NaHCO_3$ (Wako Pure Chemical Industries, Osaka, Japan), 10 mM HEPES (Sigma) and 75 μg/ml kanamycin (Sigma).

*Vitrification and warming*

The basic medium for the equilibration or dilution of cryoprotectant was HEPES-buffered Medium 199 supplemented with 20% (v/v) FCS (Gibco-BRL, Grand Island, NY, USA, HM). The COCs were transferred to HM with 7.5 μg/ml cytochalasin B (Sigma) for 15min. Then the COCs were equilibrated cryoprotectant. First, the COCs were exposed to 7.5% cryoprotectant (3.75% ethylene glycol, EG, and 3.75% dimethylsulfoxide, DMSO) in HM for 8 min and then 15% cryoprotectant (7.5% EG and 7.5% DMSO) in HM 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 HM and then five to six COCs were placed on a sheet of Cryotop (Kitazatio Supplies Co., Shizuoka, Japan) and immersed in liquid nitrogen within 1 min. For warming, the Cryotop sheet holding the COCs was immersed in HM containing 1 M sucrose at 37 °C for 1 min and was further diluted by exposure to HM containing 0.5, 0.25, and 0.125 M sucrose for 4, 2, 2 min, respectively at 37 °C. After diluting cryoprotectant, COCs were kept in HM at least 15 min.

*In vitro maturation (IVM)*

The IVM medium used was Medium 199 adjusted to 390 mosmol by changing the concentration of NaCl, KCl, $MgSO_4$ (anhydrous) and $CaCl_2{\cdot}2H_2O$ at a constant ratio with Medium 199 and supplemented with

10% (v/v) WFF, 0.33 mM sodium pyruvate (Wako), 1 mM glutamine (Wako), 100 μM cysteamine (Sigma), 25 mM HEPES, 75 mg/l kanamycin, and 10 ng/ml epidermal growth factor (Sigma). The COCs were washed three times in an IVM medium. Ten to thirty COCs were transferred to a 500 μl IVM medium in a four-well dish and cultured for 46-50 h at 37 °C in 5% $CO_2$ in air. During the first 24h culture period, 0.02 AU/ml pFSH (Antrin; Kawasaki Pharmaceutical Co., Kanagawa, Japan) and 1 μg/ml estradiol-17β (Sigma) were added into the IVM medium, and the last 22-26 h was without hormonal additive.

*Viability and maturation rate*

After IVM culture, the cumulus cells were removed by pipetting the COCs for 3-5 min in the presence of 0.1% (w/v) hyaluronidase (Sigma). The denuded oocytes were observed extruding the first polar body (PB). In the present study, the oocyte with the extruded PB was considered to be viable and matured. The oocytes without PB were evaluated viability by fluorescein diacetate staining using fluorescence microscope (excitation 495 nm, emission 520 nm; Nikon, Tokyo, Japan). After viability assessment, the status of oocyte nucleus was examined by whole-mount preparation.

*Preparation of oocytes for transmission electron microscopy*

Fresh immature, vitrified-warmed and subsequently IVM oocytes (at least four oocytes in each experimental group) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, followed by 1% osmium tetraoxide in 0.1 M phosphate buffer for 1 h at 4°C. After routine

dehydration with a graded series of ethanol, the oocytes were infiltrated successively with 3:1, 1:1 and 1:3 mixtures of absolute propylene-oxide and epoxy resin, and then with absolute epoxy resin. Oocytes were embedded in beam capsules with fresh epoxy resin. Ultrathin section were cut out with an ultramicrotome and double-stained with 4% uranyl acetate and 0.5% lead citrate. Then oocytes were examined under an H-7500 transmission electron microscope (TEM; Hitachi, Tokyo, Japan,).

*Experimental design*

In this study, additional effects of WFF and trehalose to vitrification solution using immature whale oocytes were assessed by viability and maturation rate after IVM culture. Furthermore, ultrastructural changes were observed by TEM.

To examine the additional effect of WFF to vitrification solution, 20% (v/v) WFF was supplemented to the basic medium instead of FCS. In this experiment, sugar used sucrose and Bryde's whale was not examined because not enough number of oocytes were obtained. On the other hand, to compare the effect of extracellular sugar in vitrification solution, 0.5 M trehalose (Wako) added final 30% cryoprotectant solution in place of sucrose. Furthermore, the diluting cryoprotectant solution containing sucrose was also changed to 1, 0.5, 0.25 and 0.125 M trehalose, respectively. In this case, basic medium was contained FCS.

*Statistical analysis*

The rates of viability and maturation (number of oocytes matured with

PB per number of oocytes warmed and cultured for IVM) were subjected to analysis of logistic regression, following a binominal distribution. All percentage data were analyzed using the models: ln ($\alpha$/ 1-$\alpha$) = $\beta$ + main factors (additives to basic medium), where $\alpha$ = frequency of positive outcome and $\beta$ = the intercept. Differences were considered significant when the P value was less than 0.05.

## 3. Results

*Viability and maturation rate after vitrification*

The viability and maturation rate of vitrified-warmed sei and Bryde's whale immature oocytes after IVM were shown in table 1-3. The WFF addition to vitrification solution was not significant difference on both viability and maturation rates (52.9, 29.4% respectively) compared to FCS addition (47.3, 29.1%, Table 1). Furthermore, the effects of trehalose addition on viability and maturation rate (60.0 and 30.0%, 55.0 and 5.0% for sei and Bryde's whales, respectively) were also not significantly different in sucrose addition (48.8 and 26.8%, 40.0 and 10.0% for sei and Bryde's whales, respectively, Table 2 and 3).

