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NOTE

# Effects of ovarian storage condition on *in vitro* maturation of Hokkaido sika deer (*Cervus nippon yesoensis*) oocytes

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**Abstract**

**The effects of different preservation conditions (temperature 20–25 or 10–15°C; duration 12 or 24 h) for ovaries of *Cervus nippon yesoensis* on *in vitro* maturation of oocytes were examined. When ovaries were kept for 12 h at 20–25°C, maturation rate of oocytes was highest (71%); however, it declined when the preservation time was extended to 24 h (31%). When the preservation temperature decreased to 10–15°C, the maturation rate after 12 h preservation decreased (51%) but it remained in same level even though preservation time is prolonged to 24 h (55%).**

Key Words: *in vitro* maturation, ovary storage, sika deer

At present, more than 40 subspecies of deer have been listed as animals on the brink of extinction and protected by International Union for the Conservation of Nature and Natural Resources, but the result of species conservation is not so ideal[2)]. In the view of conservation, *in vitro* production (IVP) of embryos would help to reach the goal since embryo transplantation had been started to apply and succeeded in some cervid species such as sika deer[11)], red deer[6)] and fallow deer[9,16)]. For IVP of embryos, since it is difficult to collect oocytes from live endangered species, ovaries from dead animal will be the valuable source of oocytes for IVP. However, habitats of endangered species are always far away from laboratory, in which ovaries need to be transported for long time to be subjected to IVP. Therefore, it is essential to investigate the effect of the preservation condition of ovaries on IVP. In various domestic animals, cattle[14,18)],

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horse[12,13], pig[10,25], sheep[8,20], dog[5,23] and cat[19,24], the effects of ovarian storage were well investigated and the appropriate condition differs between species. In cervid species, there is only one report that investigated about ovarian storage condition[8] and they suggested to store the ovaries at room temperature since there was no difference in terms of blastocyst rate between those oocytes derived from ovaries stored at low temperature (5–8°C) and room temperature (20–25°C) for 12 h. Certainly, it is easy to keep the ovaries at ambient temperature, but it is much lower than 20–25°C during breeding season of temperate deer (*i.e.* autumn to winter) when number of visible follicles those are possible to recover the oocytes increases[15]. Therefore, the influences of temperature (10–15°C) between room and low temperature on IVP need to be investigated in deer, because it is known to be different between species. It was preferable in cattle[14] but detrimental in pig[10,25] compared to room temperature. Further, storage duration more than 12 h also need to be examined because it sometimes takes a day to transport ovaries to laboratory. In the present study, we preliminarily investigated the effect of the ovarian preservation condition of Hokkaido sika deer (*Cervus nippon yesoensis*), as a model of cervid species, on nuclear maturation of oocytes after *in vitro* maturation (IVM), which is closely related to blastocyst development.

The totals of 178 ovaries were collected from 89 females, 10–15 animals per day, at deer meat treatment center in Akan, Kushiro City, Hokkaido, Japan and 164 ovaries were used for experiments because ovary without antral follicles or with big corpus luteum was excluded from experiments. All ovaries were collected from healthy female during late breeding season (January–April). Within 15 min after does were slaughtered, ovaries were collected and put in a thermos flask with physiological saline containing antibiotics under 10–15°C or 20–25°C, and then transported to the laboratory. The pair of ovaries from each animal randomly placed into either temperature condition and mixed in the thermos flask. Randomly, half of ovaries were used for collection of oocytes 12 h after the ovary collection. Remaining ovaries were preserved in the thermos flask for another 12 h (*i.e.* 24 h later after the ovary collection) and then used for the collection of oocytes. Before putting the ovaries in the thermos flask and taking them from the thermos, the temperature in the thermos was measured. Before collecting oocytes, ovaries were washed with sterilized physiological saline for 3 times.

