VETERINARSKI ARHIV 86 (3), 467-474, 2016

Reduced mineral oil ratio improves blastocyst yield in well-of-the-well (WOW) and polyester mesh (PM) single-embryo cultures - short communication

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PEREDA-ESPINOZA, B. E., M. E. BURROLA-BARRAZA, F. A. RODRÍGUEZ-ALMEIDA, J. ANTILLON-RUIZ, A. ANCHONDO-GARAY: Reduced mineral oil ratio improves blastocyst yield in well-of-the-well (WOW) and polyester mesh (PM) single-embryo cultures - short communication. Vet. arhiv 86, 467-474, 2016.

ABSTRACT

It is common to apply a mineral oil (MO) overlay to *in vitro* embryo cultures, even though the use of MO may be harmful to embryo development. To examine whether the volume of MO used in overlays for well-of-the-well (WOW) and polyester mesh (PM) single-embryo culture systems can be reduced, two sets of experiments were performed and the resulting blastocyst formation rates were compared with a conventional group (CG) embryo culture system. In Experiment 1, groups of 20 embryos in 100 µL microdrops of CDM-2 medium were plated in Petri dishes and then were covered with 3.5 mL of MO (resulting in a 1:35 ratio of medium to MO). Groups of 20 embryos were also placed in four-well plates in 400 µL CDM-2 medium per well, and the wells were covered with 400 µL MO (resulting in a 1:1 ratio of medium to MO). In Experiment 2, groups of 20 embryos were plated in four-well plates with 400 µL CDM-2 medium per well, and 1 mL of purified water was added to the center hole of the plate. Two sets of these plates were set up in parallel, and only one of the plates received an additional 400 µL of MO per well (resulting in a 1:1 ratio of medium to MO). A greater percentage of embryos reached the blastocyst stage when they were cultured in a 1:1 ratio of medium to MO, compared with embryos that were cultured in a 1:35 ratio of medium to MO (55 % vs. 41 %, respectively; $P<0.01$). In addition, a greater percentage of embryos reached the blastocyst stage in the WOW and PM systems that included a 1:1 ratio of medium to MO overlay than the embryos that were cultured in the presence of water in the central hole of a four-well plate without MO (48 % vs. 39 %, respectively; $P<0.01$). Therefore, a 1:1 ratio of medium to MO was found to improve blastocyst rates in both WOW and PM embryo culture systems.

Key words: blastocyst, embryo culture, mineral oil, water, polyester mesh, well-of-the-well system

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ISSN 0372-5480
Printed in Croatia

Introduction

The major goal of *in vitro* embryo cattle production systems is to successfully transfer healthy blastocysts with high genetic potential to improve cattle pregnancy rates (HANSEN, 2006). However, in dairy and beef production systems, the pregnancy rates following the transfer of embryos produced *in vitro* are generally low, and typically range from 36-40 % (FARIN et al., 1999; PONTES et al., 2010; PONTES et al., 2011; SIQUEIRA et al., 2009). In embryo microdrop cultures, it is common to overlay the medium with mineral oil (MO) to prevent the evaporation of medium and to maintain a stable pH, temperature, and osmolarity for the cultures (VAJTA et al., 2010b). In our laboratory, and other laboratories (GASPERIN et al., 2010; SIQUEIRA et al., 2009; TAE et al., 2006), the conventional microdrop system involves the addition of 100 µL aliquots of culture medium to 60 mm Petri dishes. These drops are then overlaid with 3.5 mL of MO, resulting in a volume ratio of 1:35 of medium to MO. However, this abundant presence of MO may be harmful to embryo development since it may absorb the lipophilic factors that are present in the culture medium. MO also contains toxic components, such as peroxides, which are detrimental to embryo development (OTSUKI et al., 2007; OTSUKI et al., 2009). To investigate whether a decrease in MO volume in well-of-the-well (WOW) and polyester mesh (PM) single-embryo culture systems would improve the successful production of blastocysts, two sets of individual *in vitro* cultures, WOW and PM, were set up and the resulting blastocyst formation rates were compared with a conventional group (CG) embryo culture system.

