Japanese Journal of Veterinary Research 61(4): 149–154, 2013

NOTE

In vitro maturation system for individual culture of bovine oocytes using micro-volume multi-well plate

Masashi Nagano, Sung-Sik Kang, Keisuke Koyama, Weiping Huang, Yojiro Yanagawa and Yoshiyuki Takahashi

Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Received for publication, May 29, 2013; accepted, July 18, 2013

Abstract

As a preliminary study for the development of individual *in vitro* maturation (IVM) culture of bovine oocytes, a multi-well (MW) plate was used. Maturation, fertilization and development to blastocysts were examined and compared with those of IVM oocytes cultured in 50-μl droplets in groups and in 10-μl droplets individually. The maturation rates were similar in all experimental groups. Normal fertilization rates in MW and 50-μl droplets were similar, but lower in 10-μl droplets ($p < 0.01$). The blastocyst rate in 10-μl droplets tended to be lower than those in MW ($p = 0.15$) and 50-μl droplets ($p = 0.19$). These results indicate that an IVM system using MW supports the acquisition of developmental competence by bovine oocytes the same as conventional group IVM culture.

Key Words: IVM, micro-well, oocyte

The *in vitro* production (IVP) of embryos is common technology in bovine artificial reproduction, and embryos are co-cultured with several embryos in order to obtain better development to the blastocyst stage[9,16]. The current technology of ovum pick up (OPU) and IVP of bovine embryos is intended to enable the harvesting of immature oocytes from genetically superior living cows by ultrasound-guided transvaginal aspiration, followed by *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) until embryos have reached a transferable stage (morula or blastocyst). However, it was reported that OPU yielded small numbers of oocytes (7.8 oocytes, mean) and transferable embryos (1.1 embryos, mean) per donor[12]. Therefore, low-density culture systems for bovine oocytes and embryos are required for efficient IVP of transferable embryos. Few studies have reported successful individual culture from immature oocytes to the blastocyst stage and, in most cases, a marked decrease in blastocyst development was observed[4,10,17]; however, the well of the well (WOW) culture system overcame the problem of a decrease in blastocyst development by culturing embryos in micro-wells individually[28]. This method involves the formation of small wells in four-well dishes by melting the

*Corresponding author: M. Nagano, Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku Kita 18 Nishi 9, Sapporo 060–0818, Japan Phone: +81–11–706–5232. Fax: +81–11–706–5232. E-mail: mnaga@vetmed.hokudai.ac.jp

bottom with heated steel rods[28]. Recently, commercially available WOW dishes with 25 micro-wells (diameter 290 μm, depth 170 μm) were used for bovine embryo culture and the results showed comparable blastocyst development to that in group culture[23,24]. However, in these studies, IVM of oocytes was performed by group culture. Some reports have been published about the effect of individual IVM culture on the development to blastocysts; however, the obtained results were not consistent, with some indicating low[2,8] and others no effects on the blastocyst rate[4,29]. If we can obtain IVM oocytes with high developmental competence consistently by individual or small group culture, the outcome of the OPU method will be improved. Moreover, the establishment of an individual IVM system of oocytes would greatly facilitate basic studies that require the identification of individual oocytes, for example, in study of the relationship between oocyte developmental competence and follicle parameters. There are no reported studies that used a micro-well culture system for IVM as used for embryo development. WOW is too small to introduce cumulus-oocyte complexes (COCs) for IVM culture because cumulus investments expand to more than 300 μm in diameter during IVM culture[25]. Therefore, in the present study, as a preliminary investigation, we used a micro-volume (10 μl) multi-well plate (Nunc 163118 MINI TRAYS, Thermo Fisher Scientific, Roskilde, Denmark) as an IVM culture device, and examined the effect of this IVM system on nuclear maturation, fertilization and development to blastocysts of oocytes; we also compared this approach to conventional group culture and individual culture in droplets.

