STUDY ON COLD TOLERANCE AND DEVELOPMENTAL ABILITY OF FELINE OOCYTES FROM COLD-STORED OVARIES

低温保存したネコ卵巣由来卵母細胞の低温寛容と発育能に関する研究

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March 2014

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A dissertation

Submitted to the United Graduate School of Veterinary Science

Yamaguchi University in partial fulfillment of the requirements

for the degree of doctor of philosophy

by

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March 2014

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ABSTRACT

Animal ovaries are routinely used for practical application of *in vitro* fertilization for animal production. The short-term preservation of ovary for transportation is especially important in the case of farm or endangered animals, when the ovarian donor is far away from specialized laboratories. The cooling of mammalian oocytes to sub-physiological temperatures is widely known to affect their viability through the induction of various abnormalities at all stages of meiosis [1]. The duration and temperature of ovary/oocyte storage may affect the quality of oocytes such as maturation ability, DNA fragmentation, damage of cumulus cells and/or normal development of oocytes. The objectives of this study were to compare the tolerance ability against cold storage among porcine, bovine and feline oocytes after storage of ovaries at 4°C for 5 days, and to improve the developmental ability of feline oocytes collected from cold-stored ovaries.

In the first study, we conducted a study to compared the kinetics of nuclear status and oocyte damage in porcine, bovine and feline ovaries stored at 4°C for 5 days. The cold storage of ovaries decreased the proportions of porcine and bovine oocytes that remained at the germinal vesicle stage before maturation culture. The maturation rates of oocytes decreased with increasing storage time, independent of species. None of the

porcine oocytes reached metaphase II (MII) after 1 day of storage. In contrast, bovine and feline oocytes from ovaries that were stored for 2 days and 3 days reached the MII stage. The proportion of DNA fragmentation in porcine oocytes from ovaries stored for 1 day was significantly higher than that in bovine and feline oocytes. The maturation competence of oocytes after the cold storage of ovaries could be related to the meiotic resumption competence of oocytes during storage and the occurrence of DNA fragmentation in oocytes during maturation culture. The findings demonstrate that feline oocytes maintain meiotic competence until 3 days of storage, whereas ovary storage at 4°C quickly result in a loss of the ability of porcine oocytes to reach the MII stage, and bovine oocytes lose their meiotic ability after 2 days of storage. In particular, porcine oocytes are highly sensitive to chilling. It is suggested that drastic loss in the maturation competence of porcine oocytes after cold storage relates in part from not only the meiotic resumption of oocytes before in vitro maturation (IVM) culture, but also a high occurrence of DNA fragmentation in oocytes during IVM culture.

In the second study, we investigated the effect of relaxin supplementation in maturation medium on their meiotic ability and subsequent development of feline oocytes from ovaries stored at 4°C for one day. Relaxin is a member of the insulin-like family of hormones that

promotes growth in a number of reproductive tissues including the granulosa and theca cells. It was found in the previous study that feline oocytes collected from cold-stored ovaries remain capable of maturing *in vitro*, but the developmental ability of the oocytes decreases after 24 h of cold storage. So in the present study, feline oocytes were collected from ovaries stored at 4°C for one day and cultured in maturation medium supplemented with different concentrations (0, 10, 20, and 40 ng/ml) of relaxin for 24 h. They were then fertilized *in vitro* for 12 h with frozen-thawed spermatozoa. After *in vitro* fertilization, the putative zygotes were cultured in synthetic oviduct fluid medium for 8 days. There were no significant differences in the maturation rates and glutathione contents of oocytes among the groups, irrespective of relaxin supplementation. The rate of blastocyst formation from oocytes matured with 10 ng/ml relaxin (16.0%) was higher ($p < 0.05$) than that from oocytes matured without relaxin (5.9%). Our findings indicate that supplementation of 10 ng/ml relaxin into maturation medium may improve blastocyst formation of feline oocytes after in vitro fertilization.

The results in these studies provide evidence that feline oocytes have an unusual tolerance to cold storage and have the ability to undergo maturation after a longer period of ovary storage than porcine and bovine oocytes. The addition of relaxin at a low concentration (10 ng/ml) to the

IVM medium improved the rate of blastocyst formation of feline oocytes from ovaries stored for one day at 4°C. The further study to clarify mechanism of tolerance ability of feline oocyte at low storage temperature would be a key point for improvement of developmental ability of feline oocytes collected from cold-stored ovaries.

ABBREVIATION

ANOVA	Analysis of variance
ARTs	Assisted reproductive techniques
BSA	Bovine serum albumin
CC	Condensed chromatin
COC	Cumulus-oocyte complexe
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth hormone
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle break down
hCG	Human chorionic gonadotropin
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
hMG	Human menopausal gonadotropin
Hoechst 33342	bisBenzimide H33342 trihydrochloride
IVC	*In vitro* culture

IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
MI	Metaphase I
MII	Metaphase II
mPBS	Modified phosphate-buffered saline
mSOF	Modified synthetic oviduct fluid
NADPH	Nicotinamide adenine dinucleotide phosphate
TCM 199	Tissue culture medium-199
TUNEL	Terminal deoxynucleotidyl transferase nick-end labelling

GENERAL INTRODUCTION

Biological diversity is the key to preserve life. However, rapidly growing human populations place extraordinary pressures on ecosystems, such as large-scale environmental destruction, habitat conversion, habitat fragmentation, and pollution. Virtually all conservation biologists agree that habitat preservation is the best way to conserve biodiversity. However it is well known that the habitat preservation is not easy. Ex-situ conservation is one of the choices for this objectives, where ART have been suggested as an important tool for biodiversity conservation [2]. ART including gamete cryopreservation, AI, embryo transfer and IVP allow obtaining more offspring from selected genitors to ensure genetic diversity and may reduce the interval between generations.

The application of ART such as IVF and embryo transfer are promising tools for the propagation and sustainability of endangered felid populations. In felid ART procedures, such as IVM, IVF and IVC of oocytes collected from excised ovarian tissue have reached a level of consistency, in certain species, to allow replacement of the costly and labor-intensive processes of *in vivo* embryo production and recovery [3]. The ability to grow and fertilize immature oocytes is useful for producing large numbers of embryos for developmental biology, cryopreservation and genetic studies,

as well as for live animal production. The domestic cat is a valuable model for studying human genetic diseases [4] and for developing assisted reproduction in taxonomically related endangered felids [5]. An optimal *in vitro* system producing high-quality embryos from IVM, IVF and IVC is an essential prerequisite for the successful application of ART to preserve endangered species.

