Original Article

Title: The efficacy of well of the well (WOW) culture system on development of bovine embryos in a small group and the effect of number of adjacent embryos on their development

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Running headline: Bovine embryo culture in WOW

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Summary

The aim of the present study was to clarify the efficacy of the WOW culture system for a small number of embryos and the effect of number of adjacent embryos in a WOW dish on blastocyst development. In conventional droplet culture, embryos in the small-number group (5-6 embryos/droplet) showed low blastocyst development compared with a control group (25-26 embryos/droplet). However, small and large numbers of embryos (5-6 and 25 embryos, respectively) in a WOW dish showed no significant differences in cleavage, blastocyst rates, and mean cell number in blastocysts compared to the control group (25-30 embryos/droplet). In addition, the number of adjacent embryos in a WOW dish did not affect the development to blastocysts and cell number in blastocysts. In conclusion, a WOW dish can provide high and stable blastocyst development in small group culture wherever embryos are placed in micro-wells of the WOW dish.

Keywords: Bovine, Embryonic Development, Droplet, IVC, WOW

Introduction

During recent decades, assisted reproductive technologies in cattle have achieved considerable advances, such as a combination of *in vitro* embryo production (IVP) and ovum pick up (OPU). An average of 4 to 6 available oocytes from a single cow are collected by OPU or a single slaughtered valuable oocyte donor cow (Hasler, 1998; Merton *et al*., 2009). However, most laboratories currently culture up to 20 embryos in a 20- to 50-µl droplet or up to 50 embryos in 400 to 500 µl in a well *in vitro* (Krisher and Wheeler, 2010; Vajta *et al*., 2010) to obtain transferrable embryos, and small groups of 1 to 10 embryos in a droplet or a well have been shown to have low blastocyst rate and quality compared with large groups of 20 to 25 embryos (Donnay *et al*., 1997; Ikeda *et al*., 2000; Nagao *et al*., 2008; Senatore *et al*., 2010; Ward *et al*., 2000; Vajta *et al*., 2000). Therefore, a culture system for a small group of embryos is required to improve blastocyst yield. It was reported that, if embryos were cultured in a custom-made micro-well in a large well (WOW) (Vajta *et al*., 2000; Matoba *et al*., 2010) and in a commercially available polystyrene-based WOW (25 micro-wells) (Sugimura *et al*., 2010), the blastocyst rate was significantly higher than that of droplet culture. In addition, Sugimura *et al*. (2013) demonstrated that the blastocyst rate in small group culture (5 embryos) in a WOW was higher than in a droplet.

Gopichandran & Leese (2006) reported that embryos surrounded by 3 or 8 adjacent embryos showed low blastocyst development compared with those surrounded by 5 adjacent embryos when bovine embryos were cultured in a group in a droplet. It was also reported that the number of adjacent embryos (3, 5, or 8) did not affect blastocyst development in a custom-made WOW dish (Matoba *et al*., 2010). This discrepancy may have been caused by the large depth (500 µm) of micro-wells of custom-made

WOW dishes (Matoba *et al*., 2010) because the overall diameter of embryos ranges from 150 to 190 μm (Linder & Wright, 1983). It is thought that usage of a commercially available WOW dish is preferable to achieve a high and stable blastocyst rate for culturing a small number of embryos. However, an embryo in a micro-well (169 μm in depth) in the WOW dish developed by Sugimura *et al.* (2010) can be affected by factors outside of the micro-well. Thus, we should examine the effect of the number of adjacent embryos on blastocyst production by a commercially available WOW dish.

In the present study, we examined the effects of the total number of embryos (5 vs. 25 embryos) and the number of adjacent embryos (0, 3, 5, and 8 embryos) in a WOW dish on blastocyst development.

Materials and Methods

All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation (IVM) of bovine oocytes was performed as described previously (Takahashi *et al*., 1996). In brief, cumulus-oocyte complexes (COCs) aspirated from follicles (2 to 8 mm in diameter) of slaughterhouse-derived ovaries were cultured for 22 h in a droplet (about 10 COCs/50 µl) of maturation medium under a humidified atmosphere of 5% CO_2 in air at 39˚C. Maturation medium consisted of HEPES-buffered TCM-199 (Invitrogen, Grand Island, NY, USA) supplemented with 10% FCS (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/ml follicle-stimulating hormone, 1 µg/ml estradiol-17β, and 50 µg/ml gentamicin sulfate.

