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Evaluation of different culture systems on the in vitro production of bovine embryos

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Abstract

The objective of this study was to determine the development potential and quality of in vitro produced bovine embryos cultured individually or in groups. After IVM and IVF, presumptive zygotes were cultured in groups or individually, either in drops or in the modified "well of the well" (mWOW) system. In Experiment 1, four culture systems were utilized: T1: drop in group (control); T2: mWOW in groups; T3: mWOW individually; and T4: drop individually. Cleavage and blastocyst rates at Days 6, 7 and 8 and total cell number of Day 6 blastocysts were similar ($P > 0.05$) for all treatments. However, in Day 7 blastocysts, total cell number was lower ($P < 0.05$) in embryos cultured individually in a small drop than those cultured in the mWOW. In Experiment 2, blastocysts of T1, T2 and T3 were allocated into two groups, control and vitrified. After warming, the vitrified embryos were cultured for 72 h. At 48 h, the development of the Days 6 and 7 embryos was similar ($P > 0.05$) for all treatments in the control group. For the vitrified embryos, lower hatching rates ($P < 0.05$) were observed in the T3 group. In conclusion, embryos cultured in groups in the mWOW system had the same blastocyst rates but better quality (measured by their survival after vitrification) than those cultured individually in the mWOW system.

Keywords: Bovine; Embryos; mWOW system; Vitrification

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1. Introduction

The in vitro production of bovine embryos has advanced remarkably in the past decades, especially after the incorporation of the ovum pick up technique (OPU), which allowed the commercial application of that technology. However, the percentage of blastocyst production and pregnancy rates were still lower than that obtained with in vivo-produced embryos.

Embryo development in vitro is influenced by a number of factors, such as co-culture with somatic cells [1], addition of anti-oxidants to the culture medium [2] and oxygen tension [3]. Another factor is the number of embryos present in the culture drop; embryo development was better when embryos were cultured in groups than when they were cultured individually [4–6]. The group effect in embryo development was probably due to the secretion of embryonic factors, which act in an autocrine–paracrine fashion [6].

However, in commercial IVP, it is not rare to have only one viable embryo from an oocyte donor that would require an individual culture system. In addition, it has an important role for research, since it allows the investigator to follow the development of individual embryos that have been subjected to some type of treatment, micromanipulation, or that originated from nuclear transfer. Therefore, the development of a system that allows the culture of many embryos, while maintaining the identity of individual embryos, has considerable appeal.

To overcome this problem, Vajta et al. [5] developed a new culture technique named well of the well (WOW), in which the embryos were cultured inside of a small cylindrical well with a ''V'' shape and a rounded bottom, made on a well of a four-well dish. The well is covered with culture medium, creating in each WOW a microenvironment for the embryo. This system allows the culture of embryos from different donors or treatment, maintaining individual identification of each structure. However, reports in the literature using this system are very scarce.

There are indications that the culture system and the composition of the medium, can affect embryo quality [7,8]. In fact, various studies have been shown that while the innate quality of the oocyte is the major factor that determines the blastocyst yield, the in vitro culture environment which the embryos are exposed after fertilization is the key determinant of the blastocyst quality [8–10]. Studies comparing culture conditions usually focus on the percentage of embryos that develop to the blastocyst stage. However, it is likely that in some cases, the effect of a given culture method may not manifest itself in the blastocyst yield, but in other parameters such as blastocyst quality [11]. Therefore, methods of assessing blastocyst quality such as cell counts, cryotolerance and gene expression patterns, should be associated with measurements of embryo development when different culture systems are compared.

The present study was carried out to assess the development and quality of in vitro produced embryos cultured individually or in groups. The effect of the culture system was evaluated by the blastocyst rate and by embryo viability after cryopreservation.

2. Materials and methods

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Oocyte recovery and in vitro maturation (IVM)

Ovaries from crossbreed cows (Bos indicus × Bos taurus) were collected just after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 μg/mL) at 30 °C. Cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm diameter follicles with an 18 G needle and pooled in a 15 mL conical tube. After sedimentation, the COCs were recovered and selected using a stereomicroscope. Only COCs with a homogenous cytoplasm and ≥3 layers of cumulus cells were used. The selected COCs were washed three times in maturation medium and transferred to a 200 μL drop of maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% of CO_2 in air. The maturation medium consisted of TCM-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (v/v), 24 IU/mL of LH, 10 μg/mL of FSH and antibiotics (100 IU/mL of penicillin and 50 μg/mL of streptomycin), with 5% CO_2 in air at 39 °C.