The cooling of mammalian ovaries to sub-physiological temperatures is widely known to affect their viability through the induction of various abnormalities at all stages of oocyte meiotic maturation [1, 6-9]. The effect of low temperature on meiotic maturation is varied among different species. In pigs, it has been suggested that porcine oocytes/embryos have higher sensitivity to low temperatures compared with those of other mammalian species [10, 11]. For example, porcine oocytes that were collected from ovaries stored at 15°C or lower for 6 h could not reach the MII stage [12]. Didion et al. [13] reported also that porcine oocytes at the germinal vesicle stage could not survive when the oocytes were cooled to 15°C or below. In general, oocytes appear to be much more sensitive to sub-physiological temperature than cleavage-stage embryos [11, 14, 15]. In particular, it has been suggested that porcine oocyte/embryos have more sensitivity to low temperatures compared to other mammalian species [11, 15]. In cows, ovaries obtained from slaughterhouses are routinely used for

practical application of *in vitro* fertilization for animal production and the experimental approach of nucleus transfer in cattle. Recently, bovine ovaries were obtained from slaughterhouses immediately after slaughter and transported to the laboratory in saline at 20–35°C within several hours before oocyte recovery. Storage of ovaries at 37–39°C for 5–8 h decreased the maturation rate of follicular oocytes and the potential to develop into blastocysts after *in vitro* fertilization [16, 17]. Storage of ovaries at 20°C [17] or 25°C [16] for 8 h did not reduce the maturation rate or the potential of *in vitro* fertilized oocytes to develop into blastocysts. The storage of bovine ovaries at 10°C for more than 48 h had detrimental effects on the meiotic competence of oocytes [18]. However, the storage of ovaries at 4°C or 10°C for 24 h did not reduce the meiotic maturation of oocytes [18, 19]. In cats, it has been shown that oocytes that are recovered from ovaries stored at 4°C for up to 72 h were capable of maintaining meiotic competence [20]. Our previous study demonstrated that the meiotic maturation of feline oocytes from ovaries stored at 4°C for 24 h was similar to that of ovaries that were stored at 38°C for 2 h [21]. Moreover, storage at 4°C for 24 h improved the meiotic competence of feline oocytes compared with storage at room temperature or 38°C for the same duration [22]. Prior to the present study, there has been no information concerning the effect of longer periods (>3 days) of ovary storage at low temperature and the

comparison of the quality and viability of oocytes from stored ovaries. The long-term storage of ovaries could increase the number of oocytes with DNA-fragmented nuclei and reduce the rate of oocyte maturation [12]. The first study (chapter 1) was conducted to investigate the effects of the storage of porcine, bovine, and feline ovaries for different periods of time at 4°C on the nuclear status and oocyte quality before and after maturation culture, and to compare the kinetics of nuclear status and oocyte damage during storage among the animals.

The possibility of *in vitro* produced embryos from ovaries following temporary storage at 4°C for 24 h after extirpation, combined with transfer of the resulting embryos following cryopreservation, would provide even greater opportunities for preserving valuable genetic material [23]. The domestic cat is a valuable model for development of ARTs for potential use in non-domestic cats. Approximately 50% to 65% of oocytes retrieved from excised ovaries of domestic cats are capable of *in vitro* maturation to the MII stage, and 20% to 30% of matured oocytes can develop to the blastocyst stage after IVF [24, 25]. Furthermore, unlike other species, cat oocytes have a unique ability to mature *in vitro* after temporary storage at 4°C [22, 26]. It has been shown that cat oocytes obtained from ovaries stored at 4°C for up to 72 h remain capable of maturing *in vitro* [22, 26]. Moreover, offspring have been obtained after IVF and IVC of *in*

vitro-matured oocytes recovered from ovaries stored at 4°C for 24 h [23, 27]. However, the ability of oocytes to be matured and develop after IVF declined markedly after 24 h of the cold storage [26, 28, 29]. IVM of cat oocytes depends on different factors, such as the stage of the estrous cycle of donor cats [30, 31], the quality of the cumulus-oocyte complex [24, 32], and the time of culture [4, 26, 33] and hormonal supplementation [32, 34-36].

Relaxin is a small peptide commonly known as pregnancy hormone in many mammals. Relaxin is expressed in various tissues across a broad range of mammalian species [37, 38]. Relaxin is found in a variety of body fluids and have pleiotropic actions on numerous tissue targets [38, 39]. In female reproductive tissues, relaxin is involved in a range of events such as ovarian follicular growth, ovulation, development of mammary glands, and preparation of uterus and cervix for pregnancy and delivery [38-40]. Moreover, porcine relaxin has been reported to support meiotic maturation of porcine oocytes [41] and to improve the function and penetrability of boar sperm [42]. However, it is not known whether relaxin influences oocyte maturation and/or embryo development in the cat. Thus, to improve the developmental competence of cat oocytes after cold storage, in the second study (Chapter 2), we investigated whether supplementation of relaxin in maturation medium would improve the nuclear maturation and *in*

vitro development of cat oocytes from ovaries stored at 4°C for one day.

The overall aim of the present studies was: (1) to compare the nuclear status, DNA fragmentation of porcine, bovine and feline oocytes that were stored in low temperature (4°C) for up to 5 days and (2) to improve *in vitro* development of feline oocyte stored at 4°C for 1 day by supplementation of relaxin hormone to IVM medium.

CHAPTER I; NUCLEAR STATUS AND DNA FRAGMENTATION OF OOCYTES FROM PORCINE, BOVINE AND FELINE OVARIES STORED AT 4°C FOR 5 DAYS

INTRODUCTION

The cooling of mammalian oocytes to sub-physiological temperatures is widely known to affect their viability through the induction of various abnormalities at all stages of meiosis [1]. It has been suggested that porcine oocytes/embryos have higher sensitivity to low temperatures compared with those of other mammalian species [10, 11]. In our previous study, we observed that porcine oocytes that were collected from ovaries stored at 15°C or lower for 6 h could not reach the MII stage [12]. In cows, the storage of ovaries at 4°C or 10°C for 24 h did not affect dramatically the meiotic maturation competence of oocytes [18, 19]. However, the storage of bovine ovaries at 10°C for more than 48 h had detrimental effects on the meiotic competence of oocytes [18]. In cats, it has been shown that oocytes that are recovered from ovaries stored at 4°C for up to 72 h are capable of maintaining meiotic competence [20]. Our previous study demonstrated that the meiotic maturation of feline oocytes from ovaries stored at 4°C for 24 h was similar to that of ovaries that were stored at 38°C for 2 h [21]. Moreover, storage at 4°C for 24 h improved the meiotic competence of feline oocytes compared with storage at room temperature or 38°C for the same duration [22]. Prior to the present study, there has been no information concerning the effect of longer periods (>3 days) of ovary

storage at low temperature and the comparison of the quality and viability of oocytes from stored ovaries among the animals. The long-term storage of ovaries could increase the number of oocytes with DNA-fragmented nuclei and reduce the rate of oocyte maturation [12].

The study of chapter 1 was conducted to investigate the effects of the storage of porcine, bovine and feline ovaries for different periods of time at 4°C on the nuclear status and oocyte quality before and after maturation culture, and to compare the kinetics of nuclear status and oocyte damage during storage among the animals.