The ovaries of cattle (mostly of the Holstein breed) were obtained at a local abattoir and delivered to the laboratory in plastic bags at 20°C. The COCs were collected from small antral follicles (2–8 mm in diameter) as described previously[15]. The COCs with brown-colored ooplasm[15] were washed twice in HEPES-buffered Tyrode's medium (TALP-HEPES)[1] supplemented with 3 mg/ml bovine serum albumin (BSA, fraction V, Sigma-Aldrich, St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma-Aldrich) and 50 μg/ml gentamicin sulfate (Sigma-Aldrich).

The COCs were subjected to IVM culture individually using the micro-volume multi-well plate (MW; Fig. 1): the MW had 60 wells in the plate and the volume of each well was 10 μl. The MW plate was filled with 6 ml of maturation medium and the COCs were cultured individually in a well of the plate for 22 h at 39°C in a humidified atmosphere of 5% CO_2 in air. Before IVM culture, the COCs were washed once 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, Sigma-Aldrich), 1 μg/ml estradiol-17β (Sigma-Aldrich), 0.2 mM sodium pyruvate and 50 μg/ml gentamicin sulfate. As a control of the group IVM culture, the COCs were cultured in our conventional 50-μl droplets[15,27] (10–11 COCs/droplet, group control). As a control of the individual IVM culture, 10-μl droplet culture (1

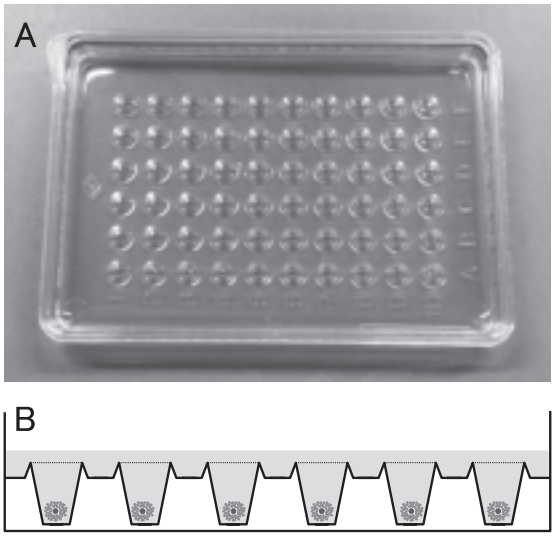

Fig. 1. Micro-volume multi-well (MW) plate used for *in vitro* maturational culture of bovine oocytes. A) MW plate filled with 6 ml of maturation medium. B) Cumulus-oocyte complexes in a well of MW plate. Volume under the dashed line is approximately 10 μl.

COC/droplet, individual control) was carried out because the comparable blastocyst yield with group culture was provided in a previous study[4]. To evaluate the maturational ability of oocytes, some COCs derived from IVM culture in MW, 10- or 50-μl droplet 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[14].

Around 10–15 COCs matured by the different IVM systems were co-incubated with motile sperm (5×10^6 sperm/ml) in a 100-μl droplet of modified Brackett and Oliphant (mBO) medium for 18 h at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (Day 0) as described previously[3]. At the end of the IVF culture, presumptive zygotes were transferred to 15-ml tubes and vortexed for 4 min in 0.5 ml of TALP-HEPES to strip cumulus investments. To evaluate the fertilizability, some presumptive zygotes derived from different IVM systems were fixed and their fertilization rates were examined[15].

After removal of the cumulus cells, presumptive zygotes were washed three times with culture medium and cultured for 150–152 h as groups of 28–30 embryos in a 30-μl droplet of culture medium covered with paraffin oil at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2, as described previously[27]. The culture medium was a modified synthetic oviduct fluid[27] with 3 mg/ml BSA instead of polyvinyl alcohol. To evaluate the developmental competence of oocytes matured by the different IVM systems, cleavage and development to blastocysts were assessed at 48 h (Day 2) and 168–170 h (Day 7) after insemination, respectively. All blastocysts were fixed and the number of cells was counted using an air-drying method reported previously[26].

For statistical analysis, all data were analyzed by one-way ANOVA followed by Tukey-Kramer's honestly significant difference test. All analyses were performed using software (JMP version 8.0, SAS Institute Inc., Cary, NC, USA).