2.2. In vitro fertilization (IVF) and embryo culture

For IVF, COCs were separated in groups of 25 to 30, washed and transferred to a 200 μL drop of fertilization medium under silicone oil. The fertilization medium was TALP [12], supplemented with penicillamine (21.1 μM), hypotaurine (10.4 μM), epinephrine (1 μM) and heparin (10 μg/mL).

Frozen semen from one ejaculate of a single bull was used for all treatments and replicates. Motile spermatozoa were obtained by the Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient method [13] and were added into the fertilization drop in a final concentration of 1×10^6 spermatozoa/mL. Spermatozoa and oocytes were co-incubated for 22 h at 39 °C with 5% CO_2 in air.

After co-incubation, presumptive zygotes were washed in embryo culture medium and randomly distributed among the different culture treatments. In all experiments embryo culture took place in SOFaaci [14], supplemented with 2.77 mM of myo-inositol and 5% FCS in a humidified atmosphere of 5% of CO_2 in air at 39 °C. Embryos were evaluated on Day 2 post-insemination for cleavage, on Days 6 and 7 for blastocyst formation, and Day 8 for hatching.

2.3. Preparation of the modified well of well system (mWOW)

The wells were produced using a steel needle, similar to that described by Vajta et al. [5], with subsequent modifications. Briefly, a steel needle was heated on a gas flame from 3 to 5 s and then it was gently pressed in the bottom of a 60 mm × 15 mm petri dish (Corning Incorporated, New York, NY, USA), creating a small well with a V-shape (mWOW). The mWOWs were created either 1 cm apart in the individual culture dishes, or 1 mm apart in the group culture dishes.

After the mWOWs were produced, they were covered either individually with a 20 μL drop or in groups under a 400 μL drop of SOFaaci medium. In both systems, the culture medium was covered with silicone oil, and then the mWOWs were washed several times

with culture medium. After preparation, the dishes were kept in the incubator for 12 h and the medium changed again just before embryo culture.

2.4. Preparation of dishes for post-vitrification culture

Preparation of dishes for embryo culture used in this study was based upon in the original description, with subsequent modifications due to the differences in culture systems conditions. Dishes for culture of vitrified and control embryos were prepared 1 week before use. After preparation of the dishes as described above, the cumulus cells that were left in the fertilization drop were washed in SOFaaci medium and placed in the culture drops. The prepared dishes were kept in the incubator until use.

2.5. Total cell number determination

Total cell number of Days 6 and 7 blastocysts was determined according to the protocol describe by Vajta et al. [5]. Briefly, blastocysts were placed in sodium citrate solution (0.9%) for 5 to 10 min, then fixed in ethanol:acetic acid:water (3:2:1). After air drying the slides they were fixed again in ethanol:acetic acid (3:1) for 12 h. Fixed embryos were stained with a 2% acetic orcein for 10 min. The cell number was determined under a light microscope (100×) and the embryos were classified into three categories: <64 cells, from 64 to 120 cells and >120 cells.

2.6. Embryos vitrification and warming

Embryo vitrification was performed using open pull straws (OPS) (Minitub, Tiefenbach, Bayern, Germany) as described earlier [15]. Days 6 and 7 Grade I (according to the International Embryo Transfer Society Standards for in vivo embryos [16]) blastocysts were used. For vitrification, embryos were first incubated for 1 min in 10% ethylene glycol, 10% dimethylsulfoxide (DMSO) dissolved in holding medium (TCM-199 supplemented with 20% of FCS), and then transferred to a 20 μL drop of 20% of ethylene glycol, 20% DMSO dissolved in holding medium. Embryos were then placed in a small drop (2 μL) of the medium and with the capillar effect loaded into the straws, which were immediately submerged into liquid nitrogen. Warming was performed by immersing the straw end into a small drop of holding medium supplemented with 0.5 M of sucrose. Then the embryos were transfer for holding medium with 0.25 M of sucrose, and finally to the original holding medium. Subsequently, the embryos were placed in the culture dishes that had been previously prepared.

2.7. Experimental design

2.7.1. Experiment 1: in vitro production of bovine embryos in different culture systems

A total of 860 in vitro matured and in vitro fertilized oocytes were used in seven replicates. After fertilization, the presumptive zygotes were randomly allocated into four different culture systems as follows: (1) T1: control: embryos were allocated to a 400 μL

drop of SOFaaci medium; (2) T2: mWOW in group: embryos were cultured in the mWOWs being several mWOWs covered with 400 μL drop of SOFaaci medium; (3) T3: mWOW individually: embryos in the mWOWs being each mWOW covered with 20 μL drop of SOFaaci medium; and (4) T4: 20 μL drop individually: culture in a 20 μL drop of SOFaaci medium.

