View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Cadernos Espinosanos (E-Journal)

Effective individual culture system for *in vitro* production of bovine embryos

Sistema eficaz de cultivo individual de embriões bovinos produzidos in vitro

Kelly ANNES[1]; Carlos Alexandre SOARES[1]; Camila Bruna de LIMA[1]; Marcella Pecora MILAZZOTTO[1]

[1] Universidade Federal do ABC, Centro de Ciências Naturais e Humanas,
Laboratório de Biologia Celular e Molecular, Santo André – SP, Brazil

Abstract

A new and effective protocol to culture bovine embryos without coculture and with individualized culture media has been established, which would allow the study of a single embryo's metabolism. For this purpose, bovine embryos were produced *in vitro* by standard protocols in three different types of media: KSOM, SOFaa, and KSOM followed by SOFaa at day 2. Presumptive zygotes were divided into six groups: control, cultured in groups (C-KSOM, C-SOFaa, and C-KS), and individual well system (W-KSOM, W-SOFaa, and W-KS). Cleavage and blastocyst rates were assessed on days 2 and 7 respectively. Relative quantification of transcripts related to important metabolic processes (GLUT1, GLUT3, GSK3, SOD1, HSPD1, G6PD) were assessed in C-KS and W-KS blastocysts. Results show that cleavage was significantly higher only in W-KSOM when compared to C-KSOM, while blastocyst rates differ only between C-SOF and W-SOF. All the other comparisons did not present statistical difference. Moreover, gene expression analysis revealed that blastocysts cultured in groups and in the individual well system present similar transcription patterns. Thus, the obtained conclusion was that the individual well system performed could be used as an effective alternative protocol for individual culture of bovine embryos, since the rates are similar to routine group culture.

Keywords: Bovine. Embryo. Individual culture.

Resumo

Estabeleceu-se um protocolo novo e eficaz de cultivo individual de embriões bovinos sem o uso de cocultivo e sem compartilhamento de meio visando à análise do metabolismo individual do embrião. Para isso, embriões foram produzidos *in vitro* por protocolos convencionais em três diferentes tipos de meio: KSOM, SOFaa e KSOM seguido por SOFaa no dia 2. Os zigotos presumíveis foram divididos em seis grupos: controles (cultivo em grupo – C-KSOM, C-SOFaa e C-KS) e sistema de poços individuais (W-KSOM, W-SOFaa e W-KS). As taxas de clivagem foram avaliadas nos dias 2 e 7, respectivamente. Além disso, a quantificação relativa de transcritos relacionados a importantes processos metabólicos (GLUT1, GLUT3, GSK3, SOD1, HSPD1 e G6PD) foi avaliada nos blastocistos dos grupos C-KS e W-KS. Os resultados mostram que as taxas de clivagem foram maiores apenas no grupo W-KSOM quando comparado ao grupo C-KSOM, enquanto a taxa de blastocistos diferiu apenas entre os grupos C e W-SOF. Além disso, a análise da expressão gênica mostrou que blastocistos cultivados em grupo ou em sistema de poços individuais são semelhantes quanto à expressão gênica. Assim, a conclusão obtida foi que o sistema individual proposto pode ser utilizado como um protocolo alternativo eficiente para o cultivo individual de embriões de bovino, uma vez que suas características permanecem semelhantes àquelas do sistema convencional de produção de embriões.

Palavras-chave: Bovino. Embrião. Cultivo individual.

Correspondence to:
Marcella Pecora Milazzotto
Universidade Federal do ABC
Avenida dos Estados, 5001, Bloco A, Torre 3,
Lab502-3, Bairro Bangu
CEP 09210-580, Santo André, SP, Brazil
e-mail: marcella.milazzotto@ufabc.edu.br

Received: 24/11/2015
Approved: 02/08/2017

Introduction

The routine practice for *in vitro* production (IVP) of bovine embryos is to handle the embryos in groups. A number of previous studies reported that *in vitro* culture is more successful when the embryos are kept in large groups, probably due to the reciprocal stimulation during their development (GOOVAERTS et al., 2009). Despite the large-scale production of bovine embryos for reduced costs

and commercial purposes, an individual system may be a promising model in order to elucidate remaining problems in IVP and also for further studies in human infertility (GOOVAERTS et al., 2012).

