Evaluation of Equine Oocytes from Preserved Ovaries Using Intracytoplasmic Sperm Injection

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Abstract: *This study was conducted to examine if oocytes obtained from one day preserved equine ovaries could be used for IVM and IVF research. Ovaries were transported at 20°C over a long distance from a slaughterhouse to a laboratory taking about 24 h. Oocytes with compact cumulus cell layers were collected from ovaries and cultured for 42 h in IVMD101 medium at 38.5°C in 5% CO_2 in air. The proportions of metaphase-II stage oocytes increased significantly between 24 and 30 h of culture and reached a plateau at 30 h. After 30 h of culture, the denuded oocytes with a first polar body were subjected to intracytoplasmic sperm injection (ICSI) using frozen-thawed stallion spermatozoa. The ICSI oocytes were activated by 10 min treatment with ionophore A23187 and 6 h treatment with cycloheximide, and cultured for up to 10 days in CR1aa medium. The proportion of ICSI oocytes fertilized normally (defined as those with 2 pronuclei and 2 polar bodies) was 44%. In vitro culture of ICSI oocytes resulted in a cleavage rate of 52% and a blastocyst development rate of 9%. These results indicate that equine oocytes derived from one day preserved ovaries can reach metaphase-II stage during 30 h of IVM, and that IVM oocytes can develop into blastocysts after ICSI.*
Key words: *Equine oocytes, ICSI, IVM, Preserved ovary*

In vitro production of bovine embryos using slaughtered materials has become more or less routine. However for horses, there have been only several foals derived from IVF [1, 2] and intracytoplasmic sperm injection (ICSI) [3–6]. Slow progress in equine IVF research is due in part to the difficulties in obtaining enough experimental oocytes and/or in conditioning stallion spermatozoa for *in vitro* capacitation. In Japan, the supply of mare ovaries is very limited both by geographical area and the numbers available, therefore equine oocytes from ovaries transported for long distances need to be examined for their normality.

In cattle, storage of ovaries for 24 h at 15 to 21°C did not affect the efficiency of blastocyst production *in vitro* [7]. In horses, Del Campo *et al.* [8] reported that intervals of 3–9 and 10–15 h from slaughter of mares to placement of equine oocytes into culture kept at 30–35°C had no effect on IVM rates, but there have been no reports about the effect of ovary storage on late embryogenesis in this species. To investigate this, IVM oocytes must be fertilized *in vitro*. However, ICSI has been used as the tool for the evaluation of cytoplasmic maturation of equine oocytes matured *in vitro* [6, 9, 10], because an equine IVF system has not yet been established.

The aim of the present study was to evaluate the usefulness of transported (preserved) mare ovaries as a source of equine oocytes. The culture period of the oocytes required for nuclear maturation was determined, and the cytoplasmic maturation of IVM oocytes was evaluated by developmental competence to blastocysts following ICSI.

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Materials and Methods

Ovary storage

Mare ovaries were collected at a local slaughterhouse by veterinarian staff of Kumamoto City Meat Inspection Office. The ovaries were placed in physiological saline containing 50 mg/L gentamycin sulfate (Sigma Chemical Co., St. Louis, MO) in thermal containers at 20°C, and were transported to a laboratory in Tsukuba by a commercial transportation service. The interval from mare slaughter to oocyte recovery was approximately 22 to 24 h.

Oocyte recovery and IVM

Cumulus-oocyte complexes (COCs) were recovered by aspirating surface follicles of 5 to 30 mm in diameter, and additional COCs were aspirated from inner follicles in 5 mm slices of ovaries and by washing the slices with Dulbecco's phosphate buffered saline. The COCs with compact cumulus layers and evenly granulated ooplasm were selected and washed three times with IVMD101 medium (Research Institute for the Functional Peptides Co., Yamagata, Japan) pre-warmed to 37°C. The COCs were cultured in 100 μl microdrops of the IVMD101 medium covered with paraffin oil (Nakalai tesque Inc., Kyoto, Japan) at 38.5°C in an atmosphere of 5% CO_2 in air (5 to 10 COCs per microdrop). At 0, 24, 30, 36 and 42 h of culture, cumulus cells were removed from COCs in M2 medium [11] containing 600 IU/ml hyaluronidase (Type I-S: From bovine testes; Sigma). The denuded oocytes were then fixed in acetic acid : ethanol (1:3, v/v) for 24 h, and stained with 1% orcein for assessing their nuclear configuration at $\times$ 200 magnification.