In vitro fertilization (IVF) was conducted according to a procedure described previously (Takahashi & Kanagawa, 1998). Briefly, after the thawing of frozen semen, motile sperm were separated using a Percoll gradient (45 and 90%). COCs were co-incubated with motile sperm (5 x 10^6 cells/ml) in droplets (10-13 COCs/100 µl) of modified Brackett and Oliphant's isotonic medium (Takahashi & First, 1992) containing 3 mg/ml fatty acid-free BSA and 2.5 mM theophylline for 18 h at 39˚C under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

In vitro culture of presumptive zygotes

In vitro culture (IVC) of presumptive zygotes was performed using the procedures that were basically the same as described previously (Takahashi & Kanagawa, 1998). After co-incubation with sperm, presumptive zygotes freed from cumulus cells by vortexing were washed 3 times and cultured for 150 h in droplets or a WOW dish using

a modified synthetic oviduct fluid medium containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 non-essential amino acids for minimum essential medium, 10 µg/ml insulin, 5 mM glycine, 5 mM taurine and 1 mM glucose (Takahashi & Kanagawa, 1998), and added 3 mg/ml fatty acid-free BSA instead of polyvinyl alcohol at 39˚C under 5% CO_2, 5% O_2 and 90% N_2. Polystyrene-based WOW dishes that have 25 micro-wells (5 x 5 micro-wells with 170-µm depth, 290-µm diameter, and 400-µm distance between them; Dai Nippon Printing Co. Ltd., Tokyo, Japan) were prepared as described previously (Sugimura *et al*., 2010). In brief, 125 µl of culture media were placed within the circular wall of WOW dish containing micro-wells and covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan). Twenty-five embryos were placed individually in each micro-well in a WOW dish. For the culturing of 5 embryos, 4 embryos were placed in the micro-well of 4 corners and the remaining one was put into the center micro-well in a WOW dish (no adjacent embryo).

To examine the effect of embryo number in a droplet and a WOW dish on the development to blastocysts, small or large numbers of embryos (5-6 or 25-26, respectively) inseminated by bull A sperm were cultured in a 40-µl droplet. Another large number of embryos inseminated by bull B sperm were cultured in a droplet (25-30 embryos/30 µl), and 5 or 25 embryos were cultured in a WOW. The effect of the number of adjacent embryos in a WOW was examined using data of 5- (0 adjacent embryos; Fig. 1A) and 25-embryo culture with different numbers of adjacent embryos (3, 5, and 8 embryos; Fig. 1B). Cleavage and blastocyst rates were assessed after 30 h and 150 h of IVC, respectively. All embryos that developed to blastocysts were subjected to counting of their cell numbers using an air-drying method (Takahashi & First, 1992).

Statistical analysis

The frequencies of cleavage and development to blastocysts, and cell numbers in blastocysts were compared by one-way ANOVA followed by Tukey–Kramer's HSD as a *post hoc* test. Proportions of blastocysts among the embryo groups placed in different positions of a WOW dish (different numbers of adjacent embryos) were compared by Chi-square test. All analysis was performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC).

Results

As shown in Table 1, when embryos were cultured in a droplet, cleavage and blastocyst rates of the large-number group (25-26 embryos/droplet) tended to be higher than those of the small-number group (5-6 embryos/droplet) ($p = 0.14$ and 0.07, respectively). In addition, the total cell number in blastocysts of the large-number group was significantly higher than that of the small-number group ($p < 0.01$). When embryos were cultured in WOW, blastocyst rates were high regardless of embryo number in the WOW dish and similar to that of the large-number group in a droplet. Furthermore, the number of adjacent embryos in a WOW dish did not affect the development to blastocysts and total cell numbers in blastocysts, as shown in Table 2.

