Well-of-the-well (WOW) versus polyester mesh (PM): a comparison of single-embryo culture systems in bovines

Pozo dentro de pozo (WOW) versus tela de poliéster (PM): una comparación de sistemas de cultivo individual de embriones bovinos

Well-of-the-well (WOW) versus malha de poliéster (PM): uma comparação de sistemas de cultura de um único embrião em bovinos

Daniel Contreras-Benicio; Beatriz Elena Castro-Valenzuela; Juan Alberto Grado-Ahuir; María Eduviges Burrola-Barraza*.

Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua. Chihuahua, Chihuahua, México.

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Abstract

Background: Mexico is innovating in the livestock industry through *in vitro* generation of bovine embryos with technologies such as well-of-the-well (WOW) and polyester mesh (PM) single-embryo culture systems. These techniques allow to maintain embryos in separate areas of a shared culture medium. **Objective:** To compare the quantity and quality of bovine embryos produced in WOW and PM culture systems versus the conventional (CG) culture system. **Methods:** In total, 345 embryos fertilized *in vitro* were evaluated for blastocyst yield in the three culture systems. To count blastocyst cell numbers, 69 embryos in each system were differentially stained for trophectoderm (TE), inner cell mass (ICM), and apoptotic cells. A qPCR gene expression analysis was performed for embryos in all three systems. **Results:** The WOW, PM and CG systems developed similar amount of blastocysts (41, 35 and 36%, respectively; $p>0.05$). Blastocysts in all three systems showed adequate amounts of ICM and apoptotic cells. Blastocysts in the PM system showed a greater number of TE cells [63.7 versus 58.6% in the CG system ($p<0.05$)]. Relative mRNA expression of the embryonic genes *POUF5F1, GNAS* and *TP53* did not differ significantly among systems ($p>0.05$). The *ATP5B* expression was higher in WOW than in PM ($p<0.05$), but similar to CG ($p>0.05$). The *TJP3* expression was higher in PM than in WOW and CG ($p<0.05$). Expression of *ID2* and *CLDN4* was higher in WOW than in PM and CG ($p<0.05$). The biplot graphic from Principal Component Analysis (PCA) revealed that CG was located near degenerated embryos, whereas PM was located near arrested embryos, larger ICM and TE, and *TJP3* expression. The WOW was located toward blastocysts, morulae, and expression of *CLDN4*, *ID2* and *GNAS*. **Conclusion:** Compared with CG, both the PM and WOW systems are good options for culturing single embryos in the bovine model. Moreover, the PCA results suggest that embryos developed in the WOW system have greater capacity for generating blastocysts with increased ability to form TE and ICM layers, which might improve implantation.

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***Corresponding author.** Perif. R. Aldama Km 1, 31031 Chihuahua, Mexico. Phone: +526 144 340 303. E-mail: mburrola1@uach.mx

Keywords: *bovine embryo; embryo quality; embryo recovery; in vitro culture; in vitro embryo production; polyester mesh culture; single-embryo culture; well-of-the-well culture.*

Resumen

Antecedentes: México está innovando en la industria ganadera a través de la generación *in vitro* de embriones bovinos con tecnologías de cultivo individual como lo son Pozo dentro de Pozo (WOW) y Malla de Poliéster (PM). Estos mantienen los embriones en áreas separadas mientras comparten un mismo medio de cultivo celular. **Objetivo:** Comparar la cantidad y calidad de embriones bovinos producidos en los sistemas WOW y PM contra el sistema de cultivo convencional en grupo (CG). **Métodos:** En total se evaluaron 345 embriones fertilizados *in vitro* para determinar la producción de blastocistos generados en los tres sistemas. Para contar el número de células por blastocisto, 69 embriones en cada sistema se tiñeron diferencialmente para trofectodermo (TE), masa celular interna (ICM) y células apoptóticas. Se realizó un análisis de expresión génica por qPCR de los embriones obtenidos en los tres sistemas. **Resultados:** Los sistemas WOW, PM y CG desarrollaron similares cantidades de blastocistos (41, 35 y 36%, respectivamente; p>0,05). Los blastocistos en los tres sistemas mostraron cantidades adecuadas de ICM y células apoptóticas. Los blastocistos en el sistema PM mostraron un mayor número de células TE [63,7% versus 58,6% en el sistema CG (p<0,05)]. La expresión relativa de mRNA de los genes embrionarios *POUF5F1, GNAS* y *TP53* no difirió significativamente entre los sistemas (p>0,05). La expresión de *ATP5B* fue mayor en WOW que en PM (p<0,05), pero similar a CG (p<0,05). La expresión de *TJP3* fue mayor en PM que en WOW y CG (p<0,05). La expresión de *ID2* y *CLDN4* fue mayor en WOW que en PM y CG (p<0,05). El gráfico de biplot del análisis de componentes principales reveló que CG se encontró cerca de embriones degenerados, mientras que PM se encontró cerca de embriones en arresto, ICM, TE, y *TJP3*. El WOW se localizó hacia blastocistos, mórulas y la expresión de *CLDN4*, *ID2* y *GNAS*. **Conclusión:** En el modelo bovino los sistemas PM y WOW son buenas opciones para cultivar embriones individuales, ya que se obtienen resultados muy similares a los obtenidos con el sistema CG. Además, los resultados de PCA sugieren que los embriones individuales desarrollados en el sistema WOW generan blastocistos con mayor capacidad de formar TE e ICM, lo que podría mejorar su éxito de implantación.