Most techniques routinely used to evaluate embryo quality and viability are still based on morphological parameters, though it is clear that they are not sufficiently accurate. Molecular analysis such as Omics studies and characterization of gene-expression patterns may represent more reliable tools to provide non-invasive markers that predict embryo potential (BUNEL et al., 2015). However, for these purposes it is of great importance that bovine embryos be cultured individually, without the intervention of any other cell. The specific requirements for a single embryo to be developed individually until blastocyst stage are still unknown and many reports described the attempts of achieving an optimal performance in individual culture systems (VAJTA et al. 2000; GOOVAERTS et al., 2012; BUNEL et al. 2015).

The first attempts of individual culture were performed with simple proportionate amounts of media and supplements. However, results showed impaired development, low blastocyst rates, and low number of cells (TAGAWA et al., 2008). Besides, growing embryos in reduced volumes of media leads to the accumulation of toxic substances that are potentially harmful and that may lead to anomalies in embryonic development. In 2000, Vajta et al. developed a more effective system, known as well-of-the-well (WOW). An apparently simple modification of the bottom of the culture dish promoted a notable improvement in bovine embryos produced *in vitro*. However, in this system the embryos were still sharing the same culture media. Some other attempts to individualize the media drops were performed without success, since blastocyst rates were very low or almost null (GOOVAERTS et al., 2012).

In 2010, Sugimura et al. demonstrated that WOW embryos did not present differences in blastocyst development, number of MCI, and trophectoderm cells when compared to controls (cultured in groups). Besides, the study also showed that the consumption of oxygen in WOW blastocysts was closer to the physiological when compared to controls and observed an increase in pregnancy rates 30 and 60 days after transfer (SUGIMURA et al., 2010). In this context, we may conclude that the individual cultivation system might lead to metabolic changes without interfering in the morphology of the cells.

Important metabolic markers are genes related to energy metabolism, such as glucose transporters (GLUT1 and GLUT3) and glucose regulators (GSK3 and G6PD), as well as stress-response genes such as superoxide dismutase 1 (SOD1) and Heat Shock Protein family D member 1 (HSPD1). GLUT1 is responsible for maintaining basal glucose levels for embryonic development; GLUT3 is responsible for glucose uptake from the external environment (PANTALEON; KAYE, 1998); G6PD encodes the enzyme that will limit the rate of the pentose phosphate pathway (PPP), which is directly linked to glucose-6-phosphate metabolism (GARCIA-HERREROS et al., 2012), and GSK3 is involved in many cellular processes such as regulation of protein synthesis, cell proliferation and differentiation, and apoptosis (FRAME; COHEN, 2001). SOD1 is important to protect the cell from free superoxide radicals and HSPD1 are chaperones that promote cell protection against damage, avoiding protein denaturation and preventing apoptosis (TAKAHASHI, 2012). Thus, the analysis of such genes may be a useful tool to predict the metabolic status of the cells.

Therefore, the aim of this study was to establish a routine protocol for single bovine embryo culture without coculture and with individualized media culture, which would allow the study of individual embryo metabolism.

Material and Methods

In vitro maturation (IVM)

Ovaries were recovered from slaughtered cows and kept in saline solution. All follicles with a diameter between 2 and 8 mm were aspirated, but only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were included in the study (three replicates, n = 640). Groups of 30 oocytes were cultured in drops of 90 µL of TCM 199 supplemented with 10% fetal bovine serum (FBS), hormones, and antibiotics. The drops were placed in Petri dishes and covered with mineral oil. *In vitro* maturation followed at 38.5°C in 5% CO_2 with saturated humidity for 24h.

In vitro fertilization (IVF)

Groups of 20 oocytes were transferred to 90 µL drops of fertilization media and covered with mineral oil. Frozen semen from three different bulls with proven fertility were thawed in warm water, mixed together and submitted to a discontinuous Percoll® gradient (90% and 45%); the final

concentration was adjusted to 1x10^{6} spermatozoa/mL. Oocytes and sperm were incubated for approximately 18 h.

In vitro culture (IVC)

At 18 hours post insemination (hpi), the presumptive zygotes were randomly divided into three different types of culture media: SOFaa (supplemented with 5% FBS, essential, and non-essential amino acids), KSOM (107-D Millipore® supplemented with 10% FBS, gentamicin, and non-essential amino acids), and KSOM followed by SOFaa on day 2; and 2 different culture systems (Group or Individual Culture). Therefore, the study groups based on (i) culture system and (ii) types of culture media were set as follows: Controls (C-SOFaa, C-KSOM, and C-KS) and Individual Well System (W-SOFaa, W-KSOM, and W-KS).