*Ultrastructural observation*

In fresh immature oocytes, vesicles were scatter throughout the oocytes but peripheral were less than central position (Fig. 1A and C). Clustered mitochondria and lipid droplets (LD) were observed in peripheral. Intercellular communications between cumulus cells projection (CCP) and the ooplasm and microvilli (MV) were also identified (Fig. 1).

Vitrified-warmed sei whale immature oocytes were shown in Figure 2. It was clear that small LD and vacuoles were increase in all treatment groups. In WFF addition group, CCP was kept and MV was not so injured (Fig. 2C). Mitochondria and endoplasmic reticulum (ER) were swollen in all groups and some of mitochondria reduced its density (Fig. 2B, D and F). After IVM sei whale oocytes, almost mitochondria were swelling with ER (Fig. 3). In the

trehalose group, there were partly normal density mitochondria (Fig. 3F). Additionally, cortical granule aligned peripheral ooplasm was observed (Fig. 3E). In the Bryde's whale, vitrified-warmed immature oocytes were shown in Figure 3. The trehalose group is more keeping CCP than sucrose one (Fig. 4A and C). In sucrose addition group, part of oocytes plasma was dissociated from membrane (Fig. 4B). After IVM Bryde's whale oocytes, in sucrose addition group, two kind of LD was observed (Fig. 5A). On the other hands, in the trehalose group, it was remarkable that there were organelle free area in peripheral (Fig. 5C). Furthermore, in the trehalose group, mitochondria were more normally density compared to sucrose group (Fig. 5B and D).

## 4. Discussion

The present study investigated the effects of WFF and trehalose addition to vitrification solution for optimized whale immature oocyte vitrification condition. This is the first attempt on observations on the ultrastructure of vitrification sei and Bryde's whale oocytes.

Asada *et al* [5, 12], ultrastructural observation of cryopreserved Antarctic minke whale follicular oocytes was performed and showed wide spread damage in the cryopreserved oocytes such as rupture of the ooplasm membrane, vacuolated mitochondria, disruption of CCP, migration of cortical granule. In this experiment, sei and Bryde's whale ultrastructural aspects were similar to those of the previous studies.

Both sucrose and trehalose are disaccharides that are commonly used as components in cryoprotectant solutions or osmotic buffers. The effect of sucrose and trehalose is attributed to dehydration of the cells at equilibration before cooling and counterbalance swelling during rehydration after warming for vitrification techniques [39, 43]. However, which sugar is better for immature oocyte vitrification was not known for certain. Therefore, present study compared sucrose and trehalose for optimization of whale vitrification solution. The viability and maturation rates were not significant difference in both sucrose and trehalose addition. In contrast, the trehalose group tended to lower injured on mitochondria than sucrose group in both sei and Bryde's whales (Figs. 3 and 5) in this study. It is known that the distribution of mitochondria within the oocytes is indicative of energy and ion requirement through various key events during oocyte maturation,

fertilization and early embryonic development [44]. Therefore, mitochondria abnormality affected embryonic developmental competence and inducing chromosomal defect [45]. Furthermore, it was also reported that osmotic injury caused disruption of gap junction which connection between cumulus cell and oolemma [25, 38]. However, CCP in trehalose group was more intact than other group (Fig. 3C). Additionally, IVM cultured oocyte in trehalose group was able to observe organelle free zoon which represented matured oocytes aspect [36, 46] (Fig. 5C). Therefore, it was indicated that trehalose was better than sucrose on whale oocytes vitrification solution.

On the other hand, the effect of WFF addition instead of FCS was not detectable on viability and maturation rate. Furthermore, there was also not clearly difference by ultrastructural observation. Which is better for whale immature oocyte vitrification was not clear. However, the WFF addition to IVM medium in this study has also beneficial effect, because similarly maturation rate (around 30%) in previous study [10] was obtained in sei whale.

In conclusion, although there were no beneficial effects on viability and maturation rates of vitrified-warmed whale immature oocytes after IVM, trehalose addition to vitrification solution may be better than sucrose on whale oocytes vitrification. On the other hand, WFF addition to vitrification solution may be not effective for whale oocytes vitrification. Additionally, information of ultrastructural observation in vitrified sei and Bryde's whale oocytes was useful for further studies.

Table 1. Effect of whale follicular fluid addition to vitrification solution on viability and *in vitro* maturation rate of vitrified-warmed sei whale oocytes

| Additive* | No. of oocytes | | |
|---|---|---|---|
| | Cultured | Survived (%) | Matured (%) |
| FCS | 55 | 26 (47.3) | 16 (29.1) |
| WFF | 34 | 18 (52.9) | 10 (29.4) |

* 20% (v/v) WFF or FCS was supplemented to the basic medium. Sucrose was added in both groups.

Table 2. Effect of trehalose addition to vitrification solution on viability and *in vitro* maturation rate of vitrified-warmed sei whale oocytes

| Additive* | No. of oocytes | | |
|---|---|---|---|
| | Cultured | Survived (%) | Matured (%) |
| Sucrose | 41 | 20 (48.8) | 11 (26.8) |
| Trehalose | 40 | 24 (60.0) | 12 (30.0) |

* 0.5 M sucrose or trehalose was added to the final 30% cryoprotectant solution. And, the diluting solution was also added with 1, 0.5, 0.25 and 0.125 M sucrose or trehalose. FCS was added in both groups.