Materials and methods

Bovine ovaries were collected from a local slaughter house and were transported to the laboratory in sterile 0.15 M saline (25-25 °C). A PrecisionGlide needle (BD 18G × 11/2") with vacuum suction (~50 mmHg pressure, WOB-L® Dry Vacuum Pumps, Standard-Duty, Welch®) was applied to the follicles with a diameter less than 10 mm. Cumulus oocyte complexes (COCs) were washed twice in chemically defined medium, CDM (710 mM NaCl, 60 mM KCl, 10 mM KH_2PO_4 and 5 mM Na-citrate.$2H_2O$, 5 mM $NaHCO_3$, 2 mM $CaCl_2.H_2O$, 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 gentamycine) for handling of oocytes, 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 cumulative compact cells were detected under a stereomicroscope (Leica MS5). COCs were cultured in four-well plates (Nunc, Thermo Scientific, Rockford, IL, USA). Briefly, groups of 50 COCs per well were maintained in 1 mL of chemically defined medium for *in vitro* maduration medium, M-CDM (CDM supplemented with 2 mM D-Fructose, 2.77 mM myo-Inositol, 0.1 mM Taurine, 5 % fatty acid free bovine serum albumin (BSA), 15

ng/mL follicle stimulating hormone (FSH), 1 μg/μL leutinizing 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 in 100 % humidity for 23 h. For *in vitro* fertilization (IVF), four-well plates 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 and 14 mM NaCl). One mL of purified water was added to the center of each four-well plate. Oocytes at the meiosis I (MI) stage were transferred in groups of 50 to each well. Semen from an Angus bull was used for *in vitro* fertilization, 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 1300 rpm for 20 min. The resulting pellet was resuspended in 4.5 mL F-CDM and was centrifuged again (1300 rpm for 5 min). After the medium was aspirated, the cell concentration was adjusted to 1×10^5 sperm per mL. Twenty-three hours after the *in vitro* maturation of the oocytes, 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 presumed 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 bovine serum albumin (BSA) and 22.5 mM NaCl) and were vortexed for 1 min to remove cumulus cells. For the CG cultures, 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 media 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, groups of 20 embryos were applied to CG, WOW, and PM culture systems to perform two sets of experiments. In the first experiment, embryos in 100 μL of chemically defined media 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) were placed on sterile 60-mm Petri dishes and then were covered by 3.5 mL of MO (resulting in a 1:35 ratio of medium to MO). In parallel, 400 μL of CDM-2 containing embryos was placed in each well of a four-well plate, and then 400 μL MO was added to each well (resulting in a 1:1 ratio of medium to MO). In the second experiment, embryos in CDM-2 were placed in each well of two 4-well plates (400 L per well). One of the plates did not receive an overlay of MO in its wells, and 1 mL of purified water was added to the center hole of the plate. For the second plate, each well received 400 μL MO (resulting in a 1:1 ratio of medium to MO), and 1 mL of purified water was added to the center hole of the plate. Both plates

were then incubated with 5 % CO_2, 5 % O_2, and 90 % N_2 in humidity-saturated air at 39 °C for four days.

Two single-embryo culture methods were used: WOW and PM. The WOW system was established according to VAJTA et al., 2008, with some modifications. Briefly, microwells were created by applying a mechanical force to the bottom of each well, using DN-09/B BLS aggregation needles, in sterile Petri dishes or four-well plates, and they contained 100 μL or 400 μL of CDM-2, respectively. The PM system was established according to SOMFAI et al. (2010) with a polyester mesh (SEFAR FEDEX) placed at the bottom of each Petri dish, or in each well of the four-well plates that received 100 μL or 400 L of CDM-2, respectively. In both systems, each embryo was limited to an area approximately 275 μm in diameter and the overlay volume of MO was present at a ratio of 1:35, 1:1, or 1:0 of medium to MO.