Palabras clave: *calidad embrionaria; cultivo de malla de poliéster; cultivo individual; cultivo in vitro; cultivo pozo-dentro-de-pozo; embriones bovinos; producción in vitro de embriones; recuperación de embriones.*

Resumo

Antecedentes: O México está inovando na indústria pecuária por meio da geração *in vitro* de embriões bovinos com tecnologias de cultura de embriões individuais, bem como em poço (WOW) e malha de poliéster (PM). Estes mantêm os embriões em áreas separadas, enquanto compartilham o mesmo meio de cultura de células. **Objetivo:** Comparar a quantidade e a qualidade de embriões bovinos produzidos nos sistemas de cultura WOW e PM com o sistema convencional de cultura em grupo (CG). **Métodos:** No total, 345 embriões fertilizados *in vitro* foram avaliados para determinar a produção de blastocistos gerados nos três sistemas. O número de células por blatocisto foi contado, 69 embriões em cada sistema foram diferencialmente corados para trofectoderme (TE), massa celular interna (ICM) e células apoptóticas. Uma análise de expressão gênica qPCR foi realizada para os embriões obtidos nos três sistemas. **Resultados:** Os sistemas WOW, PM e CG desenvolveram quantidades semelhantes de blastocistos (41, 35 e 36%, respectivamente; p>0,05). Os blastocistos nos três sistemas mostraram quantidades adequadas de ICM e células apoptóticas. Os blastocistos no sistema PM mostraram um número maior de células TE [63,7 versus 58,6% no sistema CG (p<0,05)]. A expressão relativa do mRNA dos genes embrionários *POUF5F1, GNAS* e *TP53* não diferiu significativamente entre os sistemas (p>0,05). A expressão de *ATP5B* foi maior no WOW do que no PM (p<0,05), mas semelhante ao GC (p<0,05). A expressão de *TJP3* foi maior no PM do que no WOW e CG (p<0,05). A expressão de *ID2* e *CLDN4* foi maior no WOW do que no PM e CG (p<0,05). O gráfico biplot da análise de componentes principais revelou que CG foi encontrado próximo a embriões degenerados, enquanto PM foi encontrado próximo a embriões presos, ICM, TE e *TJP3*. WOW foi encontrado para ter blastocistos, mórulas e a expressão de *CLDN4*, *ID2* e *GNAS*. **Conclusão:** Em comparação com o CG, os sistemas PM e WOW são boas opções para a cultura de embriões individuais no modelo bovino. Além disso, os resultados da PCA sugerem que embriões individuais desenvolvidos no sistema WOW têm maior capacidade de desenvolver blastocistos com maior capacidade de formar as camadas TE e ICM, o que poderia melhorar seu sucesso de implantação.

Palavras-chave: *cultura em tela de poliéster; cultura individual; cultura in vitro; cultura well-in-well; embriões bovinos; qualidade embrionária; produção de embriões in vitro; recuperação embrionária.*

Introduction

A priority for most Latin American countries, including Mexico, is to develop an agricultural sector that is capable of safely and efficiently producing enough food to meet population demands. This objective can be reached through innovation of technologies used to produce bovine embryos, thus allowing to improve the competitiveness of the meat and dairy industry. *In vitro* embryo production has become an excellent tool to investigate reproduction mechanisms and is used in procedures for gene editing, cloning by somatic cell nuclear transfer (SCNT), and production of embryonic stem cells (Hansen, 2020a). Conventional evaluation of embryos cultured *in vitro* is based on visual assessment of cell morphology, which may be subjective since it depends on the technician's experience and judgement. This is reflected in the low pregnancy rates (~50%) recorded for embryos selected under this criterion (Ealy *et al.*, 2019; Hansen, 2020b; Lopes *et al.*, 2020). Additional criteria for embryo quality include: number of cells in the inner cell mass (ICM) and trophectoderm (TE), incidence of apoptosis, eclosion capacity, chromosome abnormalities, and expression of specific genes, among others (Sugimura *et al.*, 2012). Evaluation of these features requires that the embryos are correctly identified, which is challenging to achieve in conventional group (CG) culture since it is conducted with groups of approximately 50 embryos in the same culture dish (Fujita *et al.*, 2006; Salvador *et al.*, 2011). A potential solution to this issue is single-embryo culture; however, several studies (Marianowski *et al.*, 2007; Salvador *et al.*, 2011) have been unsuccessful at single-embryo production because individual embryos do not benefit from the paracrine and autocrine factors secreted by multiple embryos and accumulated in CG culture (Fujita *et al.*, 2006; Gopichandran and Leese, 2006; Paria and Dey, 1990). Therefore, if quality selection is desired, a cell culture system that allows identifying embryos without sacrificing the benefits of CG culture is necessary. A way to achieve this is to sequester single embryos in the same culture medium; and two systems, well-of-the-well (WOW) and polyester mesh (PM), have been used with good results in species such as cattle (Sugimura *et al.*, 2010; Vajta *et al.*, 2000), pigs (Vajta *et al.*, 2008), mice (Komori *et al.*, 2012), and humans (Vajta *et al.*, 2010a; Vajta *et al.*, 2010b). Therefore, the objective of this work was to compare WOW and PM single-embryo culture systems versus CG system as alternatives for generating better-quality bovine embryos.

Materials and Methods

Ethical considerations

All experiments were conducted in accordance with the institutional code for Bioethics Regulation for Animal Welfare of Universidad Autónoma de Chihuahua, México (case number: CFTZyE-Acta-101/2015:ACUERDO 4.2).

In vitro oocyte maturation

Bovine ovaries were collected in a Mexican slaughterhouse (Tipo Inspección Federal -TIF-366) and transported within 2 hours to the laboratory in sterile sodium chloride 0.15 M (22–25 °C). The cumulus-oocyte complexes (COC) in the ovaries were aspirated from less than 10 mm diameter follicles using a BD 18G x $1\frac{1}{2}$ PrecisionGlide needle (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with ~50 mmHg pressure of vacuum suction (WOB-L® Dry Vacuum Pumps, Standard-Duty, Welch®, Denver, CO, USA). All chemical compounds used for media culture were from Sigma (St Louis, MO, USA). The chemically defined CDM medium was as follows: 710 mM NaCl, 60 mM KCl, 10 mM KH_2PO_4, 5 mM Na-citrate, 5 mM $NaHCO_3$, 2 mM $CaCl_2$, 4.9 mM glycine, 1 mM alanyl-glutamine, 20 mM HEPES, 10 mM sodium L-lactate, 0.5 mM Na-pyruvate, 0.5 mM $MgSO_4$, 67 mM non-essential amino acids, and 25 µg/mL gentamycin. The COC were washed twice in a chemically semi-defined medium for oocyte handling, H-CDM-M [CDM supplemented with 0.5 mM D-fructose, 2.5% fatty acid-free bovine serum albumin (BSA), 22.5 mM NaCl and 20 µg/mL heparin Na salt] and were selected for study if at least three coats of cumulus cells were

detected under a Leica MS5 stereomicroscope (Leica Microsystems, Wetzlar, Germany). The COC were cultured in four-well plates (Nunc, Thermo Scientific, Rockford, IL, USA). Briefly, groups of 50 COC per well were maintained in 1 mL of chemically semi-defined medium for *in vitro* maturation, M-CDM (CDM supplemented with 2 mM D-fructose, 2.77 mM myo-inositol, 0.1 mM taurine, 5% fatty acid free BSA, 15 ng/mL follicle stimulating hormone (FSH), 1 µg/µL luteinizing hormone (LH), 0.1 µg/µL estradiol-17β, 50 ng/µL epidermal growth factor (EGF), and 0.1 mM cysteamine) at 38.5 °C with 5% CO_2 at 100% humidity for 24 h.