Table 3. Effect of trehalose addition to vitrification solution on viability and *in vitro* maturation rate of vitrified-warmed Bryde's whale oocytes

| Additive* | No. of oocytes | | |
|---|---|---|---|
| | Cultured | Survived (%) | Matured (%) |
| Sucrose | 20 | 8 (40.0) | 1 (10.0) |
| Trehalose | 20 | 11 (55.0) | 2 (5.0) |

* 0.5 M sucrose or trehalose was added to the final 30% cryoprotectant solution. And, the diluting solution was also added with 1, 0.5, 0.25 and 0.125 M sucrose or trehalose. FCS was added in both groups.

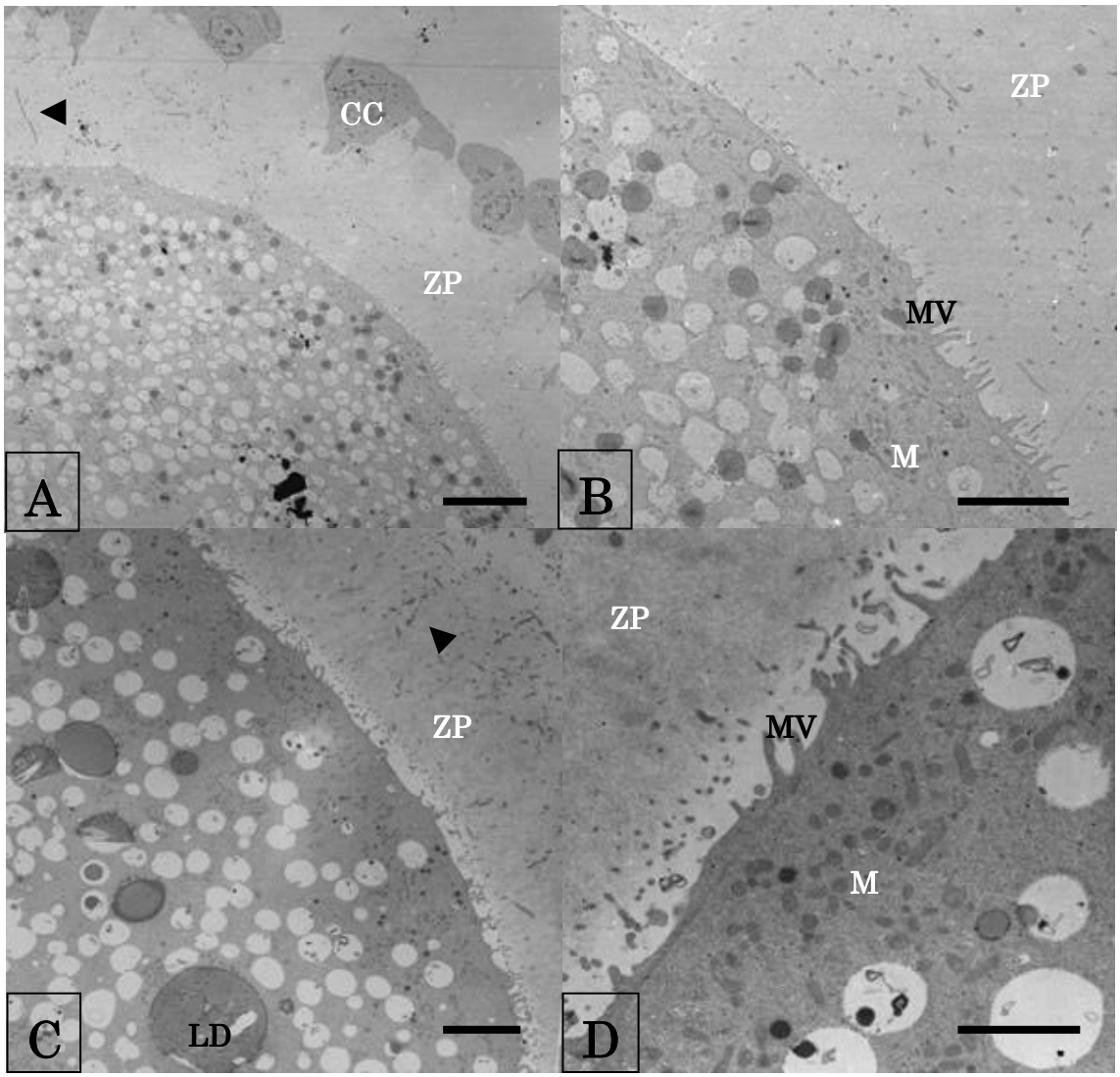


Fig. 1 A: fresh sei whale immature oocyte surrounded with cumulus cells (CC), zona pellucida (ZP). Cumulus cell projection was observed (arrow head). B: High magnification of A. Numerous microvilli (MV) were observed. C: fresh Bryde's whale immature oocytes. D: High magnification of C. Mitochondria (M) was clustered at spherical position. (bar: A:10, B: 5, C: 5, D: 2 μm)