For the Control Groups, around 20 presumptive zygotes were transferred to 90 µL drops of each culture media under mineral oil. For the Individual Well System groups, each dish was prepared with 20 µL drops of culture media. Right in the center of each drop, the bottom of the plate was gently pressed with a needle to create a small well. Then, one presumptive zygote was placed individually inside each well as it is showed on figure 1.

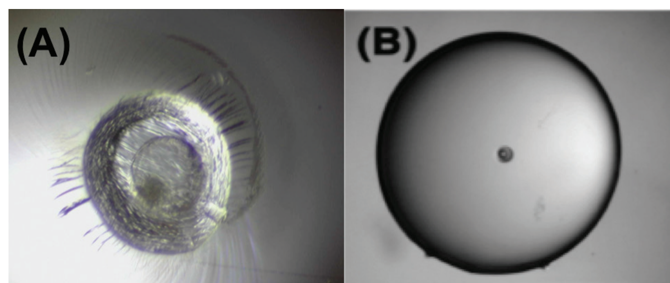

Figure 1 – (A) Augmentation of 100x shows individual well containing the developing embryo (day 7). (B) Detail of individual drop and well containing the developing embryo on a 20x augmentation

All embryos were cultured for seven days at 38.5°C in 5% CO2 and saturated humidity.

Embryo evaluation

Cleavage rates were assessed on Day 2 (cleaved/oocytes). Blastocyst rates (blastocysts/oocytes) and gene expression were evaluated on the Day 7. All data were obtained in four replicates.

We have also selected two groups of study (C-KS and W-KS) to evaluate the expression of target genes (GLUT1, GLUT3, GSK3, SOD1, HSPD1, and G6PD) by quantitative real-time PCR, using TaqMan system (Life Technologies) (Table 1). For this analysis, ACTB was used as endogenous control.

Table 1 – TaqMan assays used for gene expression analysis – Santo André – June 2017

Gene	Code (Life Technologies, Inc.)	Genebank ID
GLUT-1	Bt03215314_m1	NM_174602.2
GLUT-3	Bt03259514_g1	NM_174603.3
GSK-3	Bt03273695_m1	NM_001102192.1
G6PD	Bt03649181_m1	NM_001244135.1
HSP60	Bt04301470_g1	NM_001166608.1
SOD1	Bt03215423_g1	NM_174615.2
ACTB	Bt03279175_g1	NM_173979.3

Amplification cycles were carried out on Real Time Thermocycler (Mastercycler EP, Eppendorf, Germany) qPCR SuperMix (Invitrogen, Carlsbad, CA, USA). The reaction (total volume of 20 µL) contained Master Mix (10 µL), probe, reverse and forward primers (1 uL), and cDNA template (1 µL – equivalent to ≈ 0.15 embryo). Reaction conditions were: initial activation at 95°C for 10 min; cycling: denaturation at 94°C for 15 s; annealing and extension at 60°C for 1 min. Three technical and biological replicates were performed for each sample.

Statistical Analysis

Statistical analysis was performed by Student *t* test using the software Prism 5 (GraphPad Inc.). For embryo production rates, comparisons were performed in pairs as follows: C-SOFaa *vs.* W-SOFaa, C-KSOM *vs.* W-KSOM, and C-KS *vs.* W-KS. The alpha error was set to 5%. Differences in gene expression were based on the estimated amplification efficiency and the variation of expression (ΔCt). Reactions were normalized by the frequency of expression of the endogenous control gene (ACTB).

Results

In vitro production rates are shown on Table 2. Results show that cleavage rate was higher in W-KSOM when compared to C-KSOM. Besides, blastocyst rate was higher in C-SOF when compared to W-SOF. All the other comparison did not present statistical difference. As groups C-KS and W-KS did not present differences neither in cleavage nor in blastocyst rates, these groups were selected for a relative quantification of target transcripts.

Table 2 – Cleavage and Blastocysts rates were assessed on Day 2 and Day 7, respectively – Santo André – June 2017

	Cleavage (%) (mean ± SEM)	p-value	Blastocyst (%) (mean ± SEM)	p-value
SOFaa				
C-SOF	77.9 ± 1.115	**0.188**	35.3 ± 8.658	**0.028***
W-SOF	81.33 ± 1.856		24.07 ± 5.139	
KSOM				
C-KSOM	74.03 ± 3.353	**0.033***	36.67 ± 6.009	**0.782**
W-KSOM	86.70 ± 2.113		34.57 ± 3.795	
KSOM + SOFaa				
C-KS	67.88 ± 5.373	**0.710**	30.25 ± 3.701	**0.7134**
W-KS	70.03 ± 1.704		32.98 ± 6.198	

For all the comparisons, statistical difference was considered when $p < 0.05$

None of the evaluated transcripts presented differences in relative expression between C-KS and W-KS, suggesting that the individual system does not induce metabolic changes on these pathways (Figure 2).