For all treatments embryo morphology was evaluated on Days 6, 7 and 8 to determine stage of development. Those at the blastocyst stage in the day of evaluation were removed from culture, fixed and stained to determine total cell number.

2.7.2. Experiment 2: viability of embryos cultured in different culture systems after vitrification

In this experiment, vitrification was used to evaluate the quality of embryos cultured individually or in groups. The culture systems used were the same as those described for Experiment 1, except for T4, which was eliminated based on the previous results.

For all treatments, embryos were evaluated on Days 6 and 7, and those that were at blastocyst stage were removed from the culture drop and, randomly allocated between the vitrified and control (non-vitrified) groups. During vitrification procedure, the embryos from the control group were kept out of the incubator, on the heating plate at 41 °C. Thirty minutes after vitrification, the embryos were warmed and transferred to culture medium. Embryos from the control and vitrified groups were morphologically evaluated 48 and 72 h after vitrification, for assessment of embryo development.

2.8. Statistical analysis

The data for total cell number were examined by one-way ANOVA, and the remain data by the chi-square analysis. All statistical analyses were conducted with the Jandel SigmaStat software (Version 2.0, 1995). For all analyses, a probability of 0.05 was considered significant.

3. Results

3.1. Experiment 1: in vitro production of bovine embryos in different culture systems

Cleavage and blastocyst rates were evaluated at Days 2, 6, 7, and 8 (Table 1). There were no differences ($P > 0.05$) among treatments for cleavage or blastocyst rates. Although total cell number of Day 6 blastocysts was similar ($P > 0.05$) among treatments (Table 2), for Day 7 embryos, the total cell number was lower ($P < 0.05$) in embryos cultured individually in a small drop (T4) than those cultured in the mWOW either individually or in a group.

The beneficial effect of the mWOW system became more evident when cell number data were grouped by type of culture. The cell number of Day 6 blastocysts cultured in mWOW or drop system were similar ($P > 0.05$), independent of the treatment (individual or group). However, the Day 7 blastocysts cultured in the mWOW system had a higher number of cells ($P = 0.004$) compared to the drop system (Table 3). When embryos from

Table 1
Cleavage (Day 2) and blastocyst rates at Days 6, 7 and 8 of embryos cultured in group, in 400 μL drop (T1) and in the mWOW with 400 μL (T2), or individually in mWOW with 20 μL drop (T3) and in a 20 μL drop (T4)

Treatment	*n*	Cleavage (%)	Blastocyst (%)		
			Day 6	Day 7	Day 8
T1	215	198 (92.1)	73 (33.9)	118 (54.8)	123 (57.2)
T2	215	194 (90.2)	58 (26.9)	105 (48.8)	111 (51.6)
T3	215	188 (87.4)	52 (24.2)	107 (49.7)	111 (51.6)
T4	215	185 (86.0)	55 (25.6)	96 (44.6)	104 (48.3)

Table 2
Total cell number of Days 6 and 7 embryos cultured in group, in 400 μL drop (T1) and in the mWOW with 400 μL (T2), or individually in mWOW with 20 μL drop (T3) and in a 20 μL drop (T4)

Treatment	Total cell number			
	n	Day 6	*n*	Day 7
T1	67	131.8 ± 51.1a	54	114.3 ± 42.3a, b
T2	61	128.7 ± 46.6a	61	132.3 ± 57.0a
T3	50	138.1 ± 49.4a	65	132.5 ± 54.0a
T4	57	117.5 ± 45.5a	47	101.5 ± 48.0b

Different letters (a and b) in the same column differ (means ± S.D.; $P < 0.05$).

the various treatments were categorized as having <64 cells, from 64 to 120 cells, and >120 cells, no differences ($P > 0.05$) were observed among treatments (Table 4).

3.2. Experiment 2: viability of embryos cultured in different culture systems after vitrification

In this experiment, the quality of embryos cultured in different culture systems was evaluated by embryo viability after vitrification. Hatching rates at 48 and 72 h after warming, for the control and vitrified Days 6 and 7 embryos are presented in Table 5. At 48 h after warming, there were no differences ($P > 0.05$) among treatments in the hatching rate for Days 6 and 7 embryos of the control group. Similar results were obtained in the vitrified group for the Day 7 embryos. However, when Day 6 embryos were vitrified, hatching rates were affected by the culture system; embryos cultured in the mWOW individually had a lower ($P < 0.05$) hatching rate than those cultured in the mWOW groups.