The nuclear maturation (metaphase II) rate in MW culture was similar to those in the others ($p > 0.05$, Table 1). Oocytes matured in MW showed high normal fertilization, having two pronuclei, as well as those matured by group culture in 50-μl droplets (Table 2). On the other hand, the normal fertilization rate was lower and the percentage of enlarged sperm heads was higher in oocytes matured in 10-μl droplets than in the others ($p < 0.05$), although the total penetration rates were similar in all experimental groups. As shown in Table 3, there were no differences between cleavage rates, developmental rates to the blastocyst stage and cell numbers in blastocysts between MW and 50-μl droplets ($p = 0.99$). However, the developmental rate to the blastocyst stage of oocytes matured in 10-μl droplets tended to be lower than those matured in MW ($p = 0.15$) and in 50-μl droplets ($p = 0.19$).

In the present study, the percentages of nuclear maturation, fertilization and development

Table 1. Nuclear status after maturational culture of bovine oocytes

Maturational culture		No. of oocytes (Replicates)	No. (%) of oocytes at each nuclear status[a]		
			GVBD-MI	AI-TI	MII
Individual	Multi-well plate	61 (5)	7.1 ± 8.4	2.0 ± 4.5	90.9 ± 10.2
	10-μl droplet	49 (5)	10.2 ± 0.5	0	89.7 ± 0.5
Group[b]	50-μl droplet	52 (5)	7.0 ± 8.4	0	92.0 ± 8.4

Values are means ± SD.
[a] GVBD-MI, germinal vesicle breakdown or metaphase I; AI-TI, anaphase I or telophase I; MII, metaphase II.
[b] Around 10 oocytes were cultured in a 50-μl droplet.

Table 2. *In vitro* fertilization of bovine oocytes matured using different maturational culture systems

Maturational culture		No. of oocytes (Replicates)	No. (%) of oocytes[a] with			Total Penetration (%)
			2PN	ESH	Polyspermy	
Individual	Multi-well plate	77 (5)	79.5 ± 13.2[b]	8.9 ± 9.5[b]	4.7 ± 2.1	91.4 ± 7.9
	10-μl droplet	72 (5)	43.9 ± 11.6[c]	34.2 ± 14.7[c]	1.3 ± 3.0	79.4 ± 19.4
Group	50-μl droplet	72 (5)	77.3 ± 15.3[b]	5.3 ± 7.7[b]	7.3 ± 10.1	90.0 ± 14.1

Values are means ± SD.
[a]2PN, two pronuclei; ESH, enlarged sperm head with anaphase I or telophase I; Polyspermy, penetrated with more than two sperm.
[b,c]Values with different superscripts differ significantly ($P < 0.01$).

Table 3. *In vitro* development of embryos derived from oocytes matured using different maturational culture systems

Maturational culture		No. of oocytes (Replicates)	% of cleavage	%[a] of blastocysts	Cell no. of blastocysts (Ranges)
Individual	Multi-well plate	116 (4)	81.9 ± 4.2	43.4 ± 7.6	160.2 ± 19.5 (66–288)
	10-μl droplet	120 (4)	80.0 ± 2.7	30.3 ± 9.9	171.1 ± 13.7 (81–331)
Group	50-μl droplet	113 (4)	83.1 ± 4.9	42.4 ± 9.2	165.2 ± 35.1 (62–320)

Values are means ± SD.
[a]Based on numbers of cleaved oocytes.