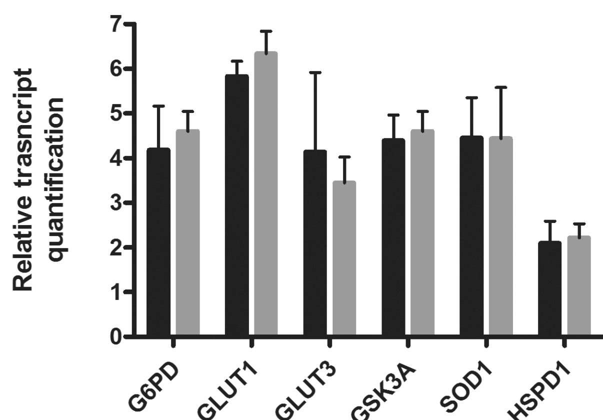

Figure 2 – Relative quantification (ΔCt) of selected genes in W-KS and C-KS groups, as determined by qPCR. For all the comparisons, statistical difference was considered when $p < 0.05$

Discussion

Monitoring a single embryo from the moment of fertilization until blastocyst stage can be truly valuable for scientific purposes as it is a non-invasive method to track background physiology, assess quality parameters and because this procedure may support studies of oocyte/embryo metabolism and variations on the pattern of gene expression (CAROLAN et al., 1996; OYAMADA; FUKUI, 2004).

In the present study the main goal was the development of fully individualized culture protocol as effectively as the usual group culture. That means similar *in vitro* production rates and minimal metabolic alterations. Despite the difference found in blastocyst rates between C-SOF and W-SOF, the individualized system produced similar rates to the ones obtained in routine group culture.

It is known that in an *in vivo* situation, the zygote is surrounded by less than 1 μL of fluid (WHEELER at al., 2007), thus it is important to optimize individual *in vitro* culture in small volumes of media. In the system applied, the development of embryos did not seem to be compromised by accumulation of toxic metabolites, which is common when smaller drops of media are used (CAROLAN et al., 1996).

Micro-well adaptation as performed in the proposed system provides the maintenance of stable conditions and an optimal microenvironment, which is more relatable to the one found in a physiological situation. The alteration of the physical environment also prevents the contact of viable embryos with blocked embryos that could be releasing inhibitory factors in culture media, and the contact with byproducts of the other embryos' metabolism.

Quantitative PCR analysis corroborated the embryo production data showing that the proposed individual system works as well as the group-culture system. The same

pattern of gene expression was also observed in all evaluated genes. It suggests that in both groups the blastocysts have the same ability to internalize energy substrates, repairing protein folding damage and eliminating degraded proteins, and to respond to the ROS effect (CHENG et al., 1990; AUGUSTIN et al., 2001; YOON et al., 2014).

In conclusion, this study determined a standard routine protocol to culture bovine embryos singularly in order to produce as many good-quality embryos as in routine group-culture practice.

Acknowledgements

The authors wish to acknowledge FAPESP (2012/10351-2 and 2013/13199-0) for providing funding for the research and a scholarship to Ms. Annes. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

The authors have no conflict of interest to disclose.

References

AUGUSTIN, R.; POCAR, P.; NAVARRETE-SANTOS, A.; WRENZYCKI, C.; GANDOLFI, F.; NIEMANN, H.; FISCHER, B. Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. **Molecular Reproduction and Development**, v. 60, n. 3, p. 370-376, 2001. doi: 10.1002/mrd.1099.

BUNEL, A.; JORSSEN, E. P.; MERCKX, E.; LEROY, J. L.; BOLS, P. E.; SIRARD, M. A. Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential. **Theriogenology**, v. 83, n. 2, p. 228-237, 2015. doi: 10.1016/j.theriogenology.2014.09.019.

CAROLAN, C.; LONERGAN, P.; KHATIR, H.; MERMILLOD, P. In vitro production of bovine embryos using individual oocytes. **Molecular Reproduction and Development**, v. 45, n. 2. p. 145-150, 1996. doi: 10.1002/(SICI)1098-2795(199610)45:2<145::AID-MRD6>3.0.CO;2-T.

