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Effect of number of oocytes and embryos on *in vitro* oocyte maturation, fertilization and embryo development in bovine

I. Salvador[1]*, A. Cebrian-Serrano[1], D. Salamone[2] and M. A. Silvestre[1]

[1] *Centro de Tecnología Animal. Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA). Apdo. 187. 12400 Segorbe (Castellón). Spain*

[2] *Laboratorio de Biotecnología Animal. Facultad de Agronomía. Universidad de Buenos Aires. Argentina*

Abstract

The aim of this study was to identify the *in vitro* development stage at which the culture of a single or low number (n = 5 or 10) of oocytes/embryos could impair development in comparison with culture in group (n = 50). In the Experiment 1, it was confirmed that single *in vitro* embryo production yielded lower cleavage and blastocyst rates than in group (49.4 *vs.* 83.0%; 0% *vs.* 37.8%, respectively; $p < 0.05$). In Experiment 2 and 3, it was observed no effect on embryo development of culturing single or low number of oocytes during maturation and fertilization, respectively. In Experiment 4, it was observed a detrimental effect on blastocyst rate when cultured single or low number of embryos during post-fertilization *in vitro* culture (2.9; 10.2-10.8; 33.2% in single, low number of embryos (5-10), and control-grouped, respectively; $p < 0.05$). In Experiment 5, it was observed that the last part of the culture period (day 3 onwards) seemed to be more affected by the low number of embryos placed in culture. In conclusion, post-fertilization culture, especially on days 3 to 7 after fertilization, seems to be the most important stage for embryo development on single and/or low number (5-10) of embryos culture.

Additional key words: single/low embryo culture.

Resumen

Efecto del número de ovocitos y embriones en la maduración, fertilización y desarrollo embrionario *in vitro* en vacuno

El objetivo de este trabajo fue identificar la etapa del proceso de producción de embriones *in vitro* en la cual, el cultivo individualizado o con bajo número de oocitos/embriones (n = 5 ó 10) penaliza al desarrollo embrionario respecto del cultivo en grupo (n = 50). En el Experimento 1, se confirmó que la producción individualizada de embriones *in vitro* resulta en una menor tasa de división embrionaria y de blastocistos que las obtenidas con el cultivo en grupo (49,4 *vs.* 83,0%; 0% *vs.* 37,8%, respectivamente; $p < 0,05$). En los Experimentos 2 y 3, no se observó efecto sobre el desarrollo embrionario del cultivo individualizado o de bajo número de embriones en las etapas de maduración y fertilización, respectivamente. En el Experimento 4, se observó un efecto negativo sobre la tasa de blastocistos durante el cultivo *in vitro* post-inseminación cuando se cultivaron los embriones individualmente o con bajo número [2,9; 10,2-10,8; 33,2% en el cultivo individual, con bajo número de embriones (5-10), y grupo control (50), respectivamente; $p < 0,05$]. En el Experimento 5, se observó que en la última parte del cultivo (día 3 post-inseminación en adelante) es donde más parece que se manifiesta el efecto negativo del cultivo con número reducido de embriones. Como conclusión, el cultivo post-inseminación, especialmente entre los días 3 y 7 de cultivo, parece ser la etapa más determinante del proceso de producción de embriones *in vitro* para el desarrollo embrionario en el cultivo individualizado y/o en número reducido de embriones (5-10).

Palabras clave adicionales: cultivo individualizado/bajo número de embriones.

* Corresponding author: salvador_ign@gva.es
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Abbreviations used: COCs (cumulus–oocyte-complexes); D (day); EGF (epidermal growth factor); FBS (foetal bovine serum); IVC (*in vitro* culture); IVF (*in vitro* fertilization); IVM (*in vitro* maturation); IVP (*in vitro* production); MII (metaphase II); mSOF aaci (modified synthetic oviductal fluid aminoacids supplemented); OPU (ovum pick-up); 6-DMAP (6-dimethylaminopurine).

Introduction

The *in vitro* production (IVP) of bovine embryos using single or low numbers of oocytes and embryos during culture is increasing among researchers linked to the production of embryos from oocytes collected by ovum pick-up (OPU) (see Carolan *et al.,* 1996; Vajta *et al.,* 2000, 2008; Ward *et al*., 2000; Goovaerts *et al.,* 2009).