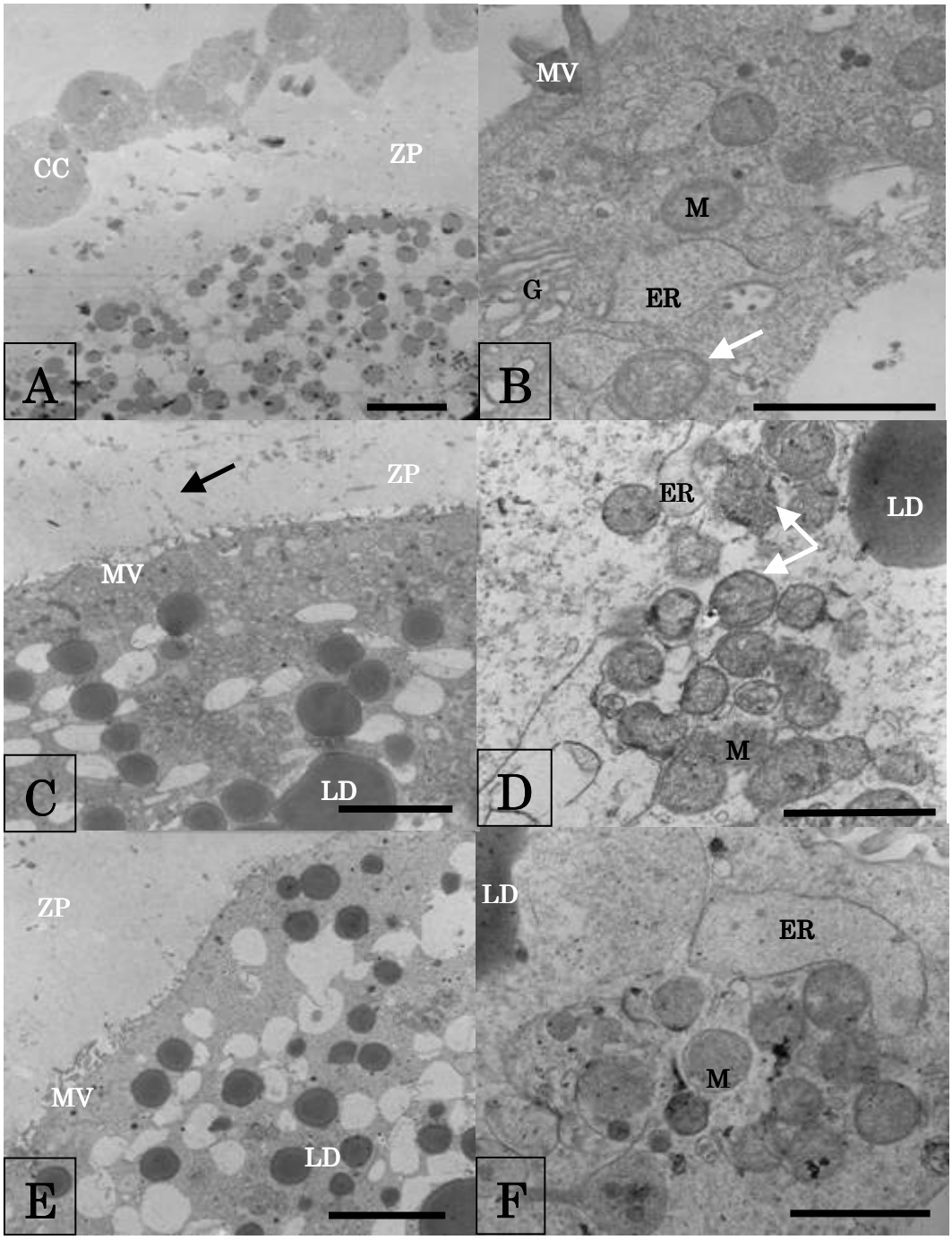


Fig. 2 Vitrified-warmed sei whale immature oocytes. A: FCS and sucrose addition group. Small lipid droplets (LD) were increased. B: High magnification of A. Golgi apparatus (G), endoplasmic reticulum (ER) were observed. Mitochondria (M) were swollen (white arrow). C and D: WFF addition group. Cumulus cell projection was remained (arrow). Mitochondria was clustered and swollen. E and F: trehalose addition group. Microvilli (MV) were attached to zona pellucida (ZP). (bar: A, C, E: 5, B, D, F: 1 μm)