ICSI and in vitro culture

Frozen-thawed stallion spermatozoa were washed twice by centrifugation at 800 g for 10 min with Hepes-buffered Hank's medium and were resuspended in Hepes-buffered Hank's medium supplemented with 3 mg/ml bovine serum albumin (Initial fractionation by cold alcohol precipitation; Sigma). Denuded oocytes with a visible first polar body after 30 h of IVM culture (presumptive metaphase-II stage oocytes) were selected for ICSI, and centrifuged at 12,000 g for 10 min to facilitate sperm injection. The ICSI was performed in M2 medium supplemented with 3 mg/ml BSA and 10% polyvinylpyrrolidone (MW 40,000; Sigma), according to the method reported for bovine ICSI [12], using a piezo micromanipulator (Prime Tech, Ibaraki, Japan) and sperm injection glass needles of 7 to 8 μm in outer diameter.

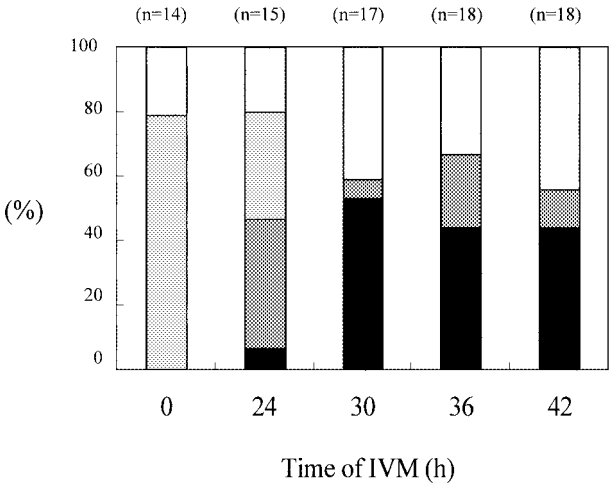

Fig. 1. Nuclear maturation of equine oocytes *in vitro*. ▨: Germinal Vesicle-stage, ▩: Resumed meiosis (GVBD to telophase-I stage), ■: Metaphase-II stage, □: Degenerated.

The ICSI oocytes were activated by treatment with 10 μM ionophore A23187 (Free acid; Calbiochem-Novabiochem Co., La Jolla, CA) for 10 min and 10 μg/ml cycloheximide (Sigma) for 6 h. The oocytes were transferred into CR1aa medium [13] and then cultured in 100 μl microdrops of the CR1aa medium at 38.5°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. At 20 h after ICSI, the oocytes were fixed and stained as described above, to evaluate their fertilization. Oocytes with both male and female pronuclei and two polar bodies were defined as having normal fertilization. An additional *in vitro* culture of presumptive zygotes was continued for 10 days. During the last 6 days, they were cultured in CR1aa medium supplemented with 5% fetal calf serum (FCS; Tissue Culture Biologicals, Tulare, CA). Development beyond 2-cell, 8-cell, and blastocyst stages was assessed after 2, 4, and 7–10 days of culture, respectively.

Statistical analysis

Data were analyzed by Chi square test, or by Fisher's exact probability test when the expected value for any parameter was less than 5.