A 2 × 2 factorial arrangement was established for each of the experiments, with eight and five independent replicates performed for the first and second experiments, respectively. In each experiment, the treatments were studied simultaneously. The percentage of embryos that reached the blastocyst stage was determined as a binominal response in each experiment, using PROC CATMOD of SAS (SAS Institute Inc., Cary, NC, USA). In addition, an adjusted model was used for the fixed effects of the culture method and MO treatments, as well as for the interactions between the culture methods and MO treatments.

Results

Experiment 1: A greater percentage of embryos reached the blastocyst stage when they were cultured with a 1:1 volume of medium to MO compared with a 1:35 volume of medium to MO (55 % vs. 41 %, respectively; $P<0.01$, Table 1). Furthermore, a higher percentage of blastocysts developed in the WOW culture system versus the PM culture system (53 % vs. 44 %, respectively; $P>0.01$). However, there was no significance difference in the percentage of blastocysts achieved between the WOW and PM systems and the GC culture system (48 %) ($P>0.01$; Table 1).

Experiment 2: Blastocyst development improved when the embryos were cultured in a 1:1 volume of medium to MO compared with culturing in the absence of MO (48 % vs. 39 %, respectively; $P<0.01$) (Table 2).

Table 1. Varying the ratio of medium culture to MO affects the blastocyst development of 160 embryos cultured in WOW and PM individual cultures systems

Ratio of medium culture to MO	Percentage of embryos at blastocyst stage ± SEM according to culture system			Average
	WOW	PM	CG	
1:35	47.0 ± 3.9	35.0 ± 3.7	41.0 ± 3.9	41.0 ± 2.2[a]
1:1	60.0 ± 3.8	52.0 ± 3.9	55.0 ± 3.9	55.0 ± 2.2[b]
Average	53.0 ± 2.7[a]	44.0 ± 2.7[b]	48.0 ± 2.7[ab]	

MO: mineral oil; SEM: standard error of the mean; WOW: well-of-the-well; PM: polyester mesh; CG: conventional group culture. [a,b] indicate a significant difference (P<0.01).

Table 2. Absence of MO layer affects the blastocyst development of 100 embryos cultured in four-well plates with water in the central hole

Ratio of medium culture to MO	Percentage of embryos at blastocyst stage ± SEM according to culture system			Average
	WOW	PM	CG	
1:1	54 ± 4.9	51 ± 5	41 ± 4.9	48 ± 2.8[a]
1:0	46 ± 4.9	35 ± 4.7	36 ± 4.8	39 ± 2.8[b]

MO: mineral oil; SEM: standard error of the mean; WOW: well-of-the-well; PM: polyester mesh; CG: conventional group culture. [a,b] indicate a significant difference (P<0.01).

Discussion

The use of MO has previously been reported to be harmful for *in vitro* embryo development, despite it preventing the rapid evaporation of culture medium and changes in pH and temperature in embryo cultures (VAJTA et al., 2010a). Therefore, in the present study, the effects of using less MO in the overlay of WOW and PM shared medium culture systems were compared with a CG culture system. Two sets of embryo cultures were set up. One involved the incubation of 100 µL microdrops of embryos covered with 3.5 mL of MO in Petri dishes (a common method for this type of culture). The second involved the incubation of embryos in four-well plates, with each well covered with 400 µL of MO. In the microdrop cultures, the ratio of MO to medium was 35:1, whereas in the four-well plate method, a 1:1 ratio of MO to medium was present. The latter method resulted in a higher percentage of embryos reaching the blastocyst stage (Table 1). In embryo cultures, secreted paracrine-autocrine factors accumulate in the medium to stimulate embryo development, and they also can protect the embryos from stress due to the presence of MO peroxides (HUGHES et al., 2010). We hypothesize that these paracrine-autocrine factors are very concentrated in the microdrop method, and this protects the embryos from the large volume of MO used in this method. However, the rate

of blastocyst formation was higher in the four-well plate method that included a larger volume of culture medium. Therefore, even though the paracrine-autocrine factors were more diluted, the blastocyst yield was superior with the four-plate method than with the microdrop method (Table 1).