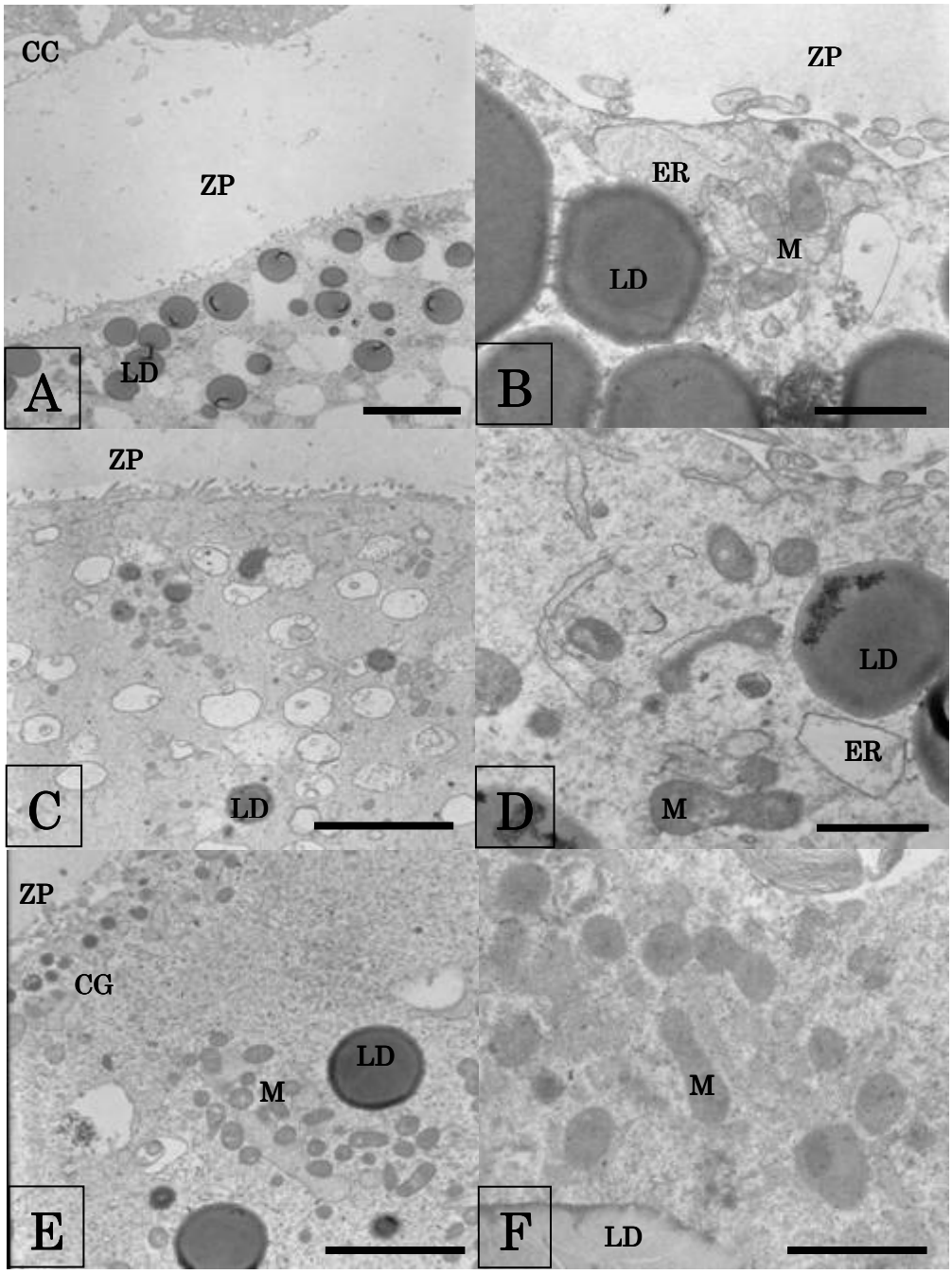


Fig. 3 Vitrified-warmed and IVM sei whale oocytes. A: FCS and sucrose addition group. Lipid droplets (LD) were aligned spherical position. B: High magnification of A. Mitochondria (M) and endoplasmic reticulum (ER) were swollen. C and D: WFF addition group. Mitochondria were swollen. E and F: trehalose addition group. Cortical granules (CG) were located beneath oolemma. Some mitochondria were normal density (bar: A, C, E: 5, B, D, F: 1 μm)