to the blastocyst stage of bovine oocytes matured individually in a micro-well culture system were similar to those in the conventional group culture system. This result indicates that IVM culture in MW seems to have no detrimental effects on bovine oocyte competence for *in vitro* development. On the other hand, normal fertilization and development to the blastocyst stage of oocytes cultured individually in 10-μl droplets tended to be low compared with those in MW and in 50-μl droplets. A possible reason for this result is the oil covering of droplets because some compounds in medium, such as steroid hormones, were absorbed by oil[13,18,21]. Progesterone secreted by cumulus cells during IVM facilitated oocyte maturation and cumulus expansion in porcine oocytes[22,30], and was also shown to be important for oocyte maturation in cattle[7]. It stimulates cumulus expansion and the production of glycosaminoglycans (GAGs) simultaneously[19,22], and the amount of GAGs was shown to be increased during IVM culture[5]. In our IVF system without GAG addition, sperm capacitation mainly depended on the amount of GAGs produced by cumulus cells[5,20]. Since a 10-μl droplet has a larger surface area/medium volume than a 50-μl droplet, the oil covering may absorb a large amount of progesterone compared with other IVM systems, resulting in inadequate production of GAGs. Thus, sperm capacitation and sperm penetration to oocytes matured in a 10-μl droplet seem to be delayed compared with those in MW or a 50-μl droplet. Delayed sperm penetration to oocytes may be the cause of the low rate of development to blastocysts in the 10-μl droplet group because fertilization did not occur at the appropriate timing in oocytes that maintained high developmental competence[6]. However, an individual IVM system using MW does not have an oil covering, and oocyte-secreted factors essential for oocyte acquisition of developmental competence[11] may accumulate in

the bottom of the micro-well, as suggested for the WOW culture system[23,24,28].

In conclusion, our preliminary results indicate that the IVM system using a micro-volume micro-well supported the acquisition of developmental competence by bovine oocytes the same as in the conventional group IVM system, and was efficient as a distinguishable culture of individual oocytes compared with the culture system using small droplets. In further study, we should transfer the blastocysts derived from oocytes matured in MW and determine their competence to develop to term.

Acknowledgments

This study was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to M. Nagano (No. 25450441). We thank the staff of Genetics Hokkaido for the donation of frozen bull sperm.

References

1) Bavister, B. D., Leibfried, M. L. and Lieberman, G. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol. Reprod.*, **28**: 235–247.
2) Blondin, P. and Sirard, M. A. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.*, **41**: 54–62.
3) Brackett, B. G. and Oliphant, G. 1975. Capacitation of rabbit spermatozoa in vitro. *Biol. Reprod.*, **12**: 260–274.
4) Carolan, C., Lonergan, P., Khatir, H. and Mermillod, P. 1996. In vitro production of bovine embryos using individual oocytes. *Mol. Reprod. Dev.*, **45**: 145–150.
5) Chen, L., Wert, S. E., Hendrix, E. M., Russell, P. T., Cannon, M. and Larsen, W. J. 1990. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol. Reprod. Dev.*, **26**: 236–247.
6) Dominko, T. and First, N. L. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol. Reprod. Dev.*, **47**: 456–467.
7) Fair, T. and Lonergan, P. 2012. The role of progesterone in oocyte acquisition of developmental competence. *Reprod. Domest. Anim.*, **47 Suppl 4**: 142–147.
8) Feng, W. G., Sui, H. S., Han, Z. B., Chang, Z. L., Zhou, P., Liu, D. J., Bao, S. and Tan, J. H. 2007. Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. *Theriogenology*, **67**: 1339–1350.
9) Ferry, L., Mermillod, P., Massip, A. and Dessy, F. 1994. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology*, **42**: 445–453.
10) Hagemann, L. J., Weilert, L. L., Beaumont, S. E. and Tervit, H. R. 1998. Development of bovine embryos in single in vitro production (sIVP) systems. *Mol. Reprod. Dev.*, **51**: 143–147.
11) Hussein, T. S., Thompson, J. G. and Gilchrist, R. B. 2006. Oocyte-secreted factors enhance oocyte developmental competence. *Dev. Biol.*, **296**: 514–521.
12) Merton, J. S., Ask, B., Onkundi, D. C., Mullaart, E., Colenbrander, B. and Nielen, M. 2009. Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology*, **72**: 885–893.
13) Miller, K. F. and Pursel, V. G. 1987. Absorption of compounds in medium by the oil covering microdrop cultures. *Gamete Res.*, **17**: 57–61.
14) Nagano, M., Katagiri, S. and Takahashi, Y. 2006. ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. *Zygote*, **14**: 299–304.
15) Nagano, M., Katagiri, S. and Takahashi, Y. 2006. Relationship between bovine oocyte morphology and in vitro developmental potential. *Zygote*, **14**: 53–61.
16) O'Doherty, E. M., Wade, M. G., Hill, J. L. and Boland, M. P. 1997. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology*, **48**: 161–169.
17) Oyamada, T., Iwayama, H. and Fukui, Y. 2004. Additional effect of epidermal growth