For experimental purposes in bovine, *in vitro* embryo production from oocytes collected from abattoir ovaries is in general quite efficient when a high number of oocytes are cultured together, although the quality of these IVP embryos continually lags behind that of embryos produced *in vivo* (Lonergan *et al.,* 2003). These *in vitro* systems usually culture approximately 40-50 oocytes/embryos in 400-500 μL of medium, obtaining blastocyst rates around 30-40% (Holm *et al.,* 1999; Gordon, 2003). However, for commercial purposes such as use in OPU, where it is usually necessary just to keep the immature oocytes from one donor together, a small number of oocytes/embryos can be cultured (approximately three to six oocytes recovered per non-stimulated cow; Rizos *et al.,* 2005; Chaubal *et al.,* 2006). It has been widely reported that *in vitro* embryo development in bovine and other mammalian species tends to be suppressed in cultures with a single or low number of embryos (in mouse: Paria and Dey, 1990; Canseco *et al.,* 1992; Lane and Gardner, 1992; Kato and Tsunoda, 1994; in bovine: Palma *et al.,* 1992; Ferry *et al.,* 1994; Keefer *et al.,* 1994; Blondin and Sirard, 1995; Carolan *et al.,* 1996; Donnay *et al.,* 1997; O'Doherty *et al.,* 1997; Ward *et al*., 2000; Oyamada and Fukui, 2004; Pereira *et al.,* 2005; Fujita *et al.,* 2006). It seems that the culture systems developed to culture embryos in groups could be unsuitable and/or incomplete for individual embryo culture, and there must be some kind of limiting factor or condition in single embryo *in vitro* culture systems which remain still without knowing (Nagao *et al.,* 2008).

Even though several reports have addressed single embryo IVP with varying levels of success (Carolan *et al.,* 1996; Hagemann *et al.,* 1998; Oyamada *et al.,* 2004), there is still lack of repeatability and high variability in the results among laboratories.

The aim of this work was to identify the stage of the *in vitro* process at which single and low number of embryo development could be impaired, in order to be able to optimise and develop an effective bovine oocyte culture system. To this end, it was studied the effect of single embryo and/or small number embryo culture during the whole *in vitro* process (maturation, fertilization and culture), and also in each process separately, on the bovine embryo development and quality.

Material and methods

In vitro maturation

Cumulus-oocyte-complexes (COCs) were aspirated from 2-8 mm follicles of slaughterhouse ovaries from heifers, and washed three times in holding medium (HM199) based on Hepes TCM199 (Sigma M7528) supplemented with 7% foetal bovine serum (FBS; GIBCO®, Invitrogen) and 75 μg mL^{-1} potassium penicillin G (P3032) and 50 μg mL^{-1} streptomycin sulphate (S6051) as antibiotics. COCs with more than three surrounding layers of cumulus cells and uniform cytoplasm were selected, washed three times in HM199, and *in vitro* matured (IVM) for 22-24 h in TCM199 medium (Sigma M4530) supplemented with 10% FBS, 10 ng mL^{-1} of epidermal growth factor (EGF), 0.1 IU mL^{-1} of recombinant human (rh)-FSH (Gonal-F 75, Serono, Madrid, Spain) and 0.1 IU mL^{-1} of rh-LH (Luveris, Serono). In the Experiment 2a (see below in experimental design), hormonal supplementation was not used. COCs were matured in humidified atmosphere at 38.5ºC and 5% CO_2 in air.

Sperm preparation, *in vitro* fertilization and artificial activation

Two straws with 0.25 mL of frozen semen from two bulls were thawed at 37°C in a water bath for 1 min and centrifuged for 20 min at 700 × *g* through a Percoll gradient of 2 mL 90%, 2 mL 45% Percoll, in 15 mL centrifuge tubes. The Percoll 90 was made according to the protocol described by Parrish *et al.* (1995). To prepare the Percoll 45, the Percol 90 was mixed 1:1 with HM199. The sperm pellet was isolated and washed in 5 mL of HM199 by centrifugation at 350 × *g* for 5 min. Approximately 50 μL of semen pellet remained after the final centrifugation and was diluted with approximately 100 μL of HM199. Final concentration of 1×10^6 sperm mL^{-1} was used for *in vitro* fertilization (IVF). After IVM, COCs were washed three times in fertilization Fert-Talp medium (Parrish *et al.,* 1988) with 10 μg mL^{-1} of heparin (Sigma H9399), and co-

incubated with the spermatozoa for 18-20 h in 5% CO_2 at 38.5°C under mineral oil (Sigma M8410).

To study a complete effect on cytoplasmic maturation and to separate the sperm interaction, artificial activated oocytes were used. In order to obtain parthenogenetic embryos, *in vitro* matured oocytes were denuded of cumulus cells and oocytes with one visible polar body were considered as metaphase II (MII) oocytes. Then, the MII oocytes were activated by exposure to 5 mM ionomycin (Sigma I0634) for 5 min, washed twice, and incubated in 2 mM 6-dimethylaminopurine (DMAP; Sigma D2629) for 3 h. After that, 6-DMAP was washed off, and the oocytes were *in vitro* cultured.