Results

The time course of nuclear maturation of equine oocytes during IVM is shown in Fig. 1. Oocytes before IVM were at the germinal vesicle stage (79%) or

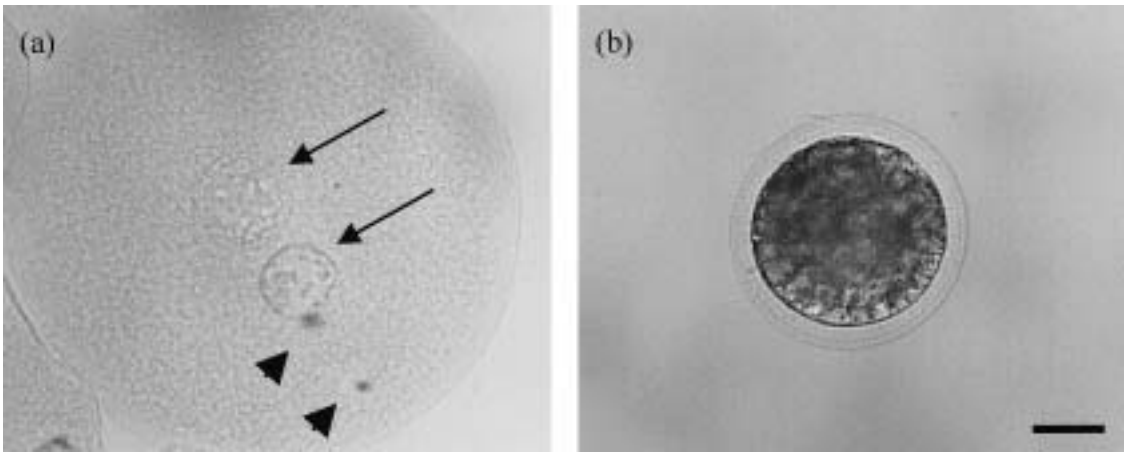

Fig. 2. (a) Normally fertilized equine oocyte by intracytoplasmic sperm injection (ICSI). Arrows: Pronucleus, Arrow heads: Polar body. (b) Equine early blastocyst derived from an IVM oocyte fertilized by ICSI. Scale bar = 50 μm.

Table 1. *In vitro* development of equine oocytes following ICSI

No. of oocytes evaluated	No.(%) of oocytes developed to		
	≥2-cell	≥8-cell	≥Blastocyst
33	17 (52)	11 (33)	3 (9)

degenerating (21%). The proportions of oocytes reaching the metaphase-II stage were 7, 53, 44, and 44% after IVM culture for 24, 30, 36, and 42 h, respectively. A significant increase of maturation rate ($p<0.01$) was observed between 24 and 30 h, and the maturation rate reached a plateau at 30 h of culture.

All oocytes (n=23) survived the process of ICSI, and the proportion of the oocytes fertilized normally (Fig. 2a) was 44% (10/23). *In vitro* development of equine oocytes after ICSI is shown in Table 1. Approximately half of the ICSI oocytes (52%) cleaved, and 9% developed into blastocysts. The three blastocysts appeared on day 7 (Fig. 2b), day 7 and day 8 of culture, respectively.

Discussion

In the present study, equine ovaries were preserved at 20°C, and were transported for approximately 22 to 24 h over a long distance before recovery of equine oocytes. We observed that 79% of the oocytes immediately after recovery from the transported ovaries had a distinct germinal vesicle. Such immature equine oocytes could be successfully cultured to the metaphase-II stage (52%) after 30 h of culture in the IVMD 101 medium. Previous authors [8, 14–16] have studied the interval from slaughter to culture (for 15 h) and the storage temperature (25 to 37°C) on subsequent maturation of equine oocytes. Three studies [8, 14, 15] reported that storage time and temperature had no effect on IVM rates, however, one [16] reported that storage of mare ovaries at 30°C for 9 h resulted in a significant decrease in IVM rates. It has also been reported that storage of ovaries up to 24 h after slaughter affected *in vitro* fertilization and embryonic development, and the effects were dependent on the storage temperature in bovine [7]. Similarly, in the horse, there may be a harmful effect of long time storage of ovaries, but we did not compare meiotic competence of equine oocytes between those from fresh and preserved ovaries.

In domestic animals, the time required for oocyte maturation *in vitro* differs among species. Our results suggest that a culture period of 24 to 30 h for equine oocytes, similar to the results of several laboratories [8, 14, 17–21], may be appropriate and this agrees with ovulation occurring at approximately 36 h after administration of gonadotropins to mares *in vivo* [22].