In vitro fertilization

After 24 h of COC *in vitro* maturation, four-well plates (Nunc, Thermo Scientific, Rockford, IL, USA) were prepared, with each well containing 430 µL of chemically defined medium for *in vitro* fertilization, F-CDM (CDM supplemented with 0.5 mM D-fructose, 2 mM caffeine, 5% BSA and 2 µg/mL heparin and 14 mM NaCl). One mL of purified water was added to the center of each four-well plate. Oocytes at the meiosis II (MII) stage were transferred in groups of 50 to each well. Semen from an Angus bull was used for IVF, as follows: 0.5 mL straws were thawed at 35 °C for 35 s. The semen was centrifuged in a Percoll gradient solution (45 and 90%) at 400 x *g* for 20 min. The resulting pellet was re-suspended in 4.5 mL F-CDM and centrifuged again (400 x *g* for 5 min). After the medium was aspirated, the cell concentration was adjusted to 1×10^6 sperm per mL, and 50 µL of this sperm dilution solution was added to each well and the plates were incubated at 38.5 °C in 5% CO_2 in humidity-saturated air. Eighteen hours post-IVF, the potential embryos were transferred to 0.5 mL microcentrifuge tubes containing 100 µL of chemically defined medium for handling of early embryos, H-CDM-1 (CDM supplemented with 0.5 mM D-fructose, 2.5% fatty acid free BSA and 22.5 mM NaCl). Then, they were vortexed for 1 min to remove cumulus cells. Then 50 embryos were placed in 500 µL of chemically defined medium for *in vitro* culture of early embryos, CDM-1 (CDM supplemented with 0.5 mM D-fructose, 2.77 mM myo-inositol, 0.1 mM taurine, 5% BSA, 0.1 mM EDTA and 1 mM NaCl) in each well of a four-well plate. One mL of distilled water was added to the center of the plate. After 60 h at 39 °C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in humidity-saturated air, embryos at the eight-cell stage were incubated and selected in chemically defined medium for handling of late embryos, H-CDM-2 (CDM supplemented with 2 mM fructose, 2.5% BSA, 1.47 mM essential amino acids and 26.5 mM NaCl). Following selection, embryos were transferred to 400 µL of chemically defined medium for *in vitro* culture of embryos, CDM-2 (CDM supplemented with 2 mM D-fructose, 2.77 mM myo-inositol, 1.47 mM essential amino acids, 5% BSA and 5 mM NaCl) in four-well plates. One mL of purified water was added to the center hole of the plate and it was then incubated with 5% CO_2, 5% O_2, and 90% N_2 in humidity-saturated air at 39 °C for four days. Subsequently, 8-cell embryos were selected and maintained in H-CDM-2 medium and were organized into 20 embryo groups to be cultured in the WOW, PM and CG systems.

Single-embryo culture with shared medium

Two *in vitro* single-embryo culture systems, PM and WOW, were used with the purpose of generating individual areas to accommodate an embryo with an approximate diameter of 75 ⌈m, and with a 165- ⌈m separation between them, as reported by Gopichandran and Leese (2006).

The PM system was implemented as reported by Somfai *et al.* (2010), with some modifications. A polyester mesh (07-300/36, Sefar, Heiden, Switzerland), whose interwoven threads generate areas with 136 ⌈m diameter, each space separated by 200 ⌈m, were cut into 1.5 mm × 2 mm pieces. These pieces of mesh were placed at the bottom of each well of the four-well dish, and each embryo was placed in the space created by the interwoven threads (Figure 1A).

The WOW experiments were conducted as reported by Vajta *et al.* (2008) with some modifications, as follows: in four-well plates containing 400 µl of CDM-2 medium, micro-

wells were created at the bottom of the well by applying mechanical pressure using an aggregation needle (DN- 09/B, BLS® Biological Laboratory Equipment Maintenance and Service Ltd., Budapest, Hungary). The resulting micro-wells were 220 ⌈m in diameter, each separated by a distance of 248 ⌈m. Twenty micro-wells were formed in each well of the four-well plate, guided by a template placed on the outside of each well. This template defines an array represented by the letters a, b, c, d and e at the top, and the numbers 1, 2, 3 and 4 on the left side (Figure 1B).

Differential staining: trophectoderm (TE) and inner cell mass (ICM)

Blastocysts were washed three times in 0.1% polyvinylpyrrolidone in phosphate-buffered saline (PBS-PVP) and permeabilized by incubation for 20 s in 0.2% Triton X-100 in PBS-PVP. The blastocysts were then washed three times in PBS-PVP, and transferred to PBS-PVP containing 100 µg/ml propidium iodide to stain the TE cells, incubating them in complete darkness at 37 °C in a humid environment for 5 min. The blastocysts were then washed three times in PBS-PVP. To fix the blastocysts and stain the ICM cells, the embryos were incubated for 30 min in 4% paraformaldehyde (PFA) solution containing 10 µg/ml Hoechst 33258, and finally were washed three times in PBS-PVP.

TUNEL procedure for cell apoptosis detection

Embryos were washed three times in PBS-PVP, then transferred to a 96-well plate containing 100 µl PBS-PVP and 100 µl 4% PFA, and incubated for 60 min at 15-25 °C. Embryos were then washed in another well with 200 µl PBS-PVP. They were then permeabilized by incubation for 5 min in ice (2-8 °C) in a freshly prepared 0.1% Triton X-100 solution in 0.1% sodium citrate, and then washed three times in PBS-PVP. For TUNEL staining of the blastocysts, an *In Situ* Cell Death Detection Kit Fluorescein (Roche Diagnostics, Indianapolis, IN, USA) was used following the manufacturer's instructions.