In vitro culture of embryos

Culture medium used was Synthetic Oviductal Fluid (Holm *et al.,* 1999) with the following modifications (mSOFaaci): 4.2 mM sodium lactate (Sigma L4263), 0.73 mM sodium pyruvate (Sigma P4562), 30 µL mL^{-1} BME amino acids (Sigma B6766), 10 µL mL^{-1} of MEM amino acids (Sigma M7145), 75 µg mL^{-1} penicillin G (Sigma P3032), 50 µg mL^{-1} streptomycin (Sigma S6501), 1 µg mL^{-1} phenol-red (Sigma P0290) and 5% FBS.

After IVF or parthenogenetic activation, oocytes were washed three times in culture medium, and cultured at 38.5°C in a humidified atmosphere with 5% CO_2 and 5% O_2, under mineral oil, for the *in vitro* culture (IVC). After IVF, oocytes were previously denuded from surrounding cumulus cells in HM199 before IVC. In Experiments 1 to 4, at day 5 (D5) of culture, IVC medium was supplemented with FBS to reach 10% concentration. In Experiment 5, this FBS supplementation was done at D3, when changing of volume culture conditions was done (see below in experimental design), in order to reduce handling of embryos. Cleavage and blastocyst formation rates were recorded at D2 (48 h post-fertilization), and at D7-8 of culture, respectively. Blastocysts were fixed and stained in ethanol with 25 µg mL^{-1} of bisbenzimide (Hoechst 33342, Sigma B2261), and the total number of cells was counted under an epifluorescence microscope.

Experimental design

Experimental design is summarised in Table 1. Experiment 1 was performed to study the effect of single

Table 1. Experimental design

Experiment	Treatment	No. oocytes or embryos/vol. medium			
		IVM[1]	IVF[2]	IVC[3]	
Exp. 1	sIVP	1/10 µL	1/10 µL	1/10 µL	
	gIVP	50/500 µL	50/500 µL	50/500 µL	
Exp. 2	sIVM	1/10 µL	50/500 µL	50/500 µL	
	r5IVM	5/50 µL	50/500 µL	50/500 µL	
	r10IVM	10/100 µL	50/500 µL	50/500 µL	
	gIVM	50/500 µL	50/500 µL	50/500 µL	
Exp. 3	r5IVF	50/500 µL	5/50 µL	50/500 µL	
	gIVF	50/500 µL	50/500 µL	50/500 µL	
Exp. 4	sIVC	50/500 µL	50/500 µL	1/10 µL	
	r5IVC	50/500 µL	50/500 µL	5/50 µL	
	r10IVC	50/500 µL	50/500 µL	10/100 µL	
	gIVC	50/500 µL	50/500 µL	50/500 µL	
				Days 1-3	**Days 4-7**
Exp. 5	gIVC	50/500 µL	50/500 µL	50/500 µL	50/500 µL
	r5IVC	50/500 µL	50/500 µL	5/50 µL	5/50 µL
	r5IVC + gIVC	50/500 µL	50/500 µL	5/50 µL	50/500 µL
	gIVC + r5IVC	50/500 µL	50/500 µL	50/50 µL	5/50 µL

[1] IVM: *in vitro* maturation. [2] IVF: *in vitro* fertilization. [3] IVC: *in vitro* culture.

embryo culture in the process of *in vitro* embryo production. Subsequently, a series of four experiments were carried out to determine in which step, or steps, of the *in vitro* embryo production the embryo viability was affected by single or low culture number (IVM, IVF, IVC, in Experiment 2, 3, 4 respectively). In the Experiment 2, *in vitro* matured oocytes were either *in vitro* fertilized (Experiment 2a) or artificially activated (Experiment 2b). Finally, in Experiment 5, the effect of changing from low number embryo (n = 5) culture to control-grouped embryo culture (n = 50), and vice-versa, at D3 of culture after fertilization, was studied. The same conditioned medium in which embryos were cultured previously was used.

Statistical analysis

At least, three to six replicates were performed in each experiment. Results of maturation, cleavage, and blastocyst rates were analysed using the χ^2 test. When a single degree of freedom was involved, the Yates' correction for continuity was carried out. Results of blastocyst cell number were analysed using analysis of variance (ANOVA). A probability of $p < 0.05$ was considered to be statistically different.

Results

In Experiment 1 (Table 2), it was observed that when the whole embryo production process is performed maintaining individually maturation, fertilization and culture (sIVP), a significantly lower cleavage and blastocyst rates are yielded compared to grouped culture (gIVP).

