

Developmental Capacity of Goat Oocytes Collected from 5 °C Preserved Ovaries

Kemampuan Perkembangan Oosit Kambing yang Dikoleksi dari Ovarium Pascapreservasi pada Suhu 5 °C

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Abstract

Research has been conducted to study the effect of ovaries preservation at 5°C on the oocytes development capacity i.e the capacity of oocytes to undergo *in vitro* maturation (IVF), *in vitro* fertilization (IVF) and *in vitro* embryo development. Goat ovaries were obtained from slaughterhouse in saline solution containing 0.1% Bovine Serum Albumine (BSA) and antibiotic and kept at 5°C for 3 and 12 hours. As control, untreated ovaries were kept for 3 hours at 30-35°C, the temperature usually used for ovaries transportation. The oocytes were aspirated from follicles with 2-5 mm in diameter using 20G needle connected to a 5 ml syringe containing modified phosphate buffered saline (mPBS). The aspirated oocytes were incubated in 100µl micro drops of tissue culture medium-199 (TCM-199) supplemented with 10% newborn calf serum (NBCS), 0.01 mg/ml follicle stimulating hormone (FSH) and 50 µg/ml gentamycine sulphate for 24 hours at 38.5°C in 5% CO₂ incubator. *In vitro* fertilization (IVF) was done in CO₂ incubator at 38.5°C for 18 hours using fresh semen. Inseminated oocytes were further cultured for 5 days. The matured and fertilized oocytes were examined by their nuclear status after aceto-orcein staining. The results showed that the average number of morphological normal oocytes collected from 5°C preserved ovaries were significantly lower than from the untreated control ovaries. After 24 h incubation the percentage of matured oocytes from the 3 and 12 h preserved ovaries were 85.3±2.8% and 75.5±2.2%, respectively ($P < 0.05$); but were not significantly different with the untreated control ovaries 80.3±3.7%. The *in vitro* fertilization and cleavage rate of oocytes collected from 3 hours preserved ovaries were not significantly different from the control untreated ovaries. However, prolong 5°C preservation until 12 hours decreased the oocytes development capacity. In conclusion, preservation of ovaries in 5°C until 12 hours can produced oocytes capable to undergo *in vitro* maturation and fertilization and support embryo development, and the oocytes development capacity were significantly reduced.

Keywords: ovary preservation, *in vitro* maturation, *in vitro* fertilization, embryo cleavage

Introduction

In vitro fertilization and embryo production in farm and wild animals has been widely reported since in cattle (Brackett *et al.* 1982), sheep (Cheng *et al.* 1986), goat (Hanada, 1985;), horse (Palmer *et al.* 1990), and felids (Pope, 2000).

One of the limiting factor on *in vitro* embryos production is the availability of viable and developmentally competence of oocytes, due to its short fertile life. Current information on transport of ovaries during one to six hours in saline solution at 30 – 37°C was reported (Pawshé *et al.* 1996; Martino *et al.* 1994; Arlotto *et al.* 1996; Byrd *et al.* 1997) and indicated that viability of oocytes lost after six

hours of transport. During ovaries transport at 30 to 37°C, metabolic process of oocytes in the ovaries continue for sometimes and ultimately it will decompose. Furthermore, Kartini *et al.* (2008) reported that the death of endangered animals in the marginal areas located far away from diagnostic or research laboratories would potentially result in the lost of genetic materials sources in the form of sperms and oocytes.

Researchers are facing undesirable problems in obtaining fresh ovaries from slaughter houses or from other low cost resources as well as from genetically valued animal. In Indonesia, some influencing factors, such as distance between research laboratory and slaughterhouses, unexpected traffic jam that might happen from time to time, and higher environmental temperature in the tropic temperature should be taken into consideration. And, therefore, delayed arrival of ovaries transport to laboratory may fail to obtain oocytes as expected. Preservation at 5°C could become one of the alternative way to be used to save the potential of germ cells materials. Preservation at 5°C could possibly minimize metabolism rate, therefore will ultimately delay the ovaries tissue decomposition and maintain oocytes viability.

Studies of *in vitro* embryo production in goat included *in vitro* maturation (IVM) and fertilization (IVF) has been reported (Crozet *et al.* 1995; De Smedt *et al.* 1992; Younis *et al.* 1992; Martino *et al.* 1995; Le Gal, 1996; Paramio, 2010). Therefore, this research used goat ovaries as model to study the developmental capacity of oocytes collected from ovaries preserved at 5°C for 3 and 12 hours.