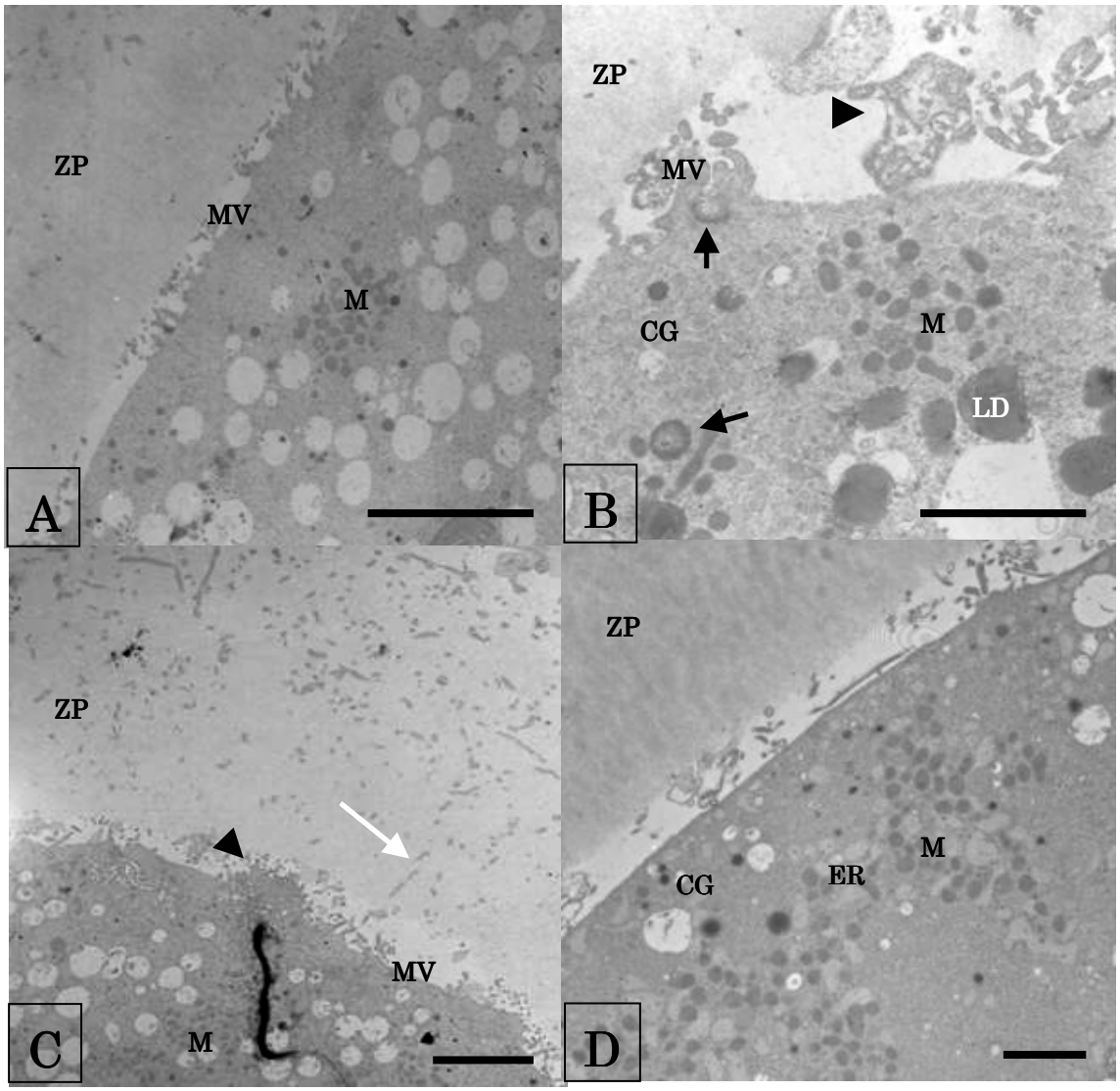


Fig. 4 Vitrified-warmed Bryde's whale immature oocytes. A: sucrose addition group. Mitochondria were clustered. B: High magnification of A. A part of mitochondria were swollen (arrow), some absolute membrane were observed (arrow head). C: trehalose addition group. Cumulus cell projection was well kept (white arrow) and a part of membrane was alteration. D: High magnification trehalose addition group. Cluster of mitochondria (M) and endoplasmic reticulum (ER) were observed. (bar: A, C: 5, B, D: 2μm)