In Experiment 2a (Table 3), no difference was observed among single maturation (sIVM), low number maturation (r5IVM, r10IVM) and control-grouped maturation (gIVM) in either nuclear maturation (ranging from 76.2 to 83.5%), cleavage rate (68.1-76.2%), blastocyst yield (30.2-46.5%) or blastocyst cell number (105-117 cells/blastocyst). In Experiment 2b (Table 3), using chemically activated parthenogenote embryos, no significant differences were observed among treatments in any of the characters studied: nuclear maturation (78.6-87.3%), rate of cleavage of MII oocytes after chemical activation (73.2-81.7%), rate of blastocyst from cleaved zygotes (20.9-25.4%) and blastocyst cell number (53-65 cells/blastocyst).

In Experiment 3 (Table 4), no significant difference was observed in cleavage, blastocyst rate or number of cells per blastocyst, between low number fertilization (r5IVF) and control-grouped fertilization (gIVF).

In Experiment 4 (Table 4), it was observed that embryos cultured in control-grouped (gIVC) conditions showed significant higher cleavage rate than those cultured single (sIVC) or in low number n = 5 (r5IVC) conditions ($p < 0.05$), although no difference reached statistical significance when compared to low number n = 10 (r10IVC) conditions. Regarding blastocyst yield, sIVC embryos showed the lowest blastocyst rates (2.9%; $p < 0.05$), whereas gIVC embryos yielded the highest blastocyst rates (33.2%; $p < 0.05$). No differences on number of cells per blastocyst were observed.

In Experiment 5 (Table 4), it was again observed that culturing embryos in r5IVC resulted in a significant lower cleavage than culturing in gIVC, and gIVC resulted in a higher blastocyst yield than any other group tested ($p < 0.05$). When variation of the culture conditions took place, embryos cultured under low number (n = 5) conditions from D1 to D3 and changed to control-grouped conditions D4 onwards (r5IVC + gIVC) showed higher blastocyst yield and number of cells per blastocyst, than when variation of the culture

Table 2. Single *in vitro* bovine embryo production (individual maturation, fertilization and culture) *vs.* group *in vitro* embryo production (Experiment 1)

Treatment[1]	No. COCs[2]	% cleavage (No.)	% blastocyst (No.)[3]	LSMean of cells/blastocyst (± SEM)
sIVP	164	49.4 (81)[a]	0 (0)[a]	—
gIVP	188	83.0 (156)[b]	37.8 (59)[b]	93 (± 4.8)

[1] See Table 1. [2] COCs: cumulus–oocyte-complexes. [3] Percentage of blastocyst is calculated from cleaved embryos. [a,b]: Different superscripts in the same column indicate a statistical difference ($p < 0.05$). No statistical analysis of blastocyst cells was performed.

Table 3. Effect of different *in vitro* maturation (IVM) conditions, varying the number of oocytes, on *in vitro* development of (a) *in vitro* fertilized bovine embryos (Experiment 2a) and (b) parthenogenetic bovine embryos (Experiment 2b)

Treatment[1]	% MII[2] (No.)	No. COCs[3]	% cleavage (No.)	% blastocyst (No.)[3]	LSMean of cells/blastocyst (± SEM)
Experiment 2a					
sIVM	76.2 (59/77)	176	68.1 (120)	38.3 (46)	116 (± 5.3)
r5IVM	77.3 (58/75)	190	73.2 (139)	30.2 (42)	117 (± 5.6)
r10IVM	83.5 (66/79)	193	76.2 (147)	33.3 (49)	113 (± 5.4)
gIVM	81.9 (95/116)	192	75.0 (144)	46.5 (67)	105 (± 4.4)
Experiment 2b					
sIVM	78.6 (88)	112	81.7 (67/82)	20.9 (14)	55 (± 4.3)
r5IVM	87.3 (76)	87	80.8 (59/73)	25.4 (15)	59 (± 5.8)
r10IVM	84.7 (72)	85	73.2 (52/71)	25.0 (13)	65 (± 6.7)
gIVM	86.6 (97)	112	79.5 (70/88)	24.3 (17)	53 (± 5.1)

[1] See Table 1. [2] MII: metaphase II oocytes. [3] COCs: cumulus–oocyte-complexes. [4] Percentage of blastocyst is calculated from cleaved embryos.

density conditions was done vice versa (gIVC + r5IVC) ($p < 0.05$). Regarding the blastocyst quality, gIVC + r5IVC group showed a significant lower number of cells per blastocyst than the other experimental groups tested ($p < 0.05$).

Discussion

This work was planned to try to identify the main step of the *in vitro* process at which single or low number embryo development could be impaired.