Materials and Methods

Oocyte Collection and In Vitro Maturation

Goat ovaries were collected from a local slaughterhouse and transported to the laboratory in saline solution containing 01.% Bovine Serum Albumine (BSA, Sigma, USA) and penicillin-G (1000U/ml) and streptomycin sulfate (0.2 ug/ml) (1) at 5°C and kept for 3 and 12 hours; and (2) 30 to 35°C (untreated control ovaries) for 3 hours. Oocytes were aspirated from 2- to 5-mm antral follicles with a

20 G needle attached to a 5-ml disposable plastic syringe containing modified phosphate buffered saline (mPBS, Gibco, USA), supplemented with 0.3% (w/v) BSA and 50 ug/ml gentamycin sulfate (Sigma, USA). Only oocytes enclosed in a compact multilayered cumulus (cumulus oocyte complexes or COCs) were recovered. The COCs were washed three times in maturation medium (TCM-199, Earle's salt with sodium bicarbonate and L-glutamine) (Gibco, USA) supplemented with 10% (v/v) inactivated new born calf serum (NBCS, Sigma, USA); 10 ug/ml follicle stimulating hormone (FSH, Denka Pharmaceutical Japan) and gentamycin sulfate 50 ug/ml. Twenty to thirty oocyte were placed in 100- μ l droplet of the maturation medium, covered with sterile mineral oil and incubated at 38.5°C in 5% CO₂ in air for 24 h.

Oocytes In Vitro Fertilization

Fresh semen of a local breed goat was obtained by artificial vagina. The spermatozoa were washed twice in 8-ml CR1aa (C.Rozenkrans 1 amino acid) medium containing 0.2 mM caffeine and 0.3% BSA by centrifugation (500G, 5 min). The sperm pellet was resuspended and diluted in CR1aa medium containing 0.2 mM caffeine, 2.0% BSA and 0.2 mM heparin to a final concentration of 10×10^6 sperm/ml. Groups of 20 matured oocytes were put into 50 μ l droplets of sperm suspension and incubated in 5% CO₂ incubator at 38.5°C for 18 h.

Embryos In Vitro Development

Eighteen hours after insemination, oocytes were washed 3 times and transferred for further development into a 50 μ l microdroplet of medium CR1aa (supplemented 3 % BSA, 5 ug/ml insulin and gentamicin 50 ug/ml). Embryos incubation was done in 4% CO₂ incubator at 38.5°C.

Assessment of Maturation and Fertilization Rate

After 24 h of maturation and 18 h of insemination, the oocyte were fixed in acetic acid-ethanol (1:3) and stained with 1% aceto-orcein as previously reported by Djuwita *et al.* (1998). The oocytes nuclear status were examined under a phase contrast microscope.

Matured oocyte was determined as nuclear at the metaphase-II (Mt-2) stage; and fertilized oocyte was determined by the presence of two pronuclei. The percentage of matured oocytes were the number of oocytes at Mt-2 by the number of oocytes cultured times 100. The percentage of fertilized oocytes were the number of oocytes with 2 pronuclei by the number of oocytes inseminated times 100.

Embryo Development

Development of the cleaved embryos up to morula was observed on day 5. The percentage of cleaved embryos were the number of embryos cleaved by the number of oocytes cultured times 100.

Experiment Design and Analysis

Each experiment is conducted in three replicates, each using 12 ovaries. The data obtained were analyzed by ANOVA and Duncan Multiple Interval Test Probability at 5% level of significant.

Results and Discussion

The results showed that the overall number of morphological normal oocytes collected from the 3 hours 5°C preserved ovaries ($n = 12$) were 133.0 ± 3.8 were not significantly different with the those collected from the control ovaries 148.0 ± 12.3 . However, prolong preservation until 12 hours significantly reduced the number of morphological normal oocytes 93.0 ± 2.0 (Tabel 1).

Preservation of ovaries at 5°C for 12 hours decreased the number of morphological normal oocytes collected compared to ovaries with short time (3 hours) 5°C preservation or the

control ovaries. Prolong 5°C preservation caused damage to the zona pellucida, plasm membrane and cytoplasmic contain which lead to oocytes degeneration. This is due to changes at the lipid protein bilayer of the plasmic membrane cause membrane destabilization (Park and Ruffing, 1992).

Most oocytes evaluated directly after collection were at the germinal vesicle (GV) stage. During 24 hours incubation, oocytes will sequensently proceed to the germinal vesicle break down (GVBD), metaphase I (Mt-I) and finally to the metaphase II (Mt-II) stages. After 24 hours incubation the percentage of matured oocytes from 3 hours preserved ovaries were $85.3 \pm 2.8\%$, not significantly higher compared to those from the control ovaries 80.3 ± 3.7 . Otoi *et al* (2001) reported similar results that the meiotic competence between oocytes from cat ovaries stored at 4°C and 38 °C for 24 hours were not significantly different. Prolong 5°C preservation until 12 hours significantly reduced the number of matured oocytes (71.4 ± 4.0) (Table 1). And incidences of chromosome clumping and metaphase-plate abberation (chromosome dispersion) were observed in oocytes of 12 hours preserved ovaries after undergo *in vitro* maturation.