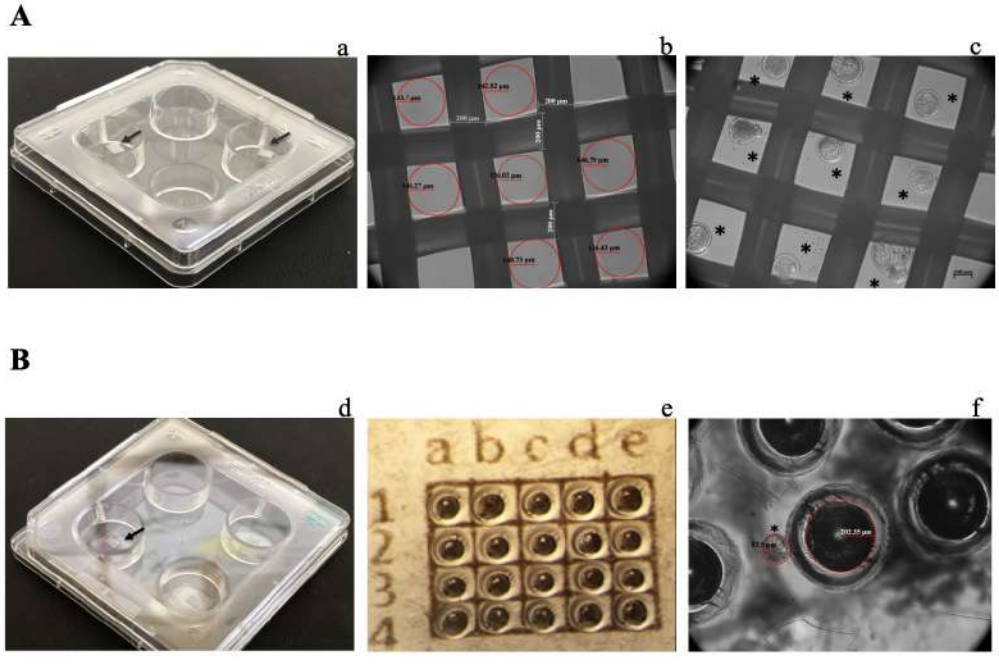

Figure 1. Prototypes of single-embryo cell culture. A. Polyester mesh (PM): a. 4-well dish, the black arrows indicate the micro-wells inside the well; b. View of PM; c. Blastocysts cultured individually in PM. B. Well-of-the-well (WOW): d. 4-well dish, the black arrow indicates the micro-wells inside the well; e. Micro-well template; f. View of micro-wells. In b and f, the red circles indicate the micro-well area. In c and f, * indicates a blastocyst. The images in b, c, e and f were taken with an Axiovert CFL40 microscope (Carl Zeiss, Oberkochen, Germany) using 10× phase contrast.

Permeabilized blastocysts were incubated in the dark in TUNEL reaction solution (1:10 dilution of terminal deoxynucleotidyl transferase enzyme in labeling solution with deoxynucleotidyl triphosphates, dNTPs) for 60 min at 37 ºC. For each TUNEL procedure, positive control embryos were treated with DNase I (3 U/mL) for 10 min at 25 ºC, then subjected to the TUNEL reaction; for the negative control, the embryos were incubated in the absence of the terminal deoxynucleotidyl transferase enzyme.

Blastocyst mounting and microscopy

Blastocysts were placed on a slide in a 10-⌈L drop of glycerol and the labeling was observed using an Axio Imager M2 fluorescence microscope (Carl Zeiss, Inc. Göttingen, Germany) at 565 nm for apoptosis, 455 nm for nuclear staining of ICM (Hoechst 33258), and 617 nm for nuclear staining of TE (propidium iodide). Images were acquired using AxioVision Software and AxioCam MRm digital Camera (Carl Zeiss, Inc. Göttingen, Germany).

Gene expression analysis via qPCR assay

Total RNA of 25 embryos was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and stored at -80 °C until used for cDNA synthesis. For all samples, the RNA concentration was determined by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Purity of nucleic acid was determined by calculating the ratio of absorbance between 260 and 280 nm. The cDNA synthesis was performed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions using a mixture of 2 µg of RNA. The reactions were placed in a thermocycler (Corbett Research, San Francisco, CA, USA) under the following program: 60 min at 37 °C, 5 min at 95 °C to deactivate the enzyme, and kept at 4 °C until use. The concentration of obtained cDNA was determined by measurement of absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and the purity of the nucleic acid was determined by calculating the ratio of absorbance between 260 and 280 nm. The cDNA was stored at -20°C until further use. For the analysis of gene expression, all reactions were performed using Real Time StepOne equipment (Applied Biosystems, Carslsbad, CA, USA). Amplification reagents for specific genes were obtained by using the TaqMan Universal Master Mix II and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for *POUF5F1* (Bt03223846_g1), *ATP5B* (Bt03216727_m1), *ID2* (Bt03220879_m1), *MATER* (Bt03218033_m1), *GNAS* (Bt03251812_g1), *TJP3* (Bt03237880_m1), *TP53* (Bt03223222_m19) and *CLDN4* (Bt04318530_s1), including the FAM reporter for the genes quantified. The reactions were performed according to the manufacturer's protocol; each reaction contained 50 ng cDNA, and the quantification of the expression of these genes was normalized to the endogenous GADPH gene (Bt3210913_g1); the results show relative abundance, calculated according to the $2^{-\Delta Ct}$ method, where ⊗Ct was generated by subtracting the reference gene Ct value from that of the target gene (Livak and Schmittgen, 2001).

Statistical analysis

All statistical analyses were performed using XLSTAT Software (Addinsoft, New York, NY, USA). The data for degenerated and arrested embryos, morulae, blastocysts, TE and ICM are expressed as percentage values. The gene expression data are given as $2^{-\Delta Ct}$ relative abundance. The normality of the data was confirmed with the Jarque-Bera test, and non-normal data were transformed with BOX-COX. Normally distributed data were subjected to one-way ANOVA and means comparison was obtained by Tukey's test. The level of statistical significance was set at $p<0.05$. All values are presented as means with their corresponding standard error.