Table 4. Effect of different culture conditions on *in vitro* development of bovine embryos i) varying the number of oocytes in fertilization (IVF) (Experiment 3), or ii) varying the number of zygotes in culture (IVC) (Experiment 4), and iii) varying the culture ratio using conditioned medium in IVC (Experiment 5)

Treatment[1]	No. COCs[2]	% cleavage (No.)	% blastocyst (No.)[3]	LSMean of cells/blastocyst (± SEM)
Experiment 3				
r5IVF	120	75.8 (91)	30.8 (28)	86 (± 6.1)
gIVF	153	85.6 (131)	29.7 (39)	82 (± 2.7)
Experiment 4				
sIVC	292	80.0 (234)[a]	2.9 (7)[a]	69 (± 25.5)
r5IVC	285	82.1 (234)[a]	10.2 (24)[b]	72 (± 15.2)
r10IVC	310	83.5 (259)[ab]	10.8 (28)[b]	108 (± 9.9)
gIVC	359	88.0 (316)[b]	33.2 (105)[c]	97 (± 5.0)
Experiment 5				
gIVC	300	86.0 (258)[a]	29.4 (76)[a]	82 (± 2.7)[a]
r5IVC	284	76.0 (216)[b]	6.0 (13)[b]	83 (± 8.6)[a]
r5IVC + gIVC	298	79.5 (237)[b]	10.5 (25)[b]	83 (± 3.2)[a]
gIVC + r5IVC	311	85.8 (267)[a]	1.8 (5)[c]	56 (± 6.6)[b]

[1] See Table 1. [2] COCs: cumulus–oocyte-complexes. [3] Percentage of blastocyst is calculated from cleaved embryos. [a,b]: Different superscripts in the same column indicate a statistical difference ($p < 0.05$).

In Experiment 1, it was observed that when the embryo production process is performed maintaining individually maturation, fertilization and culture, a significant lower cleavage and blastocyst rate was obtained, as it was observed in other works (Keefer *et al.,* 1994; Ward *et al.*, 2000; Fujita *et al.,* 2006).

Oocyte maturation environment has been demonstrated to be essential for high *in vitro* blastocyst yields (Sirard and Blondin, 1996; Jewgenow *et al.,* 1999; Rizos *et al.,* 2002). To investigate this, it was studied the effect of varying the number of oocytes placed in culture during *in vitro* maturation, but maintaining the ratio oocytes/volume of IVM medium, on their *in vitro* development of bovine embryos after IVF or parthenogenetic activation. Blastocyst yield after single, low number (n = 5-10) or control (n = 50) *in vitro* maturation was no significantly different. Results obtained here are in agreement with those reported by other authors (Carolan *et al.,* 1996; Ward *et al.*, 2000); however, it can be found some contradictory results in the bibliography, where it is also observed that single oocyte maturation could reduce the developmental capacity of oocytes compared with group maturation (Blondin and Sirard, 1995; Jewgenow *et al.,* 1999) even maintaining the same oocyte/volume ratio (1 oocyte in 10 µL *vs*. 10 oocytes in 100 µL) (Feng *et al.,* 2007). By other hand, it seems that developmental potential of oocytes following IVM, IVF and IVC could greatly depend on the presence and morphology of the surrounding cumulus cells and follicular environment (Araki *et al.,* 1998; Gordon, 2003; Feng *et al.,* 2007). Araki *et al.* (1998) observed that the tight layer of cumulus cells enhanced the developmental capacity of individual cultured oocytes, achieving higher rates of blastocyst when oocytes with homogeneous ooplasm and surrounding with more than four layers of cumulus cells were individually cultured through IVM to IVC, compared to that with two to three layers. Furthermore, Feng *et al.* (2007) showed that blastocyst rates of bovine oocytes were highly correlated with the degree of cumulus expansion. In the present work, oocytes with more than three layers of surrounding cumulus cells and homogeneous cytoplasm were used, so it could be thought that the high intrinsic quality of oocytes used could explain the fact that individual IVM did not impair blastocyst yield during *in vitro* fertilization and culture in group.

Regarding IVF process, it was observed that when five oocytes were fertilized in a drop of 50 µL (r5IVF), no significant effect was observed either in cleavage rate, blastocyst yield or blastocyst cells number compared with control-grouped fertilization when in the remaining steps (IVM, IVC) they were cultured in group. Although Carolan *et al.* (1996) observed that maintaining the control in-group oocyte/volume ratio for individual *in vitro* fertilization (1 embryo in 10 µL) seriously compromised subsequent embryo development, these authors observed that individual oocytes could be successfully fertilized by increasing the volume of IVF to 50-100 µL, with no differences in terms of embryo development compared to in-group.