After 18 hours insemination, the IVF rate were not significantly different between the oocytes from 3 and 12 hours preserved ovaries $75.6 \pm 3.8\%$ and $71.1 \pm 7.7\%$ with those of the control untreated ovaries $68.9 \pm 3.9\%$ (Table 2). However, in 12 hours preserved group, oocytes with 2 pronuclei was 37/66 (56.1%), while others showed more than 2 pronuclei (17.6%) and also the incidence of decondensed sperm head of about 11/66 (16.7%).

Table 1. Number of morphologically normal oocytes collected from ovaries after 3-12 hours 5°C preservation and the number of oocytes matured after 24 hours *in vitro* maturation (from 3 replicates)

Temperature (hours)	No. of ovaries	Total no. of oocytes	Average of oocytes per ovary	Maturation rate (%)
30-35°C (3)	12	148.0±12.3 ^a	12.3	119 (80.3±3.7) ^a
5°C (3)	12	133.0± 3.8 ^a	11.1	113 (85.0±2.8) ^a
5°C (12)	12	93.0± 2.0 ^b	7.8	66 (71.4±4.0) ^b

Within column with different superscripts means significantly different (P<0.05).

Table 2. Effect of 3-12 hours ovaries preservation 5°C on the number of oocytes fertilized and developed to cleavage stage (from 3 replicates).

Temperature (hours)	Total no of oocytes	No. of fertilized oocytes (%)	No of embryo cleaved (%)
30-35°C (3)	105	31/45(68.9±3.9)	29/60 (48.4±1.4) ^a
5°C (3)	97	34/45(75.6±3.8)	28/52(53.8±3.3) ^a
5°C (12)	91	32/45(71.1±7.7)	20/46(43.5±3.4) ^b

Within rows with different superscripts mean significantly different (P<0.05)

The embryo cleavage rate produced from oocytes of 3 hours 5°C preserved ovaries were not significantly difference with those of untreated control ovaries, 53.8±3.3.% and 48.4±1.4%, respectively. And both showed significantly differences (p <0.05) compared to those produces from oocytes of 12 hours 5°C preserved ovaries (43.5±3.4%).

The results indicated that the lower temperature provided lower rates of cellular metabolism, and consequently minimizing the metabolism need and thus increasing the the resistance of oocytes in the absen of nutrients (Santos *et al*, 2002). In this condition, the decomposition process of the ovaries can be prevented. Apparently, after the ovaries were stored in cold temperature (5°C) for 12 hours, the aspirated oocytes have capability to mature through *in vitro* maturation, fertilized and support embryo development. This results was in line with previous report that feline ovaries stored at 4 °C until 12 hours, do not show any increased of apoptosis in the granulosa cells (Jewgenow *et al* 1997). These results showed that the fertile life span of the oocytes can be prolonged and maintained at least 12 hours in transportation medium at 5°C, thus can be used for *in vitro* embryo production..

Conclusion

Preservation of ovaries at 5°C can maintain the oocytes development capacity. Prolong preservation until 12 hours decreased the oocytes development capacity.

References

- Arellano J, Illera MJ, Lorenzob P, Sanchez J. 1993. Epidermal growth factor enhances porcine oocytes maturation *in vitro* in the absence of follicular fluid or hormone. *Theriogenology*, 39:180 (Abstr).
- Arlotto T, Schwartz JL, First NL, Leibfried-Rutledge ML. 1996. Aspect of follicle and oocytes stage that affect *in vitro* maturation and development of bovine oocytes. *Theriogenology*, 45:943-956.
- Brackett BG, Bousquet D, Boice ML, Donawick WS, Evans JF, Dressel MA. 1982. Normal development following *in vitro* fertilization in the cow. *Biol. Reprod.*, 27:147-158.
- Byrd SR, Flores-Foxworth G, Applewhite AA, Westhusin ME. 1997. *In vitro* maturation of