To analyze the relationship between culture system and number of cells, type of embryo, and gene expression results, the Spearman correlation was applied, followed by a Principal Component Analysis (PCA), where the principal components (PC) were rotated with the Equamax method and the results shown in a biplot graphic.

Results

Comparison between culture systems

Figure 2 compares the WOW and PM single-embryo culture systems against the CG culture in terms of embryo development. The percentage of blastocysts was similar in all systems (41 ± 4.35% in WOW, 35 ± 3.65% in PM and 36 ± 6.0% in CG; $p > 0.05$). The same was observed for the embryos that reached the morula stage (29 ± 5.76% in WOW, 26 ± 5.5% in PM and 27 ± 6.7% in CG; $p>0.05$). The percentage of arrested embryos was similar in all systems (24 ± 6.2% in PM, 20 ± 4.11% in WOW and 20 ± 4.13% in CG; $p>0.05$). The production of degenerated embryos did show a significant difference; the WOW system produced fewer degenerated embryos compared to the other two methods (11 ± 0.54% versus 15 ± 1.8% in CG and 17 ± 2.7% in PM; $p<0.05$).

Effect of culture system on blastocyst cell numbers

To examine the effect of culture method on cellular composition of the embryo, we counted the number of cells in the ICM and TE, and the number of apoptotic cells (Table 1). Embryos cultured in all systems had similar number of total cells (81.3 ± 4.0 in PM, 74.4 ± 4.5 in WOW, 68.8 ± 3.6 in CG, $p>0.05$). Embryos in the three culture systems had similar percentage of ICM (41.4 ± 1.7 in GC, 36.3 ± 1.3 in PM and 38.8 ± 1.8 in WOW, $p>0.05$). Embryos in the PM system had a significantly higher proportion of TE cells (63.7 ± 3.1%) compared to those in the CG system (58.6 ± 2.6%, $p<0.05$), but not compared to the WOW system (61 ± 3.2%, $p>0.05$). The ICM/TE ratio was very close in all systems (0.7 ± 0.06 in GC and WOW, and 0.6 ± 0.03 in PM).

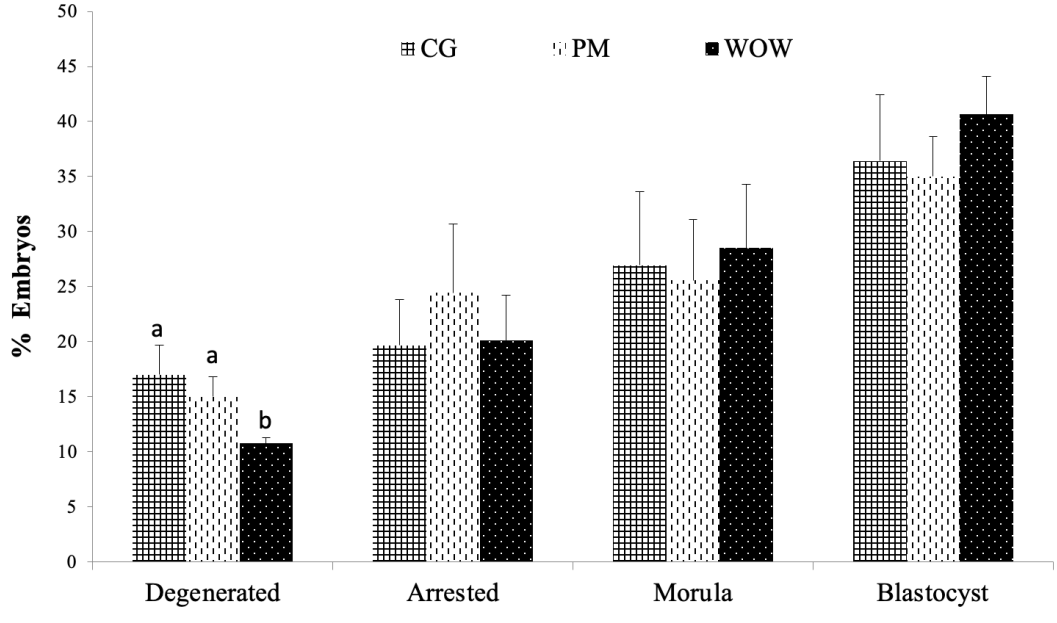

Figure 2. Comparison between the type of embryo developed as of day 7 post-IVF in each culture type: conventional culture in groups (CG), polyester mesh (PM), and well-of-the-well (WOW). Data are shown as means □ standard error of the percentage of analyzed embryos. In total, 345 embryos distributed in six independent repetitions were evaluated for the three culture types (CG = 117, PM = 115 and WOW = 113).

Regarding the formation of apoptotic cells, embryos in the three culture systems presented very similar numbers, with approximately 2 apoptotic cells for each blastocyst evaluated.

Effect of culture system on relative expression of embryonic genes

The relative mRNA expression levels of embryonic genes *POUF5F1, GNAS* and *TP53* did not show significant differences between WOW, PM and CG culture systems. However, expression of *ATP5B* gene was higher ($p<0.05$) in WOW embryos than in PM embryos. On the other hand, expression of *TPJ3* gene was higher in PM system compared to CG and WOW systems ($p<0.05$). Likewise, gene expression of *ID2* and *CLDN4* was higher in WOW culture than in PM and CG systems ($p<0.05$). Regarding the expression of *mater*nally expressed *MATER* gene (a negative control), it was negative in all three culture systems (Figure 3).

Correlation between cell culture system, type of embryo developed, apoptosis, and embryonic gene expression

Principal component (PC) analysis was used to investigate relationships between cell culture systems and the various embryo parameters studied, and two PCs were obtained: PC1 absorbed 53.16% of the variables, while PC2 absorbed 46.84%; together they absorbed 100% of the variables.

Table 1. Effect of the culture system on the number of cells in blastocysts.