In the conditions of the present work, the post-fertilization culture conditions seem to be the most important step in the whole process of *in vitro* embryo production concerning to number of oocytes/embryos. This effect has also been observed by Ward *et al.* (2000), but furthermore, in the present work it was detected that the last part of embryo culture, D3 to D7, is the stage when is more noticeable the impairment of small number of embryos in culture on blastocyst formation.

It was observed that culture of embryos in control-grouped conditions resulted in a higher cleavage and blastocyst rate than individual or low embryo number (n < 10) culture conditions. Single embryo culture results obtained here are very low, but this level is also observed in other works culturing embryos individually (sIVC), and even regrouped for the remaining steps (gIVM, gIVF) (Carolan *et al.,* 1996; Ward et *al.*, 2000; Goovaerts *et al.,* 2009). It is suggested in several animal species (mice, bovine, porcine) that suppression of early development of embryos cultured individually might be caused by a deficiency of cooperative interaction among embryos (Paria and Dey, 1990; Keefer *et al.,* 1994; Stokes *et al.,* 2005). It seems that several growth factors could act as possible embryotrophic factors reciprocally stimulating embryo development, in a paracrine/autocrine fashion, when embryos are cultured *in vitro* in groups (Paria and Dey, 1990; Palma and Brem, 1995; Thibodeaux *et al.,* 1995; Lim and Hansel, 1996; Stokes *et al.,* 2005; Contramaestre *et al.,* 2008). In addition, Nagao *et al.* (2008) concluded that the cooperative interaction among bovine early embryos during *in vitro* culture may be also mediated by the reduction of toxic factors, so at low embryo density, reduced oxygen tension or the exclusion of inorganic phosphate from the medium enhances blastocyst development. Moreover, volume of medium also seems to be an important factor for individual embryo culture. Very small volumes (< 10 µL) may accumulate toxic metabolites (Lane and Gardner, 1992; Carolan *et al.,*

1996), in contrast, larger volumes of medium or renewal of the media may dilute, or replace, autocrine embryotrophic factors (Paria and Dey, 1990). However, the mechanisms negatively affecting *in vitro* individual incubation of bovine embryos are still not understood in depth, as there is still high variability in the results among laboratories. The lower limit number of embryos from which reduced efficiency appears is not conclusive, and the comparison with other works is sometimes not easy since, apart from different culture conditions, the ratio oocyte/volume of medium may be changed even in the same work. In the present work, significant differences were observed when 10 embryos in 100 µL (1/10 oocyte μL^{-1}) were *in vitro* cultured. In this sense, Hoelker *et al.* (2009) detected significant differences in embryo development with 16 embryos (1/32 oocyte μL^{-1}) in comparison with the control group (n = 50, 1/10 oocyte μL^{-1}). However, other authors (Fujita *et al.,* 2006) detected differences only when a lower number of embryos (n = 3, 1/5 oocyte μL^{-1}) were cultured.

Some studies have addressed the use of conditioned medium to investigate the potential beneficial effect of the growth factors secreted in the media, by embryos cultured in groups or cell-coculture, on the promotion of single embryo development (Fujita *et al.,* 2006; Goovaerts *et al.,* 2009). However, to our knowledge, not in the same way as tested in the present work, in which possible depletion renewal or dilution of media was avoided, and only the effect of number of embryos could be studied. Here, it was tried to investigate the effect of changing from low number embryo culture to grouped embryo culture and vice-versa using the same conditioned media in which embryos were previously cultured, and maintaining the control embryo/volume of medium ratio. It was observed that the most deleterious change for embryo development, in terms of blastocyst formation, is when group-cultured embryos are placed in low number embryo culture conditions at D3 and onwards. From the results obtained here, it could be summarised that the last part of embryo culture is likely to be a crucial step when deficiency of cooperative interaction among embryos could arise, impairing blastocyst formation. Although the comparison with porcine embryo development should be cautiously considered, since pig are multiparous while bovine is monoparous, in porcine embryos, it was observed that the beneficial effect of grouped embryo culture, at an optimum distance of 81-160 µm separation among embryos, rose at 48-96 h after insemination and became more evident towards the last part of the culture period, for 96-144 h which corresponded to day 5-6 of culture (Stokes *et al.,* 2005). These latter authors suggested that group culture confers a greater advantage on development post genome activation, and even more so at the morula and blastocyst stage. In bovine embryos, two stages seem to be crucial for embryo development: 8- to 16-cell stage, at days 2-3 of culture after fertilization, in which developmental arrest could occur in the called "8-cell block" (Eyestone and First, 1991); and compaction and cavitation for blastocyst formation from morula, which occurs around the 32-cell stage approximately after day 5 of *in vitro* culture (Van Soom *et al.,* 1997). It was shown in the literature that a serum supplementation to the culture medium at day 5 after fertilization increased the proportion of oocytes that developed to blastocyst (De Moraes and Hansen, 1997; Paula-Lopes *et al.,* 1998; Hagemann *et al.,* 1998), and as it is widely known, growth factors, hormones and other active substances are present in FBS (Gordon, 2003). Taking these data together, it seems likely that some important autocrine/paracrine growth factor(s) and/or other components secreted to the culture media by the embryos could become more important in later stages of culture.