- ovine oocytes in a portable incubator. *Theriogenology*, 47:153-163.
- Chang MC. 1959. Fertilization of rabbit ova *in vitro*. *Nature*, 184:466-467.
- Cheng WTK, Moor RM, Polge C. 1986. *In vitro* fertilization of pig and sheep oocytes matured *in vivo* and *in vitro*. *Theriogenology*, 25:146 (Abstr).
- Chian RC, Niwa K, Sirard MA. 1994. Effect of cumulus cells on male pronucleus formation and subsequent early development of bovine oocytes *in vitro*. *Theriogenology*, 41:1499-1508.
- Cox JF, Hormazabal J, Santa Maria A. 1993. Effect of the cumulus on *in vitro* fertilization of bovine matured oocytes. *Theriogenology*, 40:1259-1267.
- Crozet N, Ahmed-Ali M, Dubos MP. 1995. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. *J. Reprod. Fertil.*, 103:192 (Abstr).
- De Smedt V, Crozet N, Ahmed-Ali M, Martino A, Cognie Y. 1992. *In vitro* maturation and fertilization of goat oocytes. *Theriogenology*, 37:1049-1060.
- I. Djuwita, Rusiyantono R, Mohamad K, Purwantara B, Sukra Y. 1998. Pengaruh serum homolog dan heterolog terhadap proses pematangan dan pembuahan oosit di dalam medium biakan *in vitro*. *Media Veteriner*, 5(1):7-10.
- Hafez ESE. 1987. *Reproduction in Farm Animals*. Lea and Febiger. Philadelphia. USA. 649pp.
- Hanada A. 1985. *In vitro* fertilization in goats. *Jpn. J. Anim. Reprod.* 31:21-26.
- Hawk HW, Nel ND, Waterman RA, Wall RJ. 1992. Investigation of means to improve fertilization rates in *in vitro* matured/ *in vitro* fertilized bovine oocytes. *Theriogenology*, 38:989-998.
- Eriani K, Boediono A, Djuwita I, Sumarsono SH, Al-Azhar. 2008. Development of domestic cat embryo produced by preserved sperms. *Hayati Journal of Biosciences*, 15 (4):155-160.
- LeGal F. 1996. *In vitro* maturation and fertilization of goat oocytes frozen at germinal vesicle stage. *Theriogenology*, 45:1177-1185.
- Martino A, Mogas T, Palomo MJ, Paramio MT. 1994. Meiotic competence of prepubertal goat oocytes. *Theriogenology*, 41:969-980.
- Martino A, Mogas T, Palomo MJ, Paramio MT. 1995. *In vitro* maturation and fertilization of prepubertal goat oocytes. *Theriogenology*, 43:473-485.
- Nagai T. 1996. *In vitro* maturation and fertilization of pig oocytes. *Anim. Reprod. Sci.*, 442:153-163.
- Palmer E, Bezard J, Magistrini M. 1990. *In vitro* fertilization in the horse: A retrospective study. *Proc. Intern'l Sym. Equine Reprod. Deauville, France*, p. 124-125.
- Pawshe CH, Paalanisamy A, Tanej M, Jain SK, Totey SM. 1996. Comparison of various maturation treatments on *in vitro* maturation of goat oocytes and their early embryonic development and cell number. *Theriogenology*, 46:971-982.
- Pawshe CH, Appa Rao KBC, Jain SK, Totey SM. 1994. Biochemical studies on goat oocytes: timing of nuclear progression, effect of protein inhibitor and pattern of polypeptide synthesis during *in vitro* maturation. *Theriogenology*, 42:307-320.
- Paramio MT. 2010. *In vivo* and *in vitro* embryo production in goats. *Small Ruminants Research* 89:144-148.
- Jewgenow K, Wood TC, Wildt DE. 1997. DNA degradation in mural granulosa cells of non- and slightly atretic follicles of

- fresh and cold-stored domestic cat ovaries. *Mol Reprod Dev* 48:350-355.
- Pinyopummintr T, Bavister BD. 1995. Optimum gas atmosphere for *in vitro* maturation and *in vitro* fertilization of bovine oocytes. *Theriogenology*, 44:471-477.
- Pope CE. 2000. Embryo technology in conservation efforts for endangered felids. *Theriogenology* 53:163-174.
- Otoi T, Murakami M, Ooka A, karja NW, T Suzuki. 2001. Effects of size and storage temperature on meiotic competence of domestic cat oocytes. *Vet Res* 148:116-118.
- Santos RR, Silva JRV, Costa SLF, et al. 2002. Effect of 0.9% saline solution and phosphate buffer saline at different temperatures and incubation times on the morphology of goat preantral follicles. *Braz J. Vet. Anim Sci*, 39(5):254-159.
- Schellander K, Fuhrer F, Brackett BG. 1990. *In vitro* fertilization and cleavage of bovine oocytes matured in medium supplemented with estrous cow serum. *Theriogenology*, 33:476-485.
- Shioya Y, Kuwayama M, Fukushima M, Iwasaki S, Hanada A. 1988. *In vitro* fertilization and cleavage capability of ovine follicular oocytes classified by cumulus cells and matured *in vitro*. *Theriogenology*, 30:489-496.
- Voekel SA, Hu YX. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology*, 37:1117-1131.
- Younis AI, Keskinetepe L, Mackie K, Brackett BG. 1992. *In vitro* maturation and fertilization of toggenburg goat oocyte. *Theriogenology*, 37:330 (Abstr).