Culture system	Blastocysts tested	Number of cells per blastocyst				
		N*	ICM (%)	TE (%)	ICM/TE	Apoptotic (%)
		68.8 ± 3.6	28.5 (41.4 ± 1.7)	40.4 (58.6 ± 2.6)[a]	0.7 ± 0.06	2.2 (3.2 ± 0.6)
PM	23	81.3 ± 4.0	29.5 (36.3 ± 1.3)	51.7 (63.7 ± 3.1)[b]	0.6 ± 0.03	2.0 (2.5 ± 0.44)
WOW	26	74.4 ± 4.5	28.8 (38.8 ±1.8)	45.6 (61 ± 3.2)[ab]	0.7 ± 0.04	2.2 (3 ± 0.46)

The data represents means ± standard error. *From 5 independent repetitions (each with 4 to 6 blastocysts).
[a, b]Values with different superscripts letters within the TE column differ significantly ($p<0.05$). ICM: internal cell mass. TE: trophectoderm.

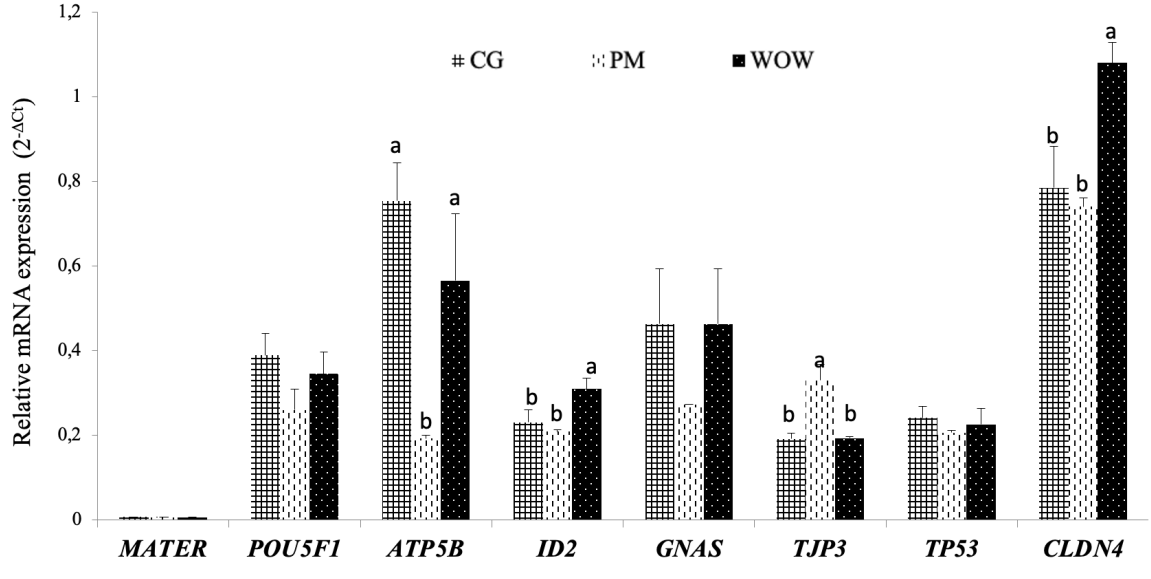

Figure 3. Effect of culture system on relative expression of *MATER, POU5F1, APM5B, ID2, GNAS, TJP3* and *CLDN4* genes. Data are presented as means ± standard error of the $2^{-\Delta Ct}$ value of three independent experiments (each with 25 embryos). For each gene, bars with different letters differ significantly ($p<0.05$).

This means that enough information was generated to interpret the most important aspects of the data set. The CG system was projected near degenerated embryos and expression of *MATER* gene; the PM system was projected near arrested embryos, higher values of ICM and TE, and expression of *TJP3* gene; and the WOW system was projected toward blastocysts and morulae, as well as toward the expression of CLDN4, *ID2* and *GNAS* genes (Figure 4).

Discussion

The most efficient way for *in vitro* embryo generation is by group culture (CG), but this approach makes it difficult to label or identify individual embryos, and a sudden movement of the culture dish can cause an embryo to be displaced from its original location. Here, we compared the alternative WOW and PM single-embryo culture protocols reported by Vajta *et al.* (2008) and Somfai *et al.* (2010), respectively, where embryos are maintained in defined areas but within the same cell culture medium, to the CG culture system for development of bovine embryos.

Initially, we found that all systems developed similar blastocyst yields. This is interesting because either WOW or PM single-embryo cultures have the ability to generate the same yields that conventional group culture. The WOW system allows each embryo to develop and maintain its own microenvironment, but still sharing autocrine and/or paracrine factors secreted by neighboring embryos (Dai *et al.*, 2012). In addition to this, the partial opening to each micro-well allows for the provision of nutrients and dilution of toxic factors such as ammonia and free radicals (Vajta *et al.*, 2008). The PM system allows embryos to stay in the same microenvironment, and the opening of polyester mesh sections allow the free pass of autocrine and/or paracrine factors (Somfai *et al.*, 2010).