MATERIALS AND METHODS

Ovary storage and oocyte collection

Porcine and bovine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% physiological saline at 30°C (porcine oocytes) and at 25°C (bovine oocytes) within 3 h of slaughter. Feline ovaries were collected from sexually mature queens by routine ovariohysterectomy at local veterinary clinics. After excision, the ovaries were stored in physiological saline and maintained at room temperature for 2 h until they were brought to the laboratory. To investigate the effects of storage period on the nuclear status and quality of oocytes, the ovaries were stored at 4°C for 0, 1, 2, 3, 4 and 5 days. After each day of storage, COCs were aspirated from 3-6 mm diameter porcine and bovine ovary follicles using an 18-gauge needle connected to a 5 mL disposable syringe. The feline ovaries were sliced repeatedly with a scalpel blade to release the COCs into a 90 mm culture dish containing mPBS (Nippon Zenyaku Kogyo, Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium (Meiji, Tokyo, Japan) and 100 μg/mL streptomycin sulphate (Meiji). The COCs were collected into mPBS. It has been demonstrated that the proportion of morphologically intact oocytes among oocytes that are collected from stored ovaries decreases with increasing storage periods

[22]. In the present study, therefore, only COCs with uniform ooplasm and a compact cumulus cell mass were used to enable the accurate assessment of the effect of the duration of storage at 4°C on oocyte quality and meiotic competence. All procedures were approved by the Animal Research Committee of Yamaguchi University.

Oocyte maturation

Porcine oocytes were matured according to the method described by Kaedei et al. [43] with minor modifications. In brief, COCs were cultured for 22 h in a maturation medium that consisted of tissue TCM 199 (Earle's salts) buffered with 25 mM HEPES(Invitrogen, Carlsbad, CA, USA), 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/mL D-sorbitol (Wako Pure Chemical, Osaka, Japan), 1 μg/mL 17 β-estradiol (Sigma-Aldrich), 50 μM β-mercaptoethanol (Wako Pure Chemical), 10 IU/mL eCG (Kawasaki, Kawasaki, Japan), 10 IU/mL hCG (Kawasaki), and 50 μg/mL gentamicin (Sigma-Aldrich). The COCs were then transferred to the maturation medium without hormones and were subsequently cultured for an additional 22 h. All incubations were performed in a 38.5°C humidified incubator containing 5% CO2 in air.

Bovine oocytes were matured according to procedures previously

described by Mori et al. [44] with minor modifications. In brief, COCs were cultured in maturation medium that consisted of TCM 199 supplemented with 25 μg/mL taurine (Sigma-Aldrich), 0.02 AU/mL FSH (Kawasaki), 5% FBS (Invitrogen), 40 μg/mL EGF (Sigma-Aldrich) and 50 μg/mL gentamicin for 22 h in a 38.5°C humidified incubator containing 5% CO2 in air.

Feline oocytes were matured according to procedures previously described by Karja et al. [25] with minor modifications. In brief, COCs were cultured in maturation medium that consisted of TCM 199 supplemented with 4 mg/mL BSA (Sigma-Aldrich), 50 μg/mL gentamicin, 0.1 IU/mL hMG (Teikokuzoki, Tokyo, Japan), 10 IU/mL hCG (Kawasaki), 1 μg/mL 17 β-estradiol (Sigma-Aldrich) and 10 ng/mL relaxin (Sigma-Aldrich) in a 38.0°C humidified incubator containing 5% CO2 in air for 24 h.

Assessment of meiotic status and DNA fragmentation of oocytes

Before and after maturation culture, the meiotic stage and DNA damage of oocytes was analyzed using a combined technique for simultaneous nuclear staining and TUNEL that was modified from the procedures previously described by Otoi et al. [45]. Briefly, oocytes were mechanically denuded from cumulus cells in DPBS (Invitrogen) supplemented with 1

mg/mL hyaluronidase (Sigma-Aldrich). Denuded oocytes were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were permeabilized in PBS containing 0.1% (v/v) Triton-X100 (Sigma-Aldrich) for 40 min. They were subsequently incubated overnight at 4°C in PBS containing 10 mg/mL BSA (blocking solution). They were then incubated in fluorescein-conjugated 2′-deoxyuridine-5′-triphosphate and TUNEL reagent (Roche Diagnostics Corp., Tokyo, Japan) for 1 h at 38.5°C. After these procedures, the oocytes were counterstained with 25 μg/mL Hoechst 33342 (Sigma-Aldrich) for 30 min. They were then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes, Eugene, OR, USA), mounted on a glass slide, and sealed with clear nail polish. Labelled oocytes were examined using a Diaphot microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with epifluorescence illumination. Two standard filter sets were used, a filter with an excitation wavelength of 450-490 nm and a barrier filter of 520 nm was used to detect FITC alone. A filter with an excitation wavelength of 330-380 nm and a barrier filter of 420 nm was used to detect the nuclear status of the oocytes that were stained with Hoechst 33342.

They were classified according to their chromatin configuration as GV, CC, MI, or MII[46]. Oocytes with the diffusely stained cytoplasmic characteristic of nonviable cells and those in which chromatin was

unidentifiable or not visible, were classified as ‘degenerated’. To assess DNA damage in oocytes before and after maturation culture, the nuclei that were labelled by TUNEL were counted.

Statistical analysis

All of the experiments were repeated 4-8 times. The data were analyzed by analysis of variance using the GLM procedure from the SAS software (SAS for Windows, version 9.1, SAS Institute Japan, Tokyo, Japan). The statistical model included the duration of storage, type of animal, and the two-way interactions. When significant interactions were not observed between the duration of storage and the type of animal, these were excluded from the model. The differences with a probability value of $p<0.05$ were considered statistically significant.

RESULTS

Nuclear status and DNA fragmentation of oocytes before IVM

No significant storage duration × animal type interactions were observed between the proportions of oocytes at the GV and MII stage and the oocytes with DNA-fragmented nuclei. The storage of ovaries at 4°C decreased the proportions of porcine and bovine oocytes, but not feline oocytes, that remained at the GV stage (Fig. 1A). Significantly more feline oocytes remained at the GV stage (72-82%) during storage for 5 days when compared to the other species ($P < 0.05$). All of the oocytes that underwent the GVBD stopped at the CC stage and did not reach the MII stage (Fig. 1B), irrespective of species. In the porcine and bovine groups, the proportions of oocytes with DNA-fragmented nuclei increased with increasing storage time (Fig. 1C). In the feline group, however, none of the oocytes with DNA-fragmented nuclei were observed until 2 days of ovary storage.

Nuclear status and DNA fragmentation of oocytes after IVM

When evaluating the maturation rates of oocytes after IVM culture, a significant storage duration × animal type interaction was observed ($P < 0.01$). However, no significant storage duration × animal type interactions

were observed for the other parameters. In the feline group, the proportions of oocytes that remained at the GV stage after IVM culture increased with increasing storage time and reached 49% after storage for 5 days (Fig. 1a). In the porcine and bovine groups, any trend of increased proportions of oocytes at the GV stage was not observed. The proportions of oocytes that reached the MII stage after IVM culture decreased with increasing storage time, irrespective of species (Fig. 1b). Notably, none of the porcine oocytes reached the MII stage after 1 day of storage. In the bovine groups, 47.4% of the oocytes from the ovaries that were stored for 1 day reached the MII stage, but the oocytes from the ovaries that were stored for 3 days lost their meiotic ability to reach the MII stage. Interestingly, 17.4% of the feline oocytes from the ovaries that were stored for 3 days could reach the MII stage, however 1% of the oocytes maintained the ability, even after 5 days of storage. The proportions of oocytes with DNA-fragmented nuclei after IVM culture increased with increasing storage time, irrespective of species (Fig. 1c). Moreover, the proportion of DNA-fragmented nuclei in porcine oocytes from ovaries that were stored for 1 day was significantly higher ($P < 0.01$) than that in bovine and feline oocytes from ovaries that were stored for the same period of time.