All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Small antral follicles (diameter 1–2 mm) in ovaries were isolated under aseptic condition using fine scissors and forceps. Isolated follicles were transferred into petri dish filled with TALP-HEPES solution[22]. Under stereomicroscope, cumulus-oocytes complexes (COCs) were collected by puncturing follicles by using forceps and 25 gauge needles, and then COCs were transferred to the maturation medium. COCs having several layers of cumulus cells and evenly granulated ooplasm were used for experiments. IVM system for bovine IVP[21] was employed in the present study. Before IVM culture, COCs were washed twice in the maturation medium, which was 25 mM HEPES-buffered TCM 199 (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Invitrogen), 0.02 units/ml follicle-stimulating hormone (from porcine pituitary), 1 μg/ml estradiol-17β, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin sulfate, and then transferred to 50-μl droplet of maturation medium covered with paraffin oil. COCs (10–12 COCs per droplet) were cultured for 22–26 h at 38.5°C under humidified atmosphere in 5% $CO_2$ in air. To evaluate the nuclear maturation, oocytes were fixed with a mixture of ethanol: acetic acid (3 : 1) and stained with 1% aceto-orcein solution. Then their nuclear statuses were examined under a phase-contrast microscope[17]. Nuclear statuses were divided into germinal vesicle (GV), germinal vesicle breakdown (GVBD),

metaphase I (M I), anaphase I/telophase I (AI/TI) and metaphase II (M II) by observation under a phase contrast microscope. Oocytes at M II were considered as mature and others as immature. If the oolemma was disrupted and/or ooplasm looked irregular, the oocytes were judged as degeneration.

To confirm the developmental competence of deer oocytes, IVP was carried out in present study, oocytes derived from ovaries preserved at 20–25°C for 12 h were subjected for *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Sperm used for IVF was collected from epididymis of stag in late breeding season (January–April) and frozen as previously described[7]. Briefly, within 30 min after the stag hunted at 5–10°C outdoor temperature, the scrotum was cut by sterilizing scissors and then transported to the laboratory within 1 h after the hunting. Under room temperature (25°C), the epididymal tails were isolated from testes. Then the sperm in epididymis tail were collected and frozen in 0.25 ml straw as described previously[7]. For IVF, COCs after IVM was fertilized and cultured *in vitro* according to a procedure described previously[21,22] with slight modification. In brief, motile sperm ($5 \times 10^6$ sperm/ml) separated from thawed semen by a Percoll gradient (45 and 90%) were co-incubated with COCs in a 100-μl droplet (about 10 COCs per droplet) of modified Brackett & Oliphant isotonic medium[4] containing 10% serum of the sheep in estrus (1.5 days after estrus started)[3] and 2.5 mM theophylline for 18 h at 39°C in a humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$. After co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in the culture medium. Cumulus-free zygotes were cultured for 8 days in a 30-μl droplet of the culture medium at 39°C under 5% $CO_2$, 5% $O_2$ and 90% $N_2$. The culture medium was a modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 non-essential amino acids for minimum essential medium and 10 μg/ml insulin, and further supplemented with 5 mM glycine, 5 mM taurine, 1 mM glucose and 3 mg/ml fatty-acid-free BSA. After 2 days (about 30 h) and 8 days (about 180 h) of IVF, the cleavage and development of presumptive zygotes to the blastocyst stage were assessed, respectively. All data were compared by Chi-square test. Statistical analysis was performed using software (JMP version 10.0.2, SAS Institute, Cary, NC, USA).

As shown in Table 1, the proportion of mature oocytes were similar irrespective of the duration of preservation under 10–15°C (around 50%); however, nuclear maturation rate declined from 71% to 31% when the preservation time was extended to 24 h at 20–25°C ($P < 0.01$). Comparing the proportions of mature oocytes between ovaries preserved at 10–15°C and 20–25°C, the proportion of mature oocytes preserved at 20–25°C for 12 h was higher than that at 10–15°C for 12 h ($P < 0.01$). However, the proportion of mature oocytes preserved at