Discussion

Culturing small numbers of embryos in a droplet reduced the blastocyst rate and the quality of blastocysts (mean cell numbers in blastocysts) compared with those for embryos cultured with large numbers of embryos in a droplet (Nagao *et al.*, 2008;

Senatore *et al*., 2010). In the present study, there was no reduction in blastocyst rate and quality in a WOW dish. This result is in agreement with previous findings that the development of embryos to blastocysts was independent of the total number of embryos in a WOW dish (Sugimura *et al*., 2012). We speculated that diffusible factors like autocrine/paracrine growth factors released by embryos can be diffused in a droplet and influence the growth of their adjacent embryos (Stokes *et al*., 2005). A small amount of autocrine/paracrine factors may be secreted by a small number of embryos, would be easily diluted in a droplet, and would show few effects on embryonic development. However, in this WOW dish, diffusible factors secreted by individual embryos probably accumulated in a micro-well, which may provide a suitable microenvironment for their development, as suggested in a previous study (Swain and Smith, 2011). Moreover, adjacent embryos in a WOW dish also did not affect blastocyst development and mean cell numbers in blastocysts in the present study, as previously described (Matoba *et al*., 2010), even though the depth of the micro-well was different.

In conclusion, the polystyrene-based WOW dish used in this study is effective for individual embryo culture of small groups, and there is no reduction in embryo developments, regardless of the number of adjacent embryos (position of embryos) in micro-wells of the WOW.

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Legends

Fig.1 Different numbers of adjacent embryos in 25 micro-wells in a WOW dish

A: Embryo with no adjacent embryos.

B: a) Embryo with 3 adjacent embryos. b) Embryo with 5 adjacent embryos. c) Embryo with 8 adjacent embryos.

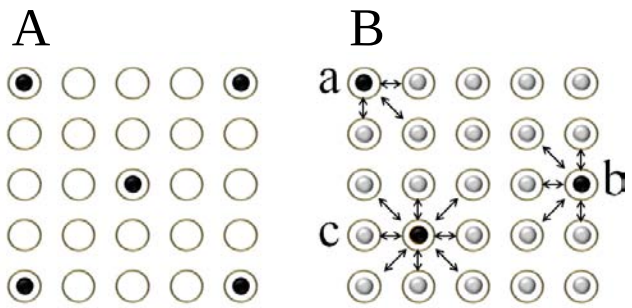

Fig. 1

Table 1 Effect of embryo number in a droplet and a WOW dish on embryonic development

Bull	Culture system	No. of embryos cultured	No. of embryos (replicates)	% cleaved	% blastocysts	Mean cell numbers in blastocysts (n)
A	droplet	25-26	102 (4)	85.4 ± 7.8	38.3 ± 1.7	186.1 ± 63.5 [a] (39)
	droplet	5-6	97 (4)	71.0 ± 15.0	28.8 ± 8.5	142.1± 64.1 [b] (28)
B	droplet	25-30	197 (7)	87.8 ± 4.4	41.2 ± 8.4	166.0 ± 59.9 (82)
	WOW	5	75 (3)	89.3 ± 10.1	41.3 ± 12.2	147.0 ± 56.7 (31)
	WOW	25	100 (4)	82.0 ± 5.2	45.0 ± 8.9	151.9 ± 45.7 (45)

[a,b]Values (means ± SD) with different superscripts differ significantly ($p < 0.01$).

Table 2 Effect of number of adjacent embryos (position of embryos) in a WOW on the development of embryos to blastocysts and their cell numbers

No. of cultured embryos in a WOW	No. of adjacent embryos	No. of embryos	% blastocysts (n)	Mean cell numbers in blastocysts (n)
25	8	36	41.7 (15)	151.3 ± 44.1 (15)
	5	48	47.9 (23)	150.9 ± 46.8 (23)
	3	16	43.8 (7)	156.6 ± 52.1 (7)
5	0	75	41.3 (31)	147.0 ± 56.7 (31)

Data were pooled from 4 replicates of culture of 25 embryos and 3 replicates of culture of 5 embryos with WOW (bull B) in Table 1.

Cell numbers in blastocysts are means ± SD.