DISCUSSION

The results that are presented here provide an evidence that feline oocytes have an unusual tolerance against cold storage and have the ability to undergo meiotic maturation after a longer period of ovary storage compared with porcine and bovine oocytes. Feline oocytes maintained meiotic competence until 3 days of storage, whereas ovary storage at 4°C quickly resulted in a loss of the ability of porcine oocytes to reach the MII stage, and bovine oocytes lost their meiotic ability after 2 days of storage.

It has been suggested that the sensitivity to low temperatures in porcine oocyte/embryos is related to their relatively high lipid content and/or lipid composition [8, 47, 48]. In fact, porcine oocytes have two-fold greater complement of fatty acids, reflecting the acyl-containing lipid mass, compared with bovine oocytes [47]. It has been reported that exposure of the follicular GV oocytes to temperatures at or below 25°C adversely affects the viability of porcine oocytes [49]. Moreover, porcine oocytes collected from ovaries that were stored at 15°C or lower for 6 h could not reach the MII stage [12]. Similarly, in the present study, we observed that porcine oocytes lost their maturation competence even after 1 day of ovary storage at 4°C. Conversely, bovine ovaries can be stored at 10°C for at least 24 h without decreasing the developmental potential of oocytes [18]. Lucci

et al. [50] demonstrated that Zebu cow ovaries were successfully stored at 4°C for up to 18 h with no morphological damage to the preantral follicles. However, storage of the oocytes at 10°C for 48 and 72 h was detrimental to their maturation competence [18]. Similarly, in the present study, we observed that although 47% of the oocytes from bovine ovaries that were stored for 1 day at 4°C could reach the MII stage, the oocytes from the ovaries that were stored for 3 days lost the meiotic ability to reach the MII stage. In cats, the meiotic competence of oocytes can be maintained for up to 72 h of ovary storage at 4°C [20]. In a previous study, we reported that the rate of completion of meiosis of oocytes from feline ovaries that were stored at 4°C for 24 h was similar to that of oocytes from ovaries that were stored at 38°C for 2 h [21]. The present study showed that the meiotic competence of feline oocytes from ovaries that were stored at 4°C for 1 day decreased, but >17% of the oocytes could reach the MII stage until 3 days of storage. Moreover, when the nuclear status and quality of feline oocytes were examined before IVM, significantly more oocytes remained at the GV stage during storage, and none of the oocytes with DNA-fragmented nuclei were observed until 2 days of ovary storage. These results were supported by the findings of Wood et al. [51] who reported that the storage of feline ovaries at 4°C inhibits taphonomic changes that are reflected by more intense pyknosis, vacuolisation, and increased loss of membrane integrity

in granulosa cells and oocytes.

In the present study, we observed that the storage of ovaries decreased the proportions of porcine and bovine oocytes that remained at the GV stage before IVM culture with increasing storage time. In the ovarian follicle, communication through gap junctions between the oocyte and the surrounding granulosa cells is essential for the regulation of its meiotic differentiation and maturation [52]. A loss of communication between the oocyte and the granulosa cells via gap junctions is responsible for meiotic resumption [53]. Therefore, the decreased proportions of oocytes remaining at the GV stage before IVM culture in the porcine and bovine group could result from the gap junctional loss within the COCs during storage. Moreover, the increased tendency in the proportions of feline oocytes at the GV stage after IVM culture may be related to the ability to undergo maturation after longer durations of ovary storage.

The storage of ovaries at low temperature may delay the accumulation of acid by-products and apoptotic processes because the metabolic enzymes that are present in warm-blooded animals function most efficiently at body temperature [54]. However, a prolonged storage of ovaries before maturation could promotes the DNA fragmentation of oocytes, resulting in a decrease in the meiotic competence of oocytes. In the present study, we observed that the proportions of oocytes with DNA-fragmented nuclei

before and after IVM culture increased with increasing storage time, irrespective of species. Notably, the proportions of porcine oocytes with DNA-fragmented nuclei after IVM culture drastically increased after 1 day of storage. Guignot et al. [55] reported that the period and temperature of ovary storage affected the quality of oocytes, in which the cytoplasmic membrane [56, 57], microtubules [1, 9, 58, 59], and the cytoskeleton [60] could be sensitive to a low temperature. Porcine oocytes are highly sensitive to chilling, resulting in a loss of membrane integrity when cooled below 16°C [61]. Therefore, a loss of the ability of porcine oocytes to reach the MII stage after storage at 4°C could result from a high chilling sensitivity including meiotic resumption as a result of the gap junctional loss within the COCs during storage and the high occurrence of DNA fragmentation during IVM culture.

The present study demonstrates that feline oocytes maintain the ability to undergo meiotic maturation after longer durations of ovary storage at 4°C compared with porcine and bovine oocytes. In particular, porcine oocytes were highly sensitive to chilling. The drastic loss in the maturation competence of porcine oocytes after cold storage resulted in part from not only the meiotic resumption of oocytes before IVM culture, but also a high occurrence of DNA fragmentation in oocytes during IVM culture. Therefore, the ability of oocytes to reach MII after cold storage could be

related to the meiotic resumption of oocytes during storage and the occurrence of DNA fragmentation in oocytes during IVM culture. Based on the differences in sensitive to chilling among the animals, further work is needed to evaluate temperature and duration of ovary storage and improve culture method of oocytes for the prevention of DNA fragmentation. This would determine whether there is desirable procedure by which ovaries should be preserved to maximize maturation competence of oocytes.

CHAPTER II; THE EFFECT OF RELAXIN SUPPLEMENTATION OF *IN VITRO* MATURATION MEDIUM ON THE DEVELOPMENT OF CAT OOCYTES FROM OVARIES STORED AT 4°C

INTRODUCTION

The domestic cat is a valuable model for development of ARTs for potential use in non-domestic cats. Approximately 50% to 65% of oocytes retrieved from excised ovaries of domestic cats are capable of reaching theMII stage *in vitro*, and 20% to 30% of matured oocytes can develop to the blastocyst stage after IVF [24, 25]. Furthermore, unlike those of other species, cat oocytes have a unique ability to mature *in vitro* after temporary storage at 4°C [22, 26]. It has been shown that cat oocytes obtained from ovaries stored at 4 °C for up to 72 h remain capable of maturing *in vitro* [22, 26]. Moreover, offspring have been obtained after IVF and *in vitro* culture of *in vitro*-matured oocytes recovered from ovaries stored at 4°C for 24 h [23, 27]. However, the ability of oocytes to be matured and develop after IVF declined markedly after 24 h of cold storage [26, 28, 29].