It could be concluded that, in the conditions tested here, post-fertilization culture conditions, regarding number of embryos in culture, seem to be the most important step determining blastocyst yield. Culture of single and/or a low number of embryos (< 10 embryos) drastically impaired the efficiency of blastocyst yield. Moreover, it seems that the final part of the culture (day 3 to day 7) could be crucial for the manifestation of this deleterious effect with a low number of embryos. More research is needed to elucidate the factors negatively affecting individual and low number embryo *in vitro* culture, and to overcome its lower efficiency.

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References

ARAKI N., SATO K., HAYASHI K., MIYAMOTO A., FUKUI Y., 1998. Relationships among follicular fluid estradiol-17β concentration, morphology of cumulus-oocyte complex and developmental capacity of individually matured fertilized and cultured bovine oocytes *in vitro*. J Reprod Dev 44, 359-365.

BLONDIN P., SIRARD M.A., 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 41, 54-62.

CANSECO R.S., SPARKS A.E., PEARSON R., GWAZDAUSKAS F.C., 1992. Embryo density and medium volume effects on early murine embryo development. J Assist Reprod Genet 9, 454-457.

CAROLAN C., LONERGAN P., KHATIR H., MERMILLOD P., 1996. *In vitro* production of bovine embryos using individual oocytes. Mol Reprod Dev 45, 145-150.

CHAUBAL S.A, MOLINA J.A., OHLRICHS C.L., FERRE L.B., FABER D.C., BOLS P.E.J., RIESEN J.W., TIAN X., YANG X., 2006. Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. Theriogenology 65, 1631-1648.

CONTRAMAESTRE A.P., SIFONTES F., MARÍN R., CAMEJO M.I., 2008. Secretion of stem cell factor and granulocyte-macrophage colony-stimulating factor by mouse embryos in culture: influence of group culture. Zygote 16, 297-301.

DE MORAES A.A., HANSEN P.J., 1997. Granulocyte-macrophage colony-stimulating factor promotes development of *in vitro* produced bovine embryos. Biol Reprod 57, 1060-1065.

DONNAY I., VAN LANGENDONCKT A., AUQUIER P., GRISART B., VANSTEENBRUGGE A., MASSIP A., DESSY F., 1997. Effects of co-culture and embryo number on the *in vitro* development of bovine embryos. Theriogenology 47, 1549-1561.

EYESTONE W.H., FIRST N.L., 1991. Characterization of developmental arrest in early bovine embryos cultured *in vitro*. Theriogenology 35, 613-624.

FENG W.G., SUI H.S., HAN Z.B., CHANG Z.L., ZHOU P., LIU D.J., BAO S., 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.

FERRY L., MERMILLOD P., MASSIP A., DESSY F., 1994. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology 42, 445-453.

FUJITA T., UMEKI H., SHIMURA H., KUGUMIYA K., SHIGA K., 2006. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. J Reprod Dev 52, 137-142.

GOOVAERTS I.G.F., LEROY J.L.M.R., VAN SOOM A., DE CLERCQ J.B.P., ANDRIES S., BOLS P.E., 2009. Effect of cumulus cell coculture and oxygen tension on the *in vitro* developmental competence of bovine zygotes cultured singly. Theriogenology 71, 729-738.

GORDON I., 2003. Laboratory production of cattle embryos, 2th ed. Biotechnology in Agriculture Series, No. 27. CAB international.

HAGEMANN L.J., WEILERT L.L., BEAUMONT S.E., TERVIT H.R., 1998. Development of bovine embryos in single *in vitro* production (sIVP) systems. Mol Reprod Dev 51, 143-147.

HOELKER M., RINGS F., LUND Q., GHANEM N., PHATSARA C., GRIESE J., SCHELLANDER K., TESFAYE D., 2009. Effect of the microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured *in vitro*. Reproduction 137, 415-425.

HOLM P., BOOTH P.J., SCHMIDT M.H., GREVE T., CALLESEN H., 1999. High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52, 683-700.

JEWGENOW K., HEERDEGEN B., MÜLLER K., 1999. *In vitro* development of individually matured bovine oocytes in relation to follicular wall atresia. Theriogenology 51, 745-756.