Previously, GASPERIN et al. (2010) concluded that water can be added to the center of four-well plates, instead of an oil overlay, and this will maintain osmolality and achieve adequate blastocyst rates. Therefore, in the second set of experiments performed in the present study, the method of GASPERIN et al. (2010) was used to establish the same conditions for groups of embryos in both the WOW and PM systems. The use of MO in a 1:1 ratio with medium achieved a higher percentage of blastocyst stage embryos than the application of water to the central hole of the four-well plates. However, the blastocyst rate of the latter was similar (although without significance) to that obtained in the cultures covered with MO (Table 2).

In conclusion, the results of the present study demonstrate that a 1:1 ratio of medium to MO can improve blastocyst rates in both the WOW and PM culture systems. However, the use of water instead of MO did produce a good rate of blastocyst formation in both the WOW and PM systems, and this may be an option for culturing embryos without the harmful effects of MO.

Acknowledgements

This research was supported by Grant 168981 from the Consejo Nacional de Ciencia y Tecnología (CONACYT), México. B. E. Pereda-Espinoza was a scholarship recipient from CONACYT, México (Registration No. 257936).

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Received: 20 March 2015
Accepted: 10 December 2015

PEREDA-ESPINOZA, B. E., M. E. BURROLA-BARRAZA, F. A. RODRÍGUEZ-ALMEIDA, J. ANTILLON-RUIZ, A. ANCHONDO-GARAY: Smanjeni omjer mineralnog ulja poboljšava prinos blastocista u sustavu mikrojažica i jednozametnim kulturama u poliesterskim mrežama - kratko priopćenje. Vet. arhiv 86, 467-474, 2016.

SAŽETAK

U *in vitro* kulturama zametaka uobičajeno je primjenjivati sloj mineralnog ulja (MU), iako to može biti štetno za razvoj embrija. U radu su provedeni pokusi s ciljem da se istraži može li se smanjiti količina primijenjenog MU u sustavu mikrojažica i u poliesterskim mrežama za uzgoj pojedinačnog zametka. Pri tome je stopa nastalih blastocista bila uspoređena s konvencionalnim načinom uzgoja zametaka. U prvom pokusu su skupine od 20 zametaka u mikrokapi od 100 µL CDM-2 medija bile stavljene na podlogu u Petrijeve zdjelice

i nakon toga prekrivene s 3,5 mL MU (omjer između medija i MU iznosio je 1:35). Skupine od 20 zametaka također su bile smještene u četverodijelne plitice s 400 µL CDM-2 medija po jažici i prekrivene s 400 µL MU (omjer između medija i MU iznosio je 1:1). U drugom pokusu, skupine od 20 zametaka bile su smještene u četverodijelne plitice s 400 µL CDM-2 medija po jažici, u čiji je središnji otvor dodan 1 mL pročišćene vode. Dvije skupine tih plitica bile su istodobno postavljene, a samo u jednu pliticu dodano je 400 µL MU po jažici (omjer između medija i MU iznosio je 1:1). Veći postotak embrija dosegao je stadij blastociste kada je u kulturi bio primijenjen omjer 1:1 između medija i MU. U usporedbi sa zametcima kod kojih je u kulturi bio primijenjen omjer između medija i MU 1:35, razlika je iznosila 55 % prema 41 %, P<0,01. Osim toga, veći postotak zametaka dosegnuo je stadij blastociste u sustavu mikrojažica i poliesterskih mrežica u kojima je omjer između medija i MU bio 1:1, nego što je to bio slučaj kad je kulturi zametaka u središnjoj jažici plitice bila dodana voda (48 % prema 39 %, P<0,01). Utvrđeno je da omjer 1:1 između medija i MU poboljšava stopu razvijenih blastocista u oba sustava.

Ključne riječi*:* blastocista, kultura zametaka, mineralno ulje, voda, mikrojažice, poliesterske mrežice