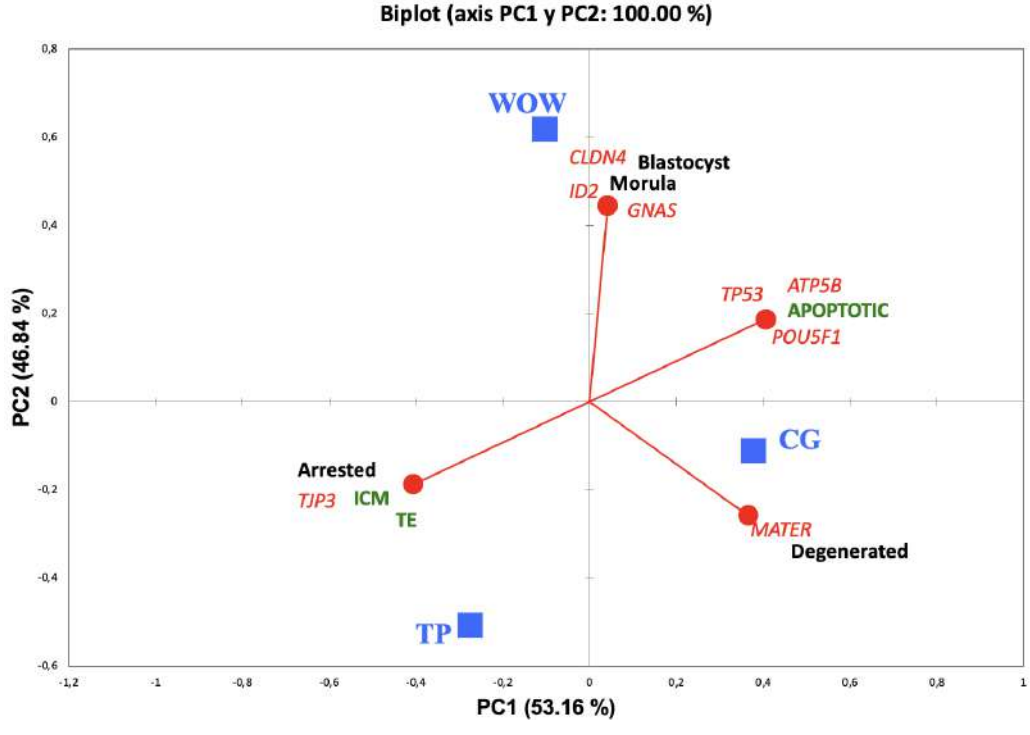

Figure 4. Relationships between culture system and class of embryos developed, type of blastocyst cells, and genes expressed 7 days post-IVF. Blue squares represent three different culture systems (CG, PM and WOW) and circles are the variables: black text designates class of embryos (blastocyst, morula, arrested and degenerated); green text designates type of cells (apoptotic, ICM, and TE). Red lines indicate direction of each variable. PC1: principal component 1; PC2: principal component 2.

The percentage of degenerated embryos was lower in WOW compared to PM and CG systems. Although WOW system yielded the highest percentage of blastocysts, this does not mean that those blastocysts had excellent cell quality. Blastocysts comprise two different cell populations, the trophectoderm (TE) and the internal cell mass (ICM). The TE gives rise to placenta and embryonic membrane development, whereas ICM, through differentiation, generates all the tissues comprising the fetus (Fouladi-Nashta *et al.*, 2005). Thus, prior to implantation, a bovine blastocyst must contain 24 to 34 ICM cells, 83 to 91 TE cells and a minimum of 2 apoptotic cells (Fouladi-Nashta *et al.*, 2005; Thouas *et al.*, 2001). The blastocysts obtained in the three evaluated systems exhibited an adequate amount of ICM and apoptotic cells; in regards to TE cells, the blastocysts obtained in PM showed higher number ($p>0.05$) of these cells compared to CG and WOW systems. A lower number of TE cells during early embryogenesis has been related with detrimental placental development (Maylem *et al.*, 2017; Meo *et al.*, 2007). An increase of TE cells has a positive effect on pregnancy establishment (Lopera-Vasquez *et al.*, 2017). This supposes an advantage of PM over WOW and GC systems.

The type of *in vitro* culture is the most critical factor in producing bovine embryos (Corcoran *et al.*, 2006), since it has a direct impact on the phenotype developed by an embryo, and is closely related to the successful development of a blastocyst viable for transfer (El-Aziz *et al.*, 2016). A phenotype modification means a shift in the expression of genes and proteins in early embryos leading to changes in fetal growth, physiological and endocrine parameters after born that affect psychosocial and psychomotor development during life (Sunde, 2019). In ruminants, *in vitro* embryo culture protocols have been related with alterations in phenotype, such as increased birth weight, gestational length, perinatal death, and large offspring syndrome (Sunde, 2019). In cattle, *in vitro* production of embryos is influenced by breed; *Bos indicus* generates greater blastocyst yield than *Bos taurus* cows (GuimarÃes *et al.*, 2020). Additionaly, *Bos indicus* embryos are more tolerant to heat shock (41 °C) than *Bos taurus* embryos (Monteiro *et al.*, 2018; Paula-Lopes *et al.*, 2013). However, in comparison with *Bos taurus*, *Bos indicus* embryos have greater amount of reactive oxygen species (ROS), and this is exacerbated after the cryopreservation process, which causes cell damage and affects embryo viability (Lopez-Damian *et al.*, 2020). Cell culture conditions may negatively affect gene expression profiles and imprinting patterns, even when a blastocyst appears to be successfully developing, based on its cleavage rate and morphological appearance (Khosla *et al.*, 2001). The environment generated is key for embryos to regulate the expression of genes that allow phenomena such as first cell divisions, activation of embryonic genome, morula compaction, and blastocyst formation and expansion (Adjaye *et al.*, 2007; Kues *et al.*, 2008). To ascertain which culture system had the highest amount of embryonic gene expression, we quantified the relative mRNA levels of genes *POUF5F1*, *ATP5B*, ID2, *GNAS*, *TJP3*, *TP53* and *CLDN4*, using the null expression of *mater*nal mRNA *MATER* as negative control.

The capacity of blastocysts to direct differentiation of two types of cell lineage, ICM and TE, is regulated by a precise molecular control that involves activation of specific genes for each lineage (Guo *et al.*, 2010). Thus, in most mammalian species, such as human, mouse, porcine and bovine, formation and pluripotent maintenance of ICM is correlated with the expression of *POUF5F1* transcription factor (Kirchhof *et al.*, 2000; Schiffmacher and Keefer, 2013; Van Eijk *et al.*, 1999); however, in bovine and porcine models, the expression of *POUF5F1* is also correlated with TE, but at a lower rate than for ICM (Kirchhof *et al.*, 2000). Therefore, expression of *POUF5F1* gene is indispensable for cell differentiation in the blastocyst (Roberts *et al.*, 2004). Another gene related to TE cell phenotype is *ID-2*, whose expression is required for maintenance and differentiation of these cells (Garcia *et al.*, 2017) and is key for blastocyst implantation (Roberts *et al.*, 2004; Xie *et al.*, 2013). In the present study, we found neither a

significant difference nor a statistical trend for *POUF5F1* expression across the three culture systems. This implies that embryos obtained from WOW and PM are very similar to those developed in CG system in terms of *POUF5F1* expression; therefore, we can be confident that embryos produced by single-embryo systems have proper differentiation of ICM and TE cell lineage. In the case of *ID2* gene, the relative expression of mRNA was higher ($p<0.05$) in WOW than in PM and CG systems; this suggests that blastocysts generated in WOW may have higher implantation capacity. The biggest challenge of *in vitro* embryo production is low pregnancy rate. The percentage of post-implantation embryo loss is 70–80% (Corcoran *et al.*, 2006; Goovaerts *et al.*, 2010; Scanavez *et al.*, 2013), thus it is possible that WOW could help improve this embryo wastage.