It has been difficult to evaluate the quality of equine IVM oocytes in the absence of a reliable IVF system. Recently, the migration of cortical granules to the oolemma [23–25] and ICSI [6, 9, 10] have been alternatively used as tools for evaluation of cytoplasmic maturation of equine oocytes matured *in vitro*. In this study, ICSI was used to evaluate the equine IVM oocytes, because we also investigated their developmental ability to the blastocyst stage *in vitro*. Our results showed that normal fertilization, cleavage

and blastocyst development rates were 44%, 52%, and 9%, respectively, and these results are within the variation range of data reported in equine ICSI: 21 to 71% of oocytes formed pronuclei; 20 to 84% cleaved; and 1 to 30% developed into blastocysts [4–6, 9, 10, 26–28, 30]. Our results suggest that the equine oocytes recovered from preserved ovaries have normal developmental competence following IVM and ICSI, although a direct comparison with those from fresh ovaries was not done in this study.

Equine oocytes are most commonly matured in TCM 199 supplemented with serum and hormones. The IVM medium (IVMD101) used in the present study contains growth factors (insulin and transforming growth factor-α) and an antioxidant-related compound (apo-transferrin), but does not contain serum. Goudet *et al.* [17] tested a chemically defined culture medium for equine oocyte maturation. After *in vitro* culture of equine oocytes in TCM 199 + crude equine gonadotropins (eFSH and eLH), oestadiol and EGF, the maturation rate was only 6%, and they suggested that fetal calf serum was essential for maturation of equine oocytes. Our results suggest that equine oocytes can be matured (52%) in serum-free medium *in vitro*, and similar proportions of nuclear maturation (45–64%) have been reported using serum-supplemented IVM medium by several studies [8, 17–21], except for one report with a higher maturation rate (>80%) [14]. However, we cannot know about the complete composition of IVM media in detail, because IVMD101 medium is a commercially available medium. At the very least, immature horse oocytes can be matured to metaphase II *in vitro* in culture conditions similar to those used in bovine.

In vitro culture systems have not yet been established for the development of equine embryos. *In vivo*-derived one- to two-cell equine embryos did not develop to the blastocyst stage unless co-cultured with uterine tubal epithelial cells [29]. In an equine ICSI study, Li *et al.* [6] reported that 30% of the two-cell embryos developed to the blastocyst stage when matured and cultured in the presence of somatic cells, while, no blastocysts developed from two-cell embryos that had been cultured in the absence of any type of cell. In contrast, 18% (3/17) of the two-cell embryos obtaining by ICSI developed to the blastocyst stage in our system without co-culture. Generally, equine ICSI has been conducted by conventional ICSI (an injection needle is inserted into the ooplasm, and small amounts of cytoplasm with a spermatozoon are drawn into the needle and then injected) [3–6, 9, 10, 26, 27]. Recently, two studies reported using a piezo micromanipulator for equine ICSI which resulted in high pronucleus formation and cleavage rates [28, 30]. When the piezo-ICSI procedure was compared with conventional ICSI, increases in efficiency were reported in mice, humans and cows [31–33]. In bovine ICSI, piezo-ICSI was useful, because the oolemma was broken readily and a spermatozoon could be injected without causing lysis of the oocyte, and live offspring were produced efficiently [12]. We made it also used a piezo maicromanipulator for sperm injection, and this, in part, possible to produce blastocysts without co-culture.

Developmental kinetics of equine ICSI oocytes *in vitro* (blastocysts at 7–8 days of culture) were not comparable with those of zygotes in the oviducts (blastocysts at 5.5–6.0 days after ovulation), but delayed development of equine embryos has always been observed *in vitro* [6, 28, 29]. Ball and Miller [29] reported that 4- to 8-cell embryos after co-culture with oviduct epithelial cells *in vitro* were more delayed and smaller in size compared with Day 7 uterine blastocysts. Choi *et al.* [28] reported that the number of nuclei in ICSI-derived embryos cultured 96 h *in vitro* was significantly lower than that in those transferred to the oviduct. Further studies are needed to improve the equine IVC-system to support embryonic development.

In conclusion, equine oocytes recovered from preserved ovaries could be matured in a serum-free IVM medium over 30 h, and such oocytes appeared to be normal as judged by development to the blastocyst stage after ICSI.

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