KATO Y., TSUNODA Y., 1994. Effects of the culture density of mouse zygotes on the development *in vitro* and *in vivo.* Theriogenology 41, 1315-1322.

KEEFER C.L., STICE S.L., PAPROCKI A.M., GOLUEKE P., 1994. *In vitro* culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors. Theriogenology 41, 1323-1331.

LANE M., GARDNER D.K., 1992. Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*. Hum Reprod 7, 558-562.

LIM J.M., HANSEL W., 1996. Roles of growth factors in the development of bovine embryos fertilized *in vitro* and cultured singly in a defined medium. Reprod Fertil Dev, 1199-1205.

LONERGAN P., RIZOS D., KANKA J., NEMCOVA L., MBAYE A.M., KINGSTON M., WADE M., DUFFY P., BOLAND M.P., 2003. Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 126, 337-46.

NAGAO Y., IIJIMA R., SAEKI K., 2008. Interaction between embryos and culture conditions during *in vitro* development of bovine early embryos. Zygote 16, 127-33.

O'DOHERTY E.M., WADE M.G., HILL J.L., BOLAND M.P., 1997. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. Theriogenology 48, 161-169.

OYAMADA T., FUKUI Y., 2004. Oxygen tension and medium supplements for *in vitro* maturation of bovine oocytes cultured individually in a chemically defined medium. J Reprod Dev 50, 107-117.

OYAMADA T., IWAYAMA H., FUKUI Y., 2004. Additional effect of epidermal growth factor during *in vitro* matura-

tion for individual bovine oocytes using a chemically defined medium. Zygote 12, 143-150.

PALMA G.A., BREM G., 1995. Effect of growth factors IGFI, TGF-α, EGF and PDGF on development of *in vitro* produced bovine blastocysts. Theriogenology 43, 291.

PALMA G.A., CLEMENT-SENGEWALD A., BERG U., BREM G., 1992. Role of the embryo number in the development of *in vitro* produced bovine embryos. Theriogenology 37, 271.

PARIA B.C., DEY S.K., 1990. Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. J Reprod Dev 87, 4756-4760.

PARRISH J.J., SUSKO-PARRISH J.L., WINER M.A., FIRST N.L., 1988. Capacitation of bovine sperm by heparin. Biol Reprod 38, 1171-80.

PARRISH J.J., KROGENAES A., SUSKO-PARRISH J.L., 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of *in vitro* fertilization and early embryonic development. Theriogenology 44, 859-869.

PAULA-LOPES F.F., DE MORAES A.A., EDWARDS J.L., JUSTICE J.E., HANSEN P.J., 1998. Regulation of preimplantation development of bovine embryos by interleukin-1beta. Biol Reprod 59, 1406-1412.

PEREIRA D.C., DODE M.A.N., RUMPF R., 2005. Evaluation of different culture systems on the *in vitro* production of bovine embryos. Theriogenology 63, 1131-1141.

RIZOS D., WARD F., DUFFY P., BOLAND M.P., LONERGAN P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro versus in vivo:* implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 61, 234-248.

RIZOS D., BURKE L., DUFFY P., WADE M., MEE J.F., O'FARRELL K.J., MACSIURTAIN M., BOLAND M.P., LONERGAN P., 2005. Comparisons between nulliparous heifers and cows as oocyte donors for embryo production *in vitro*. Theriogenology 63, 939-949.

SIRARD M.A., BLONDIN P., 1996. Oocyte maturation and IVF in cattle. Anim Reprod Sci 42, 417-426.

STOKES P.J., ABEYDEERA L.R., LEESE H.J., 2005. Development of porcine embryos *in vivo* and *in vitro*; evidence for embryo "cross talk" *in vitro*. Dev Biol 284, 62-71.

THIBODEAUX J.K., MYERS M.W., HANSEL W., 1995. The beneficial effects of incubating bovine embryos in groups are due to platelet-derived growth factor. Theriogenology 43, 336.

VAJTA G., PEURA T., HOLM P., PÁLDI A., GREVE T., TROUNSON A.O., 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.

VAJTA G., KORÖSI T., DU Y., NAKATA K., IEDA S., KUWAYAMA M., NAGY Z.P., 2008. The well-of-the-well system: an efficient approach to improve embryo development. Reprod Biomed Online 17, 73-81.

VAN SOOM A., YSEBAERT M.T., KRUIF A., 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in *in vitro*-produced bovine embryos. Mol Reprod Dev 47, 47-56.

VAN WAGTENDONK-DE LEEUW A.M., 2006. Ovum pick up and *in vitro* production in the bovine after use in several generations: a 2005 status. Theriogenology 65, 914-925.

WARD F.A., LONERGAN P., ENRIGHT B.P., 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.