Relaxin is small peptides commonly known as pregnancy hormones in many mammals. It is expressed in various tissues across a broad range of mammalian species [37, 38]. Relaxin is found in a variety of body fluids and have pleiotropic actions on numerous tissue targets [38, 39]. In female reproductive tissues, relaxin is involved in a range of events such as ovarian follicle growth, ovulation, development of mammary glands, and preparation of uterus and cervix for pregnancy and delivery [38-40].

Moreover, porcine relaxin has been reported to support meiotic maturation of porcine oocytes [41] and to improve the function and penetrability of boar sperm [42]. However, it has not known yet whether relaxin influences oocyte maturation and/or embryo development in the cat.

Thus, to counterbalance the lower developmental competence of cat oocytes after cold storage, in the chapter 2, we investigated whether supplementation of *in vitro* maturation medium with relaxin would improve the nuclear maturation and *in vitro* development of feline oocytes from ovaries stored at 4°C for one day.

MATERIALS AND METHODS

Collection of oocyte

The methods used for *in vitro* embryo production were modified from those described previously [25]. Cat ovaries were recovered from sexually mature queens by routine ovariohysterectomy performed at local veterinary clinics. Ovaries were kept in physiological saline and maintained at 4°C for 24–28 h before oocyte collection. Ovaries were placed in mPBS (Embryo-tech; Nihon-zenyaku, Fukushima, Japan) supplemented with 50 μg/ml gentamicin (Sigma-Aldrich), and were sliced repeatedly in a 90-mm culture dish at 37°C to release COCs. Only good-quality COCs with uniform dark-pigmented ooplasm and intact cumulus cell investment were transferred to the IVM medium.

Oocyte maturation and experiment design

The good quality of COCs were selected and transferred to IVM which consisted of 25 mM HEPES in TCM 199 (Invitrogen) supplemented with 4 mg/ml BSA (Sigma-Aldrich), 0.1 IU/ml hMG (Teikokuzoki), 10 IU/ml hCG(Teikokuzoki), 1 μg/ml estradiol-17β (Sigma-Aldrich), and 50 μg/ml gentamicin. The COCs were washed and cultured for 24 h in 100-μl droplets of IVM medium under mineral oil at 38°C in a humidified

atmosphere of 5% CO_2 in air (≤10 oocytes per droplet). To evaluate the effects of relaxin on nuclear maturation and development of cat oocytes after IVF, the IVM medium was supplemented with different concentrations (0, 10, 20, and 40 ng/ml) of relaxin (#R2156, Sigma-Aldrich). All procedures were approved by the Animal Research Committee of the Yamaguchi University.

Assessment of meiotic status of oocytes

At the end of IVM culture, some oocytes were denuded using small glass pipettes, and fixed and permeabilized for 15 min at room temperature in DPBS (Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich). Then, they were placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred into a small drop comprising PBS supplemented with 90% (v/v) glycerol (Wako) and 1.9 μM Hoechst 33342 (Sigma-Aldrich) on a slide. Subsequently, the oocytes were overlaid with a coverslip supported by four droplets of vaseline/paraffin and incubated overnight at 4°C. The oocytes were examined using fluorescence microscopy with a 355-nm wavelength excitation filter. They were classified according to their chromatin configuration as GV, CC, MI, or MII. Oocytes with the diffusely stained cytoplasmic characteristic of

nonviable cells and those in which chromatin was unidentifiable or not visible, were classified as ‘degenerated’.

Measurement of intracellular glutathione content

At the end of IVM culture, some oocytes were denuded and then washed 3 times in 0.2 M PO_4 buffer, and groups of 10–15 oocytes in 5 µl of PO_4 buffer were transferred to 1.5 ml microfuge tubes. The samples were then stored at –80 °C until the GSH assay was done. The intracellular concentration of GSH in oocytes was determined using the 5,5’-dithio-*bis* (2-nitrobenzoic acid)-glutathione disulphide (DTNB-GSSG; Sigma-Aldrich) reductase recycling assay [62]. Briefly, 5 µl of 1.25 M H_3PO_4, 175 µl of 0.33 mg/ml NADPH in the stock buffer, 25 µl of 6 mM DTNB in the stock buffer, and 40 µl of distilled water were added and mixed in a microfuge tube. Five microliters of 250 units/ml glutathione reductase (Wako) was added to initiate the reaction. The absorbance was monitored continuously at 412 nm using a spectrophotometer (U2001; Hitachi, Tokyo, Japan) for 3 min, and the amount of GSH was determined according to the standard curve. The GSH concentration per oocyte was calculated by dividing the total concentration per sample by the number of oocytes present in the sample.

Spermatozoa collection and cryopreservation for IVF

Testes were collected from adult male cats following castration at local veterinary clinics. They were kept in physiological saline and maintained at room temperature for 3–5 h before spermatozoa collection. The epididymis was separated, removed from the testes, and sliced repeatedly with a scalpel blade in a 90-mm culture dish containing mPBS at 37 °C to release spermatozoa. The released spermatozoa were washed in mPBS by centrifugation at 500 × *g* for 5 min. After centrifugation, the supernatant was discarded and the resultant sperm suspension (~50 μl) was diluted with 450 μl first extender, which consisted of 8.8% (w/v) lactose (Wako), 200 μg/ml ampicillin (Mitaka, Tokyo, Japan), and 20% (v/v) egg yolk in distilled water. The tube containing the diluted sperm suspension was transferred to a 500-mL glass beaker containing 350–400 mL of water at room temperature, which was then kept at 4°C for 2 h. After equilibration, 250-μl second extender (first extender supplemented with 6% [v/v] glycerol and 1.48% [v/v] Equex STMpaste [Miyazaki-kagaku, Tokyo, Japan]) was added to the sperm suspension at 4°C. After 5 min at 4°C, an additional 250 μl of the second extender was added to the sperm suspension at 4°C. Aliquots of sperm suspension were then immediately loaded into 0.25-ml plastic straws (Fujihira Co., Tokyo, Japan), which were placed on a styrofoam plate in liquid nitrogen vapour (4 cm above the

surface of the liquid nitrogen and frozen. The straws were kept on the plate for 20 min and then plunged into the liquid nitrogen for storage.

In vitro fertilization and culture

The straws of frozen sperm were thawed in a 38°C water bath and washed in IVF100 medium (IFP9630, Research Institute for the Functional Peptides Co., Yamagata, Japan) by centrifugation at 500 × *g* for 5 min at room temperature. The sperm pellet was resuspended in IVF100 medium, and the concentration was adjusted to 2×10^6 sperm/ml. The COCs cultured in the IVM medium with or without relaxin supplement were transferred to 100-µl sperm droplets of IVF100 medium (≤5 COCs per droplet) and coincubated for 12 h at 38°C in a humidified atmosphere of 5% CO_2 in air. The day of IVF was defined as day 0. After co-incubation, the COCs were denuded from cumulus cells by pipetting through a fine pipette and then cultured in a mSOF medium [63] supplemented with 4 mg/ml BSA and 50 µg/ml gentamicin for 72 h at 38°C in a humidified atmosphere of 5% CO_2 in air. After 72 h of culture, only cleaved embryos were further cultured in mSOF supplemented with 5% (v/v) FBS (Invitrogen) for additional 5 days to evaluate their ability to develop to the blastocyst stage. The embryos that cleaved and reached the blastocyst stage were evaluated morphologically on day 8.