The environment created in *in vitro* culture systems may alter genomic imprinting patterns, with deleterious consequences for embryo development (Thurston *et al.*, 2008). After fertilization, epigenetic reprogramming generates DNA methylation patterns that are necessary for activation or silencing of specific genes to drive normal embryonic development (Urrego *et al.*, 2014). Specifically, largescale genome demethylation occurs after fertilization, where most methyl groups -except those in the imprinting control regions (ICRs)- are eliminated from the DNA before the morula stage. Subsequently, *de novo* methylation occurs in the blastocyst, coinciding with ICM and TE differentiation, where ICM is hypermethylated while TE is hypomethylated (Piedrahita, 2011). The *GNAS* is an imprinting gene located in an ICR. It codes for the □ subunit of guanine nucleotide-binding protein Gs, which stimulates adenylate cyclase activation after hormonal stimulation and also cyclic adenosine monophosphate (cAMP) production for downstream cellular signal transduction pathways (Khatib, 2004; Sikora *et al.*, 2011). In bovines, *GNAS* relative expression is maintained at a low level from 2-cell until morula stage and then it increases to high expression levels in blastocyst stage (Jiang *et al.*, 2015; Ruddock *et al.*, 2004). In the present study, we found that relative *GNAS* expression was not significantly different among the three culture systems, although its expression appeared to be consistently lower in PM. This could imply that the PM system creates a more detrimental environment for *GNAS* expression; if so, this system could have a negative influence on the genomic imprinting process.

A key point for embryonic development is the energy contribution by mitochondria, which, by means of oxidative phosphorylation, generate most of the ATP required by the growing embryo (Roth, 2018). Gene *ATP5B* codes for a subunit of the F1 component of ATP synthase, comprising complex V of the oxidative phosphorylation machinery in the electron carrier chain in the mitochondrial membrane (Bougarn *et al.*, 2011; Gad *et al.*, 2012). Our results show that the expression of this gene was lower in PM than in WOW and CG systems; this suggests that embryos may be affected in their mitochondrial metabolism in PM, which could lead to diminished energy capacity.

Apoptosis is considered a normal event in early embryogenesis, necessary to eliminate abnormal cells that may compromise embryo viability (Betts and King, 2001). During bovine embryonic development there is an initial wave of apoptosis at the 9- to 16-cell stage, which later decreases in morula stage and resumes in blastocyst stage (Byrne *et al.*, 1999). Damaged embryo DNA can be reflected by increased expression of *TP53* gene, whose protein (p53) acts as a transcription factor to activate expression of the apoptotic gene *BAX*. In turn, *BAX* protein acts directly on mitochondrial membrane permeability to liberate cytochrome c, which in turn activates caspase 9, triggering apoptosis (Betts and King, 2001). Thus, in order for an embryo to develop normally it must have an adequate amount of p53 (Liang *et al.*, 2008). The results of the present study show that relative expression of *TP53* was very similar in the three culture systems. This indicates that embryos grown in both WOW and PM have adequate expression of *TP53*, similar to that of embryos cultured in the CG system. This

matches the number of apoptotic blastomeres observed by TUNEL.

The first epithelial structure that arises during early embryo development is TE, which covers the blastocyst surface, surrounds the ICM, and generates the blastocoel cavity by transporting ions, water and other small molecules across its epithelium (Fleming *et al.*, 2001). The TE allows the passage of these molecules to the interior of the blastocoel through the action of transmembrane carriers such as Na^+K^+-ATPase, aquaporins and tight junctions (Furuse and Moriwaki, 2009). In this way, TE works as a barrier isolating ICM from the uterine environment (Moriwaki *et al.*, 2007). Tight junctions become visible around the late morula stage, and in the blastocyst stage they circumscribe TE cells as belts to seal the intercellular space of adjacent cells and create permeability barriers that regulate passage of molecules and ions through a paracellular pathway (González-Mariscal *et al.*, 2011; Moriwaki *et al.*, 2007). Tight junctions are comprised of transmembrane proteins, such as ocludin and claudins, that join inside the cell with peripheral membrane proteins TJP1, TJP2, and *TJP3*, located toward the cytoplasm, which in turn interact with the actin cytoskeleton and also recruit factors involved in signal transduction and regulation of proliferation and differentiation (Moriwaki *et al.*, 2007). These tight junction transmembrane proteins couple in homo- and/or heterotypic interactions with cognate proteins on the surface of adjacent cells to form the paracellular barrier (Kiener *et al.*, 2007). Tight junctions with claudin-4 appear to seal the intercellular space of TE, where they form aqueous and ion-selective aqueous pores, and contribute to fluid accumulation in the blastocoel cavity, which becomes expanded by elevated hydrostatic pressure (Furuse and Moriwaki, 2009; Serafini *et al.*, 2009). Here, we examined the expression of *CLDN4* and *TJP3* genes, which code for tight junction proteins. Our results show that *CLDN4* expression was significantly higher in WOW, whereas *TJP3*expression was higher in the PM system. This implies that both single-embryo culture systems have the capacity for developing blastocysts with adequate TE conformation. However, since claudin-4 is necessary for blastocyst expansion, it is possible that blastocysts developed in WOW may have higher capacity for adequate TE generation to allow adequate delimitation of the ICM zone.

In conclusion, compared with the standard CG system, both PM and WOW systems are good options for culturing single, identifiable embryos in the bovine model. This can be considered as an opportunity to improve selectivity of *in vitro* produced embryos.

Declarations

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Conflict of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

M.Eduviges Burrola-Barraza designed the study; Daniel Contreras-Benicio and Beatriz Elena Castro-Valenzuela conducted the study; and J.A. Grado-Ahuir performed the statistical analysis.

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