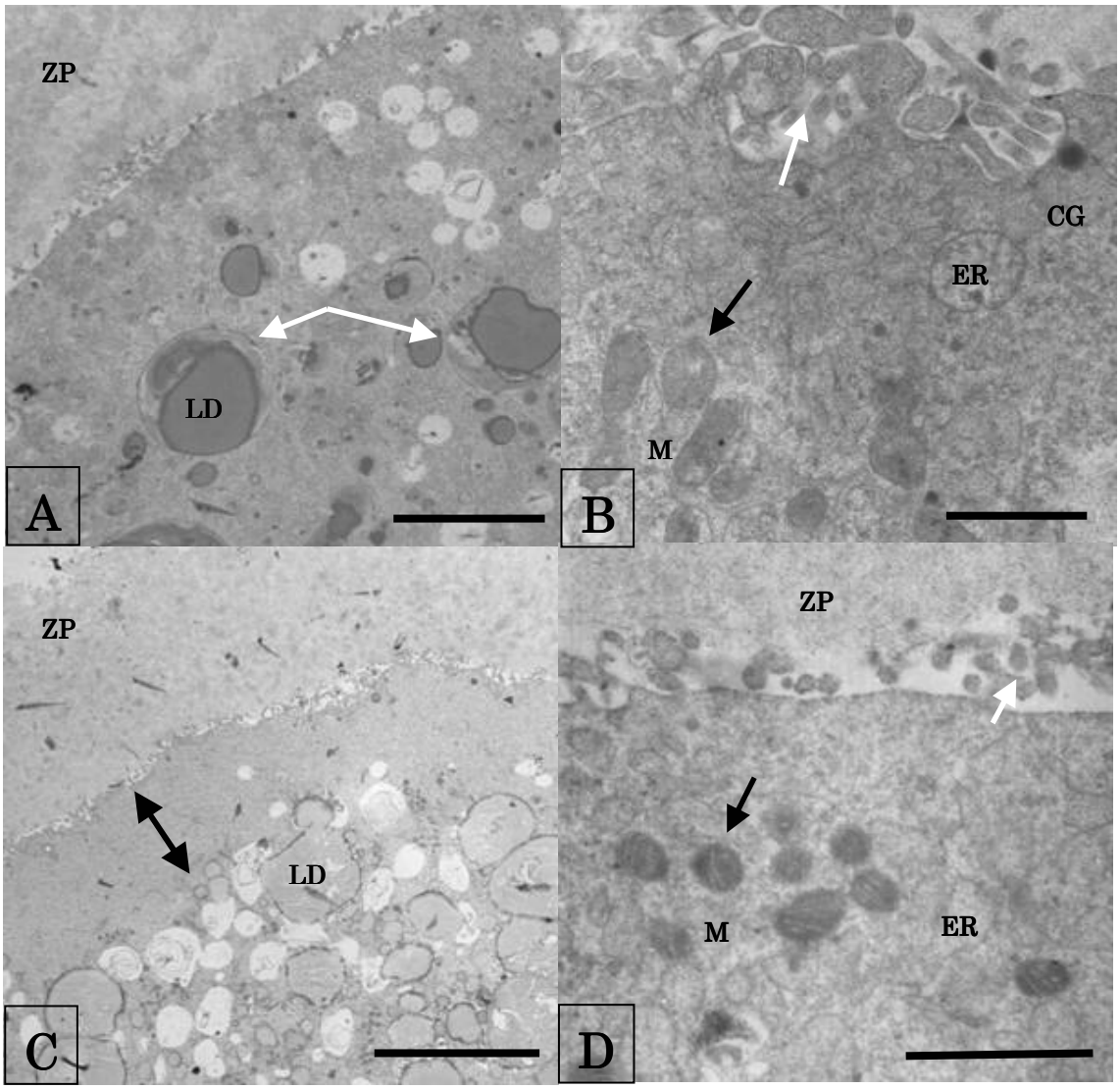


Fig. 5 Vitrified-warmed and IVM Bryde's whale oocytes. A: sucrose addition group. Blight lipid droplets were observed (white arrow). B: high magnification of A. Mitochondria (M) was swollen (arrow) and oolemma disruptions (white arrow) were observed. C: organelle free zone were observed (arrow) in trehalose group. D: in trehalose group. With high density and matrix mitochondria was observed (arrow). Microvilli were flattened (white arrow) (bar: A: 5, C: 10, B, D: 1μm)

# General Discussion

## 1. Improvement of vitrification method for whale oocytes

Until now, cryopreservation of whale oocytes is still limited [5, 7, 9, 11, 12]. Post-warming viability and maturation rate was not satisfactory for further IVF or ICSI experiment. Therefore, improvement of cryopreservation methods are quite importance for producing whale embryos.

Firstly, the conventional slow freezing method was applied [5] and not successful. Iwayama *et al.* [9] compared cryodevice types and showed that the cryotop method was beneficial for whale immature oocytes vitrification than the open pulled straw procedure. It was worth that applied another cryodevice or manner for whale immature oocyte vitrification such as solid surface vitrification [22, 47]. The solid surface vitrification not needs cryodevice, therefore able to save container space. It is very useful for whale immature oocyte vitrification, on the base ship.

Whale oocytes seems to very sensitive to low temperature, because whale oocytes contain large amounts of lipid [48]. In general, domestic animals especially porcine immature oocytes were extremely sensitive to the low temperature due to the high proportion of lipid droplets in cytoplasm [32, 48, 49]. Nagashima *et al.* [50] reported about delipidation of porcine embryos and succeed in cryopreservation. However, lipids play important roles in metabolism during maturation, fertilization and embryonic development along with mitochondria [51, 52]. Furthermore, there is a possibility that lipid droplets distribution reflected oocyte maturation [52]. Therefore, the cryopreserved method without delipidation for whale immature oocytes should be established.

In this study, cryoprotectants equilibration step was compared in Chapter 1. In general, step wise equilibration was beneficial to membrane integrity and viability [19, 20, 23, 24]. However, in this experiment, vitrified-warmed maturation rate in three step group was significantly higher than five step group on sei whales, and Bryde's and minke whales were not significantly different. Therefore, it was indicated that step wise equilibration used in this study was not effective for whale immature oocyte vitrification. Additionally, there is possibility that equilibration step was able to reduce two step method [53]. If equilibration step can be reduced, vitrification protocol was more simplified for whale oocytes.

The vitrification solution contain polymer such as serum and sugar to reduce toxicity of cryoprotectants and protect membrane integrity. In this study compared additional effect of FCS and WFF in Chapter 1 and 2, sucrose and trehalose in Chapter 2 for optimize whale oocytes vitrification solution. Although both Chapters 1 and 2, did not show beneficial improvement on vitrified-warmed oocyte maturation rate, trehalose addition seemed likely superior to sucrose on cytoplasmic organelle levels. Further study was strongly needed about vitrification solution compositions for whale immature oocytes.

## 2. Vitrification of western north pacific whale oocytes

To date, whale IVP studies were mainly performed in Antarctic minke whales. On the other hand, studies on whale IVP using sei, Bryde's and common minke whales captured at western north pacific oocyte has limited

[14-18]. Furthermore, vitrification of immature oocytes derives from sei, Bryde's and common minke whales has not been reported before. The present study firstly showed that the oocytes from three baleen whales could be vitrified and matured after IVM similar to those in Antarctic minke whales. Therefore, it could be applicable the vitrification technologies to other large baleen whales such as an endangered whale species, blue whale.

### 3. The future view in reproductive technologies for whales

The present study has shown an approximately 30% of maturation rate after IVM culture of vitrified immature sei, Bryde's and common minke whale oocytes. Also, the ultrastructural observation was performed. Therefore, the present study could be a basical study for three baleen whales IVP, especially cryopreservation in the future. Further experiment about ICSI, zygote activation methods and *in vitro* culture conditions are needed to obtain whale blastocyst stage embryos. Applying these technologies, it will be possible to reproduce artificially and to bank the genes of cetaceans including these three baleen whales.