Statistical analysis

All experiments were repeated from 5 to 7 times. The percentages of nuclear maturation, cleavage, and blastocyst formation were subjected to arcsine transformation before ANOVA. The transformed data and GSH concentrations were tested by ANOVA, followed by Fisher's protected least significant difference test using the StatView program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences with a probability value (P) <0.05 were considered to be significant.

RESULTS

There were no beneficial effects of relaxin supplementation of IVM medium on the nuclear maturation of cat oocytes from ovaries stored at 4°C for 1 day (Table 1). There were no differences in the GSH concentrations among the groups of cat oocytes after IVM culture, regardless of relaxin supplementation (Table 1). However, the rate of blastocyst formation from oocytes matured with 10 ng/ml relaxin (16.0%) was higher ($p < 0.05$) than that from oocytes matured without relaxin (5.9%). The supplementation of IVM medium with 40 ng/ml relaxin significantly decreased the rates of cleavage and blastocyst formation of oocytes after IVF compared with supplementation of 10 ng/ml relaxin ($p < 0.05$) (Table 2).

DISCUSSION

Temporal storage of ovaries can provide opportunities to rescue oocytes from ovaries of endangered felids, which sometimes die suddenly in the field, or to rescue ovaries after ovariohysterectomy for medical reasons. In this study, we confirmed that the relaxin supplementation can improve the ability of feline oocytes from ovaries stored at 4°C to develop to the blastocyst stage. To our knowledge, the current study is the first to report the effects of exogenous relaxin supplementation during maturation culture on the development of cat oocytes. However, the relaxin supplementation had no beneficial effect on meiotic competence of oocytes. These results agree in part with experiments of Kim et al. [41], who reported that relaxin supplementation during IVM culture did not enhance nuclear maturation of porcine oocytes, regardless of concentration, but did have a positive effect on blastocyst formation after IVF. They suggested that the higher blastocyst rate of porcine oocytes matured with the relaxin was possibly due to an increase of GSH contents in the oocytes. Higher GSH concentrations in oocytes have been regarded as a marker of cytoplasmic maturation [64]. The level of GSH in oocytes increases as the oocyte resumes meiosis, and higher concentrations are found in mature oocytes than in immature ones [65]. In the present study, however, the relaxin supplementation did not

increase the GSH concentrations in cat oocytes after IVM culture, indicating that the relaxin did not enhance cytoplasmic maturation of these oocytes. The reason for this discrepancy between the actions of relaxin in porcine and feline oocytes remains unclear. It has been suggested that variations in oocyte synthesis of ooplasmic GSH during IVM culture may arise according to the source of oocytes and the composition of maturation media [66, 67]. Moreover, cumulus cells play an important role in GSH synthesis, and the increase of GSH levels in oocytes is dependent on the presence of cumulus cells [68]. Jewgenow et al. [69] reported that DNA degradation in granulosa cells began within 12 h in cold-stored cat ovaries and increased in a time-dependent manner. Therefore, we speculated that the lack of a positive effect of relaxin on the cytoplasmic maturation of cat oocytes may have resulted in part from alterations of cumulus cells and damage to gap junctions between oocytes and cumulus cells which occurred during the storage of the ovaries at 4°C.

The present study indicated that supplementation of IVM medium with 40 ng/ml relaxin did not decrease the rate of blastocyst formation compared to the control without relaxin supplementation, but neither did it show the increase observed with the other concentrations of relaxin. These results are in agreement with the findings of Kim et al. [41], who reported that when porcine oocytes were matured with relaxin at a high concentration (100

ng/ml), the rates of development to blastocysts after IVF decreased as compared with oocytes matured with a lower concentration (10 ng/ml). The authors suggested that the reduction of developmental competence of the oocytes resulted from an auto/paracrine negative feedback action induced by the high concentration of relaxin. On the other hand, we found that the supplementation of 10 ng/ml relaxin into the IVM medium improved the blastocyst formation rate of feline oocytes after IVF. Kim et al. [41] also reported that supplementation of 10 ng/ml relaxin during IVM improved development of porcine oocytes after IVF. However, we observed that the supplementation of 20 ng/ml relaxin did not significantly increase the rate of blastocyst formation. The effect of the intermediate relaxin concentration on the embryonic development was not clear and may be due, at least in part, to low numbers of examined oocytes and the overall poor percentage of blastocyst formation.

In conclusion, our findings indicate that the addition of relaxin at a low concentration (10 ng/ml) to the IVM medium improved the rate of blastocyst development of cat oocytes from ovaries stored for 1 day at 4°C, but not the meiotic and cytoplasmic maturation of the oocytes.

GENERAL DISCUSSIONS

Two studies described in this thesis were designed (1) to compare the kinetics of nuclear status and oocyte damage in porcine, bovine and feline ovaries stored at 4°C for 5 days, and (2) to investigate the effect of relaxin supplementation In maturation medium on the meiotic ability and subsequent development of oocytes collected from ovaries stored at 4°C for one day.

In the first study, our results showed that feline oocytes maintain the ability to undergo maturation after longer durations of ovary storage at 4°C when compared with porcine and bovine oocytes. We found that porcine oocytes lost their maturation ability even after 1 day of ovary storage at 4°C. It has been reported that porcine oocytes from ovaries stored at 15°C or lower for 6 h could not reach the MII stage [12]. Moreover, porcine oocytes have more sensitivity to low temperatures compared with other mammalian species [11, 15], in which the sensitivity may be related to their relatively high lipid content and/or lipid composition [8, 47, 48]. Porcine oocytes have been reported to reduce the membrane integrity when stored at < 15°C [13, 70]. The storage of porcine oocytes at a low temperature may affect subsequent nuclear and cytoplasmic reorganization of oocytes at the GV stage [71] and damage the actin [72], mitochondria [73] and

microtubules, and meiotic spindle in the cytoplasm [74]. In contrast, bovine ovaries can be stored at 10°C for at least 24 h without decreasing the developmental competence of oocytes [18]. Lucci et al. [50] demonstrated that Zebu cow ovaries were successfully stored at 4°C for up to 18 h with no morphological damage in the preantral follicles. In fact, bovine oocytes have two-fold smaller complement of fatty acids, reflecting the acyl-containing lipid mass, compared with porcine oocytes [47]. In the present study, we observed that although 47% of the oocytes from bovine ovaries stored for 1 day at 4°C could reach the MII stage. However, the oocytes from the ovaries stored for 3 days lost the meiotic ability to reach the MII stage. In contrast, feline oocytes have been shown to have a unique ability to mature *in vitro* even after ovary storage at 4°C for 24 h [21]. In this study, the meiotic competence of oocytes from ovaries that were stored at 4°C for 1 day decreased, but >17% of the oocytes could reach the MII stage until 3 days of storage. These results indicate that the meiotic competence of oocytes can be maintained for up to 72 h of ovary storage at 4°C, which was supported by the finding of Wolf et al. [20]. Moreover, when the nuclear status and quality of feline oocytes were examined before IVM, significantly more oocytes remained at the GV stage during storage, and none of the oocytes with DNA-fragmented nuclei were observed until 2 days of ovary storage. These results are supported by the findings of