Table 3
Total cell number of Days 6 and 7 embryos cultured in drop system and in the mWOW system

System	Blastocysts cell number			
	n	Day 6	*n*	Day 7
mWOW	111	133.0 ± 47.9a	126	132.5 ± 55.3a
Drop	124	125.3 ± 48.9a	101	108.4 ± 45.3b

Different letters (a and b) in the same column differ (means ± S.D.; $P < 0.05$).

Table 4
Distribution of in vitro produced embryos cultured in 400 μL drop (T1) and in the mWOW with 400 μL (T2), or individually in mWOW with 20 μL drop (T3) and in a 20 μL drop (T4), according to classes of cell number (<64, 64–120 and >120 cells)

	Treatment	*n*	% Embryos and mean cell number ± S.D.					
			% Embryos	<64 cells	% Embryos	64–120 cells	% Embryos	>120 cells
Day 6	T1	67	5.6	42.0 ± 17.3	38.8	100.0 ± 16.0	55.6	153.0 ± 43.8
	T2	61	8.2	56.0 ± 10.9	39.4	97.2 ± 16.1	52.4	159.0 ± 31.4
	T3	50	10.0	49.0 ± 13.5	26.0	102.8 ± 12.3	64.0	160.5 ± 30.9
	T4	57	14.0	57.0 ± 6.7	45.6	96.9 ± 16.9	40.4	149.0 ± 30.1
Day 7	T1	54	11.2	47.8 ± 9.4	51.8	95.0 ± 16.6	37.0	147.5 ± 31.8
	T2	61	4.7	51.3 ± 5.7	44.3	89.0 ± 16.1	51.0	168.0 ± 45.8
	T3	65	4.6	54.6 ± 7.1	43.1	94.5 ± 14.7	52.3	162.0 ± 43.8
	T4	47	27.6	44.8 ± 11.9	38.4	99.0 ± 16.3	34.0	147.0 ± 32.2

Table 5
Hatching rate of Days 6 and 7 embryos produced in vitro cultured in group, 400 μL drop (T1) and in the mWOW with 400 μL (T2), or individually in mWOW with 20 μL drop (T3) at 48 and 72 h after warming

Treatment	Group	48 h				72 h			
		% Hatching				% Hatching			
		n	D6	*n*	D7	*n*	D6	*n*	D7
T1	Control	39	79.5a	36	83.3a	39	97.4a	36	88.8a
T2		34	70.6a	40	85.0a	34	88.2a, b	40	90.0a
T3		31	61.3a	40	80.0a	31	77.4b	40	87.5a
T1	Vitrified	40	67.5a	37	78.3a	40	92.5a, b	37	83.7a, b
T2		33	63.6a, b	41	70.7a	33	96.9a	41	90.2a
T3		34	41.2b	40	62.5a	34	73.5b	40	70.0b

Different letters in the same column differ ($P < 0.05$).

After 72 h of culture, a lower ($P < 0.05$) percentage of hatched blastocyst was observed for Day 6 embryos culture individually in the mWOW. An influence of the treatment on the hatching rate of Day 7 embryos was only observed in the vitrified group; mWOW individual treatment had the lowest rate.

4. Discussion

With the advent of IVF associated with OPU, the culture of individual embryos is an important procedure in the commercial application of that technology. In addition, it has an important role for research, since it enables the investigator to uniquely identify individual embryos.

Several reports were designed to identify methods to achieve an optimal individual culture system, including culture medium [1,17], volume of the drop [18], type of

supplement [19], and O_2 concentration [18]. However, blastocyst rates obtained when IVP embryos were cultured individually were contradictory; better developmental rates were frequently observed when embryos were cultured in groups [4,5,20]. In that regard, Gardner and Lane [6] affirmed that during culture, embryos produced factors that stimulated their own development as well as that of surrounding embryos.

In the present study, we investigated the effect of individual or in group culture on embryo production, using drops and mWOW systems. Cleavage and blastocyst rates were similar for all treatments (Table 1). In contrast, Vajta et al. [5] observed that the WOW system improved embryo development, resulting in higher blastocyst/oocyte rates when compared to the drop system. Different experimental conditions could at least in part, explain the discrepancy between our results and those of the previous study [5]. In their study, embryos were cultured in an atmosphere of low O_2 tension and with no co-culture, while in our experiment, in spite of the high O_2 atmosphere, we used a co-culture with cumulus cells. It is well established that somatic cells have a beneficial effect on the development of bovine embryos, mainly because they secrete embryotrophic factors [14,21,22]; these effects were confirmed by Donnay et al. [1], who evaluated the influence of somatic cells on development of embryos cultured individually or in a group. These authors found the presence of somatic cells increased the blastocyst rate in embryos cultured individually.