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## Summary

### Chapter I

### Effects of equilibration steps and addition of whale follicular fluid to vitrification solution on *in vitro* maturation of vitrified baleen whale oocytes

The present study was conducted to investigate whether or not equilibration step (3 or 5 step) and whale follicular fluid (WFF) adding to vitrification solution were effective for viability and *in vitro* maturation (IVM) of immature oocytes in three baleen whales (sei, Bryde's and common minke whales) captured at the western-north pacific. The viability of the 5 step group (89.1%) in Bryde's whales was significantly higher than the 3 step group (68.3%), but the IVM rate of the 5 step group in sei whales (17.8%) was significant lower than the 3 step group (32.0%). In sei whales, the viability was significantly higher when WFF was added to vitrification solution than the use of FCS (90.6 and 75.7% respectively). But, the IVM rates were not significantly different between WFF and FCS adding into vitrification solution in both sei and Bryde's whales. The present step number of cryoprotectant equilibration and WFF addition did not improve IVM rate of vitrified whale oocytes. However, the present study showed that immature oocytes derived from three baleen whales at the western-north pacific could be vitrified and matured *in vitro* about 30% levels.

## Chapter II

## Ultrastructural observation of vitrified sei (*Balaenoptera borealis*) and Bryde's (*B. edeni*) whales immature oocytes

In the present study, the effect of extracellular sugars and WFF addition to vitrification solution on post-warmed IVM ability and cytoplasmic organelle changes were examined using sei and Bryde's whale oocytes.

The WFF addition to vitrification solution was not significantly different on both viability and maturation rates (52.9, 29.4% respectively) compared with FCS addition (47.3, 29.1%). Furthermore, the effects of trehalose addition on viability and maturation rate (60.0 and 30.0%, 55.0 and 5.0% for sei and Bryde's whales, respectively) were also not significantly different in sucrose addition (48.8 and 26.8%, 40.0 and 10.0% for sei and Bryde's whales, respectively).

From ultrastructural observations of vitrified-warmed sei whale immature oocytes, it was clear that small lipid droplets and vacuoles were increase in all treatment groups. In WFF addition group, cumulus cell projection (CCP) was kept and MV was not so injured. Mitochondria and endoplasmic reticulum (ER) were swollen in all groups and some of mitochondria reduced its density. After IVM of sei whale oocytes, almost mitochondria were swelling with ER. In the trehalose group, there were partly normal density mitochondria. In the Bryde's whale, the trehalose group is more keeping CCP than sucrose one. In the sucrose group, part of oocytes plasma was dissociated from membrane. In the trehalose group, it was remarkable that there were organelle free area in peripheral.

Furthermore, in the trehalose group, mitochondria were more normally density compared with the sucrose group.

In conclusion, trehalose addition to vitrification solution may be better than sucrose in whale immature vitrification. On the other hand, WFF addition to vitrification solution may be not effective for whale oocytes vitrification. Additionally, information of ultrastructural observation of vitrified sei and Bryde's whale oocytes was useful for further studies.

# 第一章 平衡段階数とガラス化溶液へのクジラ卵胞液添加がガラス化クジラ未成熟卵子の成熟率に及ぼす影響

クジラ未成熟卵子のガラス化保存後の成熟率は低く、クジラ体外生産胚作出のためにも成熟率の改善が必要である。そこで本研究では耐凍剤の平衡段階数（３段階と５段階)、及びクジラ卵胞液（ＷＦＦ）のガラス化溶液への添加が体外成熟率と生存率に及ぼす影響を北西太平洋で捕獲された３鯨種（イワシ、ニタリ、ミンククジラ）を用いて調査した。

生存率ではニタリクジラの５段階平衡区（89.1%）が３段階平衡区（68.3%）に比べ有意に高くなったが、成熟率では差が観られなかった。イワシクジラでは５段階平衡区（17.8%）で３段階平衡区（32.0%）に比べ有意に低い成熟率が得られた。またイワシクジラのＷＦＦ添加区で有意に高い生存率が得られた。しかし成熟率ではどの鯨種でもＷＦＦ添加区と対照区の間に有意な差は観られなかった。

本研究の平衡段階数、及びクジラ卵胞液のガラス化溶液への添加では成熟率の改善には至らなかった。しかし本研究により北西太平洋で捕獲された３鯨種の未成熟卵子のガラス化保存後の体外成熟培養により約 30%の成熟卵子が得られた。

# 第二章 イワシクジラ及びニタリクジラ未成熟卵子のガラス化保存後の微細構造の観察

本研究ではイワシクジラ及びニタリクジラを用いて、ガラス化溶液へ添加する糖類（トレハロース 及びスクロース）及びクジラ卵胞液がガラス化加温後の成熟率に与える影響と細胞内の微細構造に与える影響を検討した。

卵胞液添加の有無及び糖類間では生存率、成熟率に有意差は見られなかった。微細構造の観察ではガラス化加温後の卵子では全ての区において、脂肪滴(LD)と腔形成の増加が目立った。また卵胞液添加区では卵丘細胞突起と微絨毛への傷害は少なかった。成熟培養後の卵子ではトレハロース添加区以外でミトコンドリアの損傷が目立った。

本研究より、トレハロースのガラス化溶液の添加は細胞へのダメージがスクロース添加区よりも少なく、クジラ未成熟卵子のガラス化保存に適している可能性が示唆された。またクジラ卵胞液のガラス化溶液への添加による有効な微細変化は観察されなかった。