CHENG, M. Y.; HARTL, F. U.; HORWICH, A. L. The mitochondrial chaperonin hsp60 is required for its own assembly. **Nature**, v. 348, n. 6800, p. 455-458, 1990. doi:10.1038/348455a0.

FRAME, S.; COHEN, P. GSK3 takes centre stage more than 20 years after its discovery. **Biochemical Journal**, v. 359, n. 1, p. 1-16, 2001. doi: 10.1042/bj3590001.

GARCIA-HERREROS, M.; APARICIO, I. M.; RATH, D.; FAIR, T.; LONERGAN, P. Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced in vitro. **Reproduction, Fertility, and Development**, v. 24, n. 2, p. 344-352, 2012. doi: 10.1071/RD11080.

GOOVAERTS, I. G. F.; LEROY, J. L. M. R.; LANGBEEN, A.; JORSSEN, E. P. A.; BOSMANS, E.; BOLS, P. E. J. Unravelling the needs of singly in vitro-produced bovine embryos: from cumulus cell co-culture to semi-defined, oil-free culture conditions. **Reproduction Fertility, and Development**, v. 24, n. 8, p. 1084-1092, 2012. doi: 10.1071/RD11286.

GOOVAERTS, I. G. F.; LEROY, J. L. M. R.; VAN SOOM, A.; DE CLERCQ, J. B. P.; ANDRIES, S.; BOLS, P. E. J. Effect of cumulus cell co-culture and oxygen tension on the in vitro developmental competence of bovine zygotes cultured singly. **Theriogenology**, v. 71, n. 5, p. 729-738, 2009. doi: 10.1016/j.theriogenology.2008.09.038.

OYAMADA, T.; FUKUI, Y. Oxygen tension and medium supplements for in vitro maturation of bovine oocytes cultured individually in a chemically defined medium. **Journal of Reproduction and Development**, v. 50, n. 1, p. 107-117, 2004. doi: doi.org/10.1262/jrd.50.107.

PANTALEON, M.; KAYE, P. L. Glucose transporters in preimplantation development. **Reviews of Reproduction**, v. 3, n. 2, p. 77-81, 1998. doi: 10.1530/ror.0.0030077.

SUGIMURA, S.; AKAI, T.; SOMFAI, T.; HIRAYAMA, M.; AIKAWA, Y.; OHTAKE, M.; HATTORUI, H.; KOBAYASHI, S.; HASHIYADA, Y.; KONISHI, K.; IMAI, K. Time-lapse cinematography-compatible polystyrene-

based microwell culture system: a novel tool for tracking the development of individual bovine embryos. **Biology of Reproduction**, v. 83, n. 6, p. 970-978, 2010. doi: 10.1095/biolreprod.110.085522.

TAGAWA, M.; MATOBA, S.; NARITA, M.; SAITO, N.; NAGAI, T.; IMAI, K. Production of monozygotic twin calves using the blastomere separation technique and Well of the Well culture system. **Theriogenology**, v. 69, n. 5, p. 574-582, 2008. doi: 10.1016/j.theriogenology.2007.11.003.

TAKAHASHI, M. Oxidative stress and redox regulation on *in vitro* development mammalian embryos. **Journal of Reproduction and Development**, v. 58, n. 1, p. 1-9, 2012. doi: 10.1262/jrd.11-138N.

VAJTA, G.; PEURA, T. T.; HOLM, P.; PÁLDI, A.; GREVE, T.; TROUNSON, A. O.; CALLESEN, H. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. **Molecular Reproduction and Development**, v. 55, n. 3, p. 256-264, 2000. doi: 10.1002/(SICI)1098-2795(200003)55:3<256::AID-MRD3>3.0.CO;2-7.

WHEELER, M. B.; WALTERS, E. M.; BEEBE, D. J. Toward culture of single gametes: the development of microfluidic platforms for assisted reproduction. **Theriogenology**, v. 68, p. S178-S189, 2007. Supplement 1. doi: 10.1016/j.theriogenology.2007.04.042.

YOON, S. B.; CHOI, S. A.; SIM, B. W.; KIM, J. S.; MUN, S. E.; JEONG, P. S. Developmental competence of bovine early embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. **Biology of Reproduction**, v. 90, n. 5, p. 104, 2014. doi: 10.1095/biolreprod.113.113480.