Wood et al. [51] who reported that the storage of feline ovaries at 4°C inhibits taphonomic changes that are reflected by more intense pyknosis, vacuolisation, and increased loss of membrane integrity in granulosa cells and oocytes. On the other hand, our results showed that the increasing storage time of ovaries leads to decrease the proportions of porcine and bovine oocytes that remained at the GV stage before IVM. The increasing tendency in the proportions of feline oocytes at the GV stage after IVM culture may be related to the ability to undergo maturation after longer durations of ovary storage. Phillips et al. [52] has reported that the communication in the ovarian follicle through gap junctions between the oocyte and the surrounding granulosa cells is essential for the regulation of its meiotic differentiation and maturation. A loss of communication between the oocyte and the granulosa cells via gap junctions is responsible for meiotic resumption [53]. Therefore, the decreased proportions of oocytes remaining at the GV stage before IVM culture in the porcine and bovine group could result from the gap junctional loss within the COCs during storage.

We found that the proportions of oocytes with DNA-fragmented nuclei before and after IVM culture increased with increasing storage time, irrespective of species. It has been hypothesised that long-term storage may induce acidosis of follicular fluid by ischemia in the ovary, leading to DNA

fragmentation of oocytes in follicles [12]. The ATP breakdown and the shift from aerobic to anaerobic stage of the cell metabolism during the ischemic condition may induce the accumulation of acid by-products such as lactic and phosphoric acids, which increase the accumulative number of H^+ [75]. The accumulated H^+ in the ovarian cell may be released into follicular fluid and induce acidosis in the environment surrounding oocytes. Dale et al. [76] suggested that the plasma membrane of the oocyte is highly permeable to H^+ ions and that no regulation of the concentration of H^+ occurs. Thus, when oocytes are placed in fluids with more acidic than the oocyte cytoplasm, the pH of oocytes might fall to the pH of the external medium leading to DNA fragmentation of oocytes. In this study, the proportions of porcine oocytes with DNA-fragmented nuclei after IVM culture drastically increased after one day of storage. Guignot et al. [55] reported that the period and temperature of ovary storage affected the quality of oocytes, in which the cytoplasmic membrane [56, 57], microtubules [1, 9, 58, 59], and the cytoskeleton [60] could be sensitive to a low temperature. Porcine oocytes are highly sensitive to chilling, resulting in a loss of membrane integrity when cooled below 16°C [61]. Therefore, a loss of the ability of porcine oocytes to reach the MII stage after storage at 4°C could result from a high chilling sensitivity including meiotic resumption as a result of the gap junctional loss within the COCs during storage and the high

occurrence of DNA fragmentation during IVM culture.

In the second study, our results showed that the supplementation of relaxin in IVM can improve the ability of cat oocytes from ovaries stored at 4°C to develop to the blastocyst stage. It has been demonstrated that storage of feline ovaries at 24°C within 24 h could produce blastocyst after *in vitro* fertilization [26]. It remains unclear mechanism of relaxin supplementation in IVM on blastocyst development of feline oocytes. However, Kim et al. [41] has reported that porcine oocytes exposed to relaxin during IVM have a high developmental rate following IVF due to increasing of GSH content. In this study, the supplementation of 10 ng/ml relaxin in IVM could improve the blastocyst rate. However, there are no benefits by addition of relaxin on the meiotic maturation of oocytes. Moreover, the supplementation of relaxin did not increase GSH content of feline oocytes stored at 4°C for 1 day. GSH has been shown to be one of the markers for ooplasm quality. It regulates sperm nuclear de-condensation and male pronuclear formation [77] and it protects cells against oxidative damage [78]. The level of GSH in oocyte increases as the oocyte resumes meiosis, and higher concentrations of GSH are found in mature oocytes than in immature oocytes [65]. The low intracellular GSH may be responsible, in part, for the low developmental competence of porcine oocytes [79, 80]. Yang et al. [81] reported that intracellular GSH content

decreased after cold preservation of porcine oocytes. In addition, Jewgenow et al. [82] suggested that DNA degradation in granulosa cells began within 12 h in feline cold-stored ovaries and increased in a time-dependent manner. Therefore, we speculated that the lack of a positive effect of relaxin on the cytoplasmic maturation of feline oocytes may have resulted in part from alterations of cumulus cells and damage to gap junctions between oocytes and cumulus cells which occurred during the storage of ovaries at 4°C. On the other hand, our results showed that the relaxin supplementation of 40 ng/ml in IVM medium significantly decreased the rates of cleavage and blastocyst formation of oocyte after IVF when compared with relaxin supplementation of 10 ng/ml. This result agreed with the finding of Kim et al. [41] who suggested that the reduction of cleavage and blastocyst rates was due to an auto/paracrine negative feedback action induced by high concentration of relaxin supplementation.

In conclusion, our findings in the present study indicate that feline oocytes maintain the ability to undergo maturation after longer durations of ovary storage at 4°C compared with porcine and bovine oocytes. The addition of relaxin at a low concentration (10 ng/ml) to IVM medium improved the rate of blastocyst formation of cat oocytes from ovaries stored for 1 day at 4°C, but not the meiotic and cytoplasmic maturation of the oocytes. The further research is needed to evaluate temperature and

duration of ovary storage and to improve culture method of feline oocytes for the prevention of DNA fragmentation. Especially, the improvement of *in vitro* production of feline embryos derived from cold-stored ovaries can provide opportunities to rescue endangered felids.

TABLES AND FIGURES

Table 1. Effect of relaxin supplementation during maturation culture on *in vitro* nuclear maturation and GSH contents of domestic cat oocytes from ovaries stored at 4 °C for one day*

Relaxin concentration (ng/ml)	Number of examined oocytes	No (mean±SEM) of oocytes reaching MII	Concentration of GSH per oocytes (pmol)
0	108	45 (46.4 ± 4.7)	6.3 ± 0.5
10	119	52 (48.5 ± 5.0)	6.0 ± 0.8
20	126	51 (47.6 ± 7.2)	5.4 ± 0.7
40	102	44 (44.9 ± 7.0)	6.1 ± 0.5

*5–7 replicate trials were performed. Percentages are presented as mean ± SEM.