factor during in vitro maturation for individual bovine oocytes using a chemically defined medium. *Zygote*, **12**: 143–150.
18) Reinsberg, J., Ackermann, D. and van der Ven, H. 2004. Pitfalls in assessment of progesterone production by granulosa cells cultured in contact with silicone rubber or paraffin oil. *Arch. Gynecol. Obstet.*, **270**: 174–178.
19) Salhab, M., Tosca, L., Cabau, C., Papillier, P., Perreau, C., Dupont, J., Mermillod, P. and Uzbekova, S. 2011. Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. *Theriogenology*, **75**: 90–104.
20) Salustri, A., Yanagishita, M., Underhill, C. B., Laurent, T. C. and Hascall, V. C. 1992. Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev. Biol.*, **151**: 541–551.
21) Shimada, M., Kawano, N. and Terada, T. 2002. Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of in vitro maturation media. *Reproduction*, **124**: 557–564.
22) Shimada, M., Nishibori, M., Yamashita, Y., Ito, J., Mori, T. and Richards, J. S. 2004. Down-regulated expression of A disintegrin and metalloproteinase with thrombospondin-like repeats-1 by progesterone receptor antagonist is associated with impaired expansion of porcine cumulus-oocyte complexes. *Endocrinology*, **145**: 4603–4614.
23) Sugimura, S., Akai, T., Hashiyada, Y., Aikawa, Y., Ohtake, M., Matsuda, H., Kobayashi, S., Kobayashi, E., Konishi, K. and Imai, K. 2013. Effect of Embryo Density on In Vitro Development and Gene Expression in Bovine In Vitro-fertilized Embryos Cultured in a Microwell System. *J. Reprod. Dev.*, **59**: 115–122.
24) Sugimura, S., Akai, T., Somfai, T., Hirayama, M., Aikawa, Y., Ohtake, M., Hattori, H., Kobayashi, S., Hashiyada, Y., Konishi, K. and Imai, K. 2010. Time-lapse cinematography-compatible polystyrene-based microwell culture system: a novel tool for tracking the development of individual bovine embryos. *Biol. Reprod.*, **83**: 970–978.
25) Sutton-McDowall, M. L., Gilchrist, R. B. and Thompson, J. G. 2004. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. *Reproduction*, **128**: 313–319.
26) Takahashi, Y. and First, N. L. 1992. In vitro fertilization of bovine oocytes in the presence of theophylline. *Anim. Reprod. Sci.*, **37**: 963–978.
27) Takahashi, Y., Hishinuma, M., Matsui, M., Tanaka, H. and Kanagawa, H. 1996. Development of in vitro matured/fertilized bovine embryos in a chemically defined medium: influence of oxygen concentration in the gas atmosphere. *J. Vet. Med. Sci.*, **58**: 897–902.
28) Vajta, G., Peura, T. T., Holm, P., Paldi, A., Greve, T., Trounson, A. O. and Callesen, H. 2000. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol. Reprod. Dev.*, **55**: 256–264.
29) Ward, F. A., Lonergan, P., Enright, B. P. and Boland, M. P. 2000. Factors affecting recovery and quality of oocytes for bovine embryo production in vitro using ovum pick-up technology. *Theriogenology*, **54**: 433–446.
30) Yamashita, Y., Kawashima, I., Gunji, Y., Hishinuma, M. and Shimada, M. 2010. Progesterone is essential for maintenance of Tace/Adam17 mRNA expression, but not EGF-like factor, in cumulus cells, which enhances the EGF receptor signaling pathway during in vitro maturation of porcine COCs. *J. Reprod. Dev.*, **56**: 315–323.