In the present study, embryos produced individually or in group in the mWOW system had similar blastocyst yield and total cell number, whereas those cultured in a small drop had a lower number of cells. Therefore, embryos produced in a 20 μL drop had lower quality when compared to the other systems, especially the mWOW individually, in which the same volume of medium was used. It is possible that the mWOW system provided a microenvironment, where the factors originating from embryo and cumulus cells remain in the medium in a higher concentration then in a small drop. Perhaps individual culture can have a detrimental effect on quality by producing embryos with a lower cell number, without affecting embryo production. Further evidence that effect of a given culture method may not manifest itself in the blastocyst yield but in other parameters such as blastocyst quality, were demonstrated by Rizos et al [8]. These authors showed that culturing zygotes totally in vitro or the oviduct of an ewe (in vivo) did not affected blastocyst yield, but in contrast there was a marked difference between the two systems in blastocyst quality.

In the first experiment we were able to detect the lower quality of the embryos, by the total cell number, in those cultured individually in small drop. However, to confirm the value of the mWOW to culture individual embryos, in Experiment 2 we combined measurements of developmental competence (cleavage and blastocyst rates), with a more strict qualitative measure (cryotolerance).

It is well established that embryos produced in vitro differ from those produced in vivo in several aspects; one aspect is higher susceptibility to cryopreservation injuries. The ability of in vitro produced embryos to survive to cryopreservation, depend on their quality, which can be affected by culture conditions. Furthermore, tolerance to cryopreservation has been used as an indicator of embryo quality [8–10,23,24]. According to Lonergan et al. [11], in addition to improving culture conditions to increase blastocyst rate, it is essential to improve blastocyst quality as a means of improving pregnancy rates.

In Experiment 2 the developmental potential of in vitro produced bovine embryos cultured individually or in groups was evaluated by blastocyst rate and embryo quality was measured in terms of tolerance to cryopreservation (i.e. vitrification). When Days 6 and 7 blastocysts from the control group were compared, there was no effect of treatment on hatching rates at 48 h of culture. Similar results were observed at 72 h for Day 7 embryos. However, for Day 6 embryos, the hatching rate was lower for those cultured in mWOW individually. We inferred that the effect of culture environment can be manifested later in development, since embryos cultured individually in the mWOW only revealed their lower quality when they were kept in culture for a longer period of time. Furthermore, the effect of culture conditions on embryo quality became evident when blastocysts from different culture systems were vitrified; after warming, the hatching rate was lower for embryos cultured in mWOW individually. At 48 h the treatment effect was only observed in Day 6 embryos; however it was clear when the embryos were kept in culture for 72 h.

The ability of an embryo to withstand freezing and thawing has been used as a useful indicator of quality; ranking treatments (based on cryotolerance) reflected differences at the level of mRNA expression. The expression of genes involved in various physiological process during embryo development (e.g. apoptosis, oxidative stress, gap junction formation and differentiation) are affected by post-fertilization conditions [10,11,25]. Studies comparing embryos cultured in conditions that resulted in both good- and bad-quality embryos demonstrated that lower quality was associated with reduced tolerance, which was accompanied by alteration of mRNA patterns and abundance of some specific genes [9,24]. Therefore, variation in cryotolerance was attributed to the alteration of gene expression caused by culture conditions.

In Experiment 2, culture of an embryo individually, either in a drop or in the mWOW system, had a negative effect on blastocyst cryotolerance. However, cryotolerance was increased by group culture in the mWOW system, which still enabled individual identification of cultured embryos.

That Day 6 blastocysts had lower tolerance to cryopreservation earlier than Day 7 blastocyst was unclear. Embryos that reached the blastocyst stage on Day 6 were expected to have higher quality, since there are indications that embryos that cleave earlier [23], develop faster [26], and have a higher total cell number [27], are more tolerant to cryoinjuries [23,27] and have higher pregnancy rates [28]. Therefore, the results of the present study, wherein Day 6 embryos were more affected by culture conditions, can be associated with the variation in pattern and abundance of gene transcripts, such as the apoptosis regulator box-α, connexin 43, sarcosina oxidase or mitocondrial Mn-superoxide dismutase, which are involved in physiological process and differ in terms of transcripts abundance according to the culture environment [9–11,24,25]. The inappropriate conditions during development could then cause changes in gene transcription, which could lead to alterations on embryo metabolism. These could explain the lower quality (measured by hatching rate) of embryos in the individual mWOW treatment. However, additional studies are needed to confirm that hypothesis.

In conclusion, embryos cultured individually were of lower quality, resulting in blastocysts with reduced tolerance to cryopreservation. In contrast, the mWOW system improved embryo quality while concurrently enabling individual embryo identification.

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