MII: metaphase II, GSH: glutathione

Table 2. Effect of relaxin supplementation during maturation culture on subsequent *in vitro* development of domestic cat oocytes from ovaries stored at 4 °C for one day*

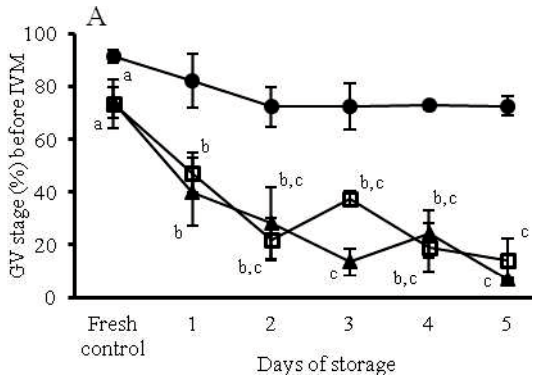
Number of examined oocytes	No (mean±SEM) of embryos	
	Cleaved	Developed to blastocysts
126	93 (72.8 ± 2.9)[a]	7 (5.9 ± 1.5)[a]
119	82 (75.2 ± 4.4)[a]	15 (16.0 ± 3.1)[b]
111	87 (72.5 ± 4.1)[a]	12 (10.7 ± 4.6)[ab]
90	51 (55.4 ± 3.4)[b]	2 (2.5 ± 1.3)[a]

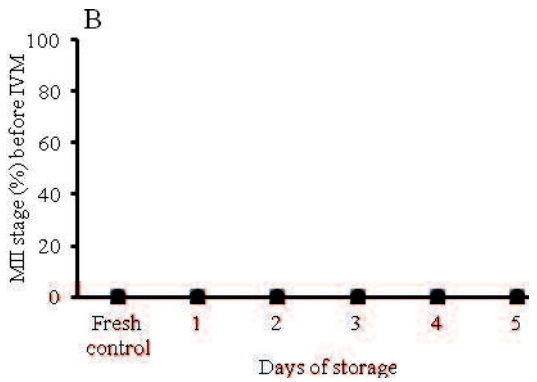
*5–7 replicate trials were performed.

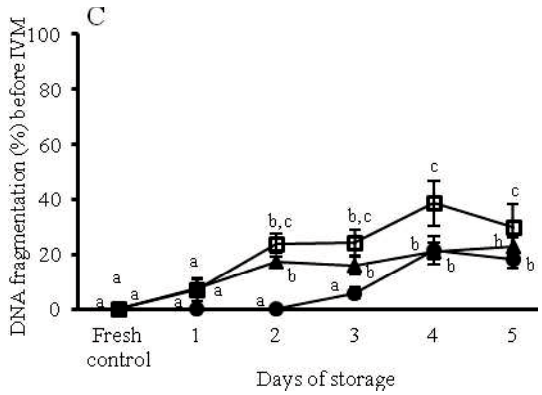
[a-b]Values with different superscripts in the same column are significantly different ($p < 0.05$).

Fig. 1. The kinetics of GV stage (A and a), MII stage (B and b), and DNA fragmentation (C and c) of porcine (▲), bovine (□) and feline (●) oocytes before and after IVM culture during the storage of ovaries at 4°C for 5 days. Oocytes from non-stored ovaries served as fresh controls and 4-8 replicated trials were carried out. The numbers of oocytes examined before and after IVM culture in each storage group were 50–77 and 100–170, respectively. The letters adjacent to a point within each species indicate significantly different values ($P < 0.05$).

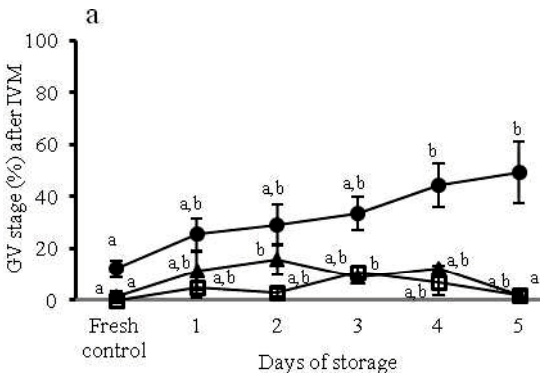
Before IVM

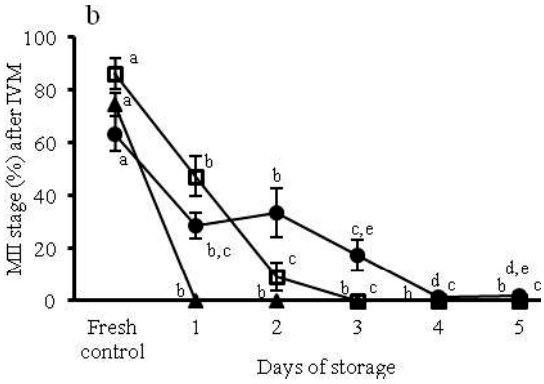


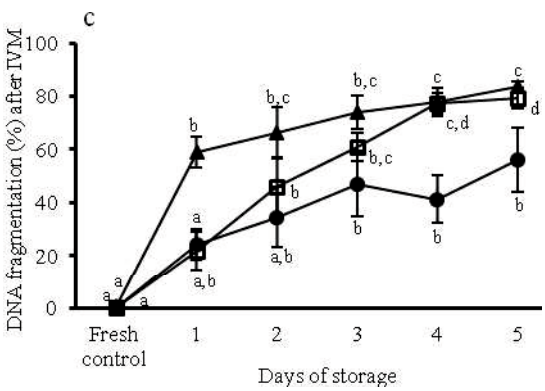




After IVM







ACKNOWLEDMENTS

This research would not have been possible without the tremendous support of my advisors, Professor Takeshige OTOI for the patient guidance, encouragement and advices he has provided throughout my time as his student. I appreciate his ideas and suggestions for my research works and also for the daily life during my time in Japan.

I have greatly benefited from weekly biochemistry seminar of Prof. Yoshimi YAMAMOTO in my first year student. My intellectual debt is to Dr. Yoko SATO who gives insightful comments, suggestions and for her positive presence in the laboratory. I would also like to thank Dr. Masayasu TANIGUCHI, Dr. Zhao Namula and all in past and present that I have had the pleasure to work with or alongside of are grad students in the Prof. Otoi laboratory for their help, cooperating and kindness during my time in Japan.

I am indebted to the following local veterinarians for donating the cat ovaries from routine ovariohysterectomy to my experiments: T. TERAZONO, H. KISHIMOTO, I. YAMAMOTO, N. NITTA. And also, I would also like to thank the staffs of the Meat Inspection Office of Kitakyusyu city for supplying bovine and porcine ovaries for use in the study.

For this dissertation I would like to thank my reading committee member: Professors Yoshimi YAMAMOTO, Mitsuhiro TAKAGI, Kazuhiro KIKUCHI and Hiroshi SATO for their time, interests and helpful comments

I would like to take this opportunity to express my gratitude to Ministry of Education, Culture, Sports, Science and Technology Japan (Monbukagakusho) for the financial support for my five year (2009-2014) PhD program in Japan.

Lastly, I would like to thank families, friends, especially my wife Takako and my daughter Ami for all their love and encouragement. Thank you.

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