**Tab1e 1. The effect of ovary preservation conditions on *in vitro* maturation of Hokkaido sika deer (*Cervus nippon yesoensis*) oocytes**

| Ovary preservation condition | | Oocytes used (n) | Proportion of oocyte after maturation culture (%) | | |
|---|---|---|---|---|---|
| Temperature (°C) | Duration (h) | | Mature (n) | Immature (n) | Degeneration (n) |
| 10–15 | 12 | 92 | 51 (47) | $38^{a*}$ (35) | $11^{a}$ (10) |
| | 24 | 96 | 55* (53) | $15^{b}$ (14) | $30^{b}$ (29) |
| 20–25 | 12 | 128 | $71^{a**}$ (91) | 23 (29) | $6^{a}$ (8) |
| | 24 | 52 | $31^{b}$ (16) | 27 (14) | $42^{b}$ (22) |

[a, b]Values with superscripts differ significantly within same temperature ($P < 0.01$).
* and **Values with superscript higher within same storage duration ($P < 0.05$ and $P < 0.01$, respectively).
This experiment was replicated three times.

20–25°C for 24 h was lower than that at 10–15°C for 24 h ($P < 0.05$). Proportion of immature oocytes after the storage of ovaries at 10–15°C for 12 h was higher compared to those storage duration extended to 24 h ($P < 0.01$) and those stored at 20–25°C for 12 h ($P < 0.05$). The degeneration rate increased as the storage duration extended in both temperature condition ($P < 0.01$), but it did not differ between the same storage duration at different temperature ($P > 0.05$). The cleavage rate and the developmental rate to blastocyst stage after storing ovaries at 20–25°C for 12 h in the present study were 65% (62/95) and 23% (14/62), respectively.

The cleavage rate was almost same as the nuclear maturation rate (71%) when ovaries were stored at 20–25°C for 12 h in the present study. Moreover, the cleavage rate and the developmental rate of present study was equivalent to previous study[8] that used oocytes derived from ovaries of red deer stored at 20–25°C for 12 h (64.9% and 13.9%, respectively). Since present culture system was possible to produce blastocyst comparable to previous study, developmental competence of oocytes derived from another storage condition need to be investigated to know the appropriate storage condition in further study. In a previous study[8], although the nuclear maturation rate was not examined, the cleavage and blastocyst rates were similar between different storage temperature at 5–8 and 20–25°C for 12 h. It may indicate that the nuclear maturation rate seems to be similar in both storage temperatures. Conversely nuclear maturation rate of oocytes derived from the storage of ovaries at 10–15°C was lower than that at 20–25°C in the present study. One possible reason of the decrease of nuclear maturation rate in oocytes derived from ovaries preserved at 10–15°C is damaged oocyte membrane, since it is reported that the phase transition of the membrane lipids of bovine oocytes at GV stage occurred between 13–20°C and fewer oocytes retained their membrane integrity after storing at 13°C compared to those stored at 4 and 38°C[1]. In further study, the phase transition of the membrane lipids of deer oocytes should be examined.

Nuclear maturation rate at 10–15°C after 24 h storage did not decrease compared to that after 12 h storage, although it decreased when ovaries were preserved at 20–25°C. In feline, it was reported that oocytes reached M II stage decreased gradually to around 20% as the duration of storage at 23–25°C prolonged to 24 h[19]; however, maturation rate of oocytes from ovaries stored at 4°C for 24 h was similar to oocytes without ovarian preservation (around 50%). In cattle, it is reported that maturational competence started to decrease after storing ovaries more than 48 h at 10°C[14]. The present results seem to indicate that the ability of nuclear maturation of deer oocytes well preserve under low temperature. There is no report that examined the effect of 24 h preservation of deer ovaries on the developmental competence after IVF. To confirm this hypothesis, maturation, fertilization and blastocyst rates after 24 h storage of ovaries at 10–15, 20–25 and 5–8°C need to be examined.

In conclusion, even though the ovary preservation at 10–15°C is not ideal for 12 h compared to 20–25°C, it might be better when preservation time is prolonged up to 24 h since it prevented the decrease of nuclear maturation rate. In further study, the developmental competence of deer oocytes preserved more than 24 h need to be investigated.

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