Oil-free culture system for in vitro bovine embryo production

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Abstract

The use of oil to avoid water evaporation from cell culture has several disadvantages, amongst which there is the migration of compounds from media to oil and from oil to media. The aim of this study was to evaluate the osmolality of a culture system using four-well plates with water in the central hole as an alternative to in vitro bovine embryo production (IVP). In addition, the osmolality changes of the oocyte washing medium were assessed in 35 mm dishes with or without 2 mL of silicon oil overlay. Osmolality of oocyte washing medium changed a great deal over time after 60 minutes on a 39°C heated plate (291 mOsm kg⁻¹), which was not detected when the medium was overlaid with silicon oil (280 mOsm kg⁻¹; P<0.05). During the maturation period, the presence of water in the central hole of four-well plates maintained the osmolality in the same pattern as oil overlay (293±0.8 to 294±1.8 mOsm kg⁻¹; P>0.05). Blastocyst rates were higher when embryos were cultured in presence of water or oil (29.7 and 28.9%) for water and 33% in oil conventional microdrop system, except in the group that oocytes were washed in hyperosmotic washing medium (15.1%; P<0.05). Groups cultured in absence of water in the central hole had lower blastocyst rates (P<0.05) independently of exposure (15.5%) or not (16.2 and 16.8%) to hyperosmotic washing medium. In conclusion, four-well plates with water in the central hole can be an alternative to oil overlay for bovine IVP, maintaining stable osmolality and embryo development rates.

Introduction

In embryo production systems, the amount of media used for cell culture is very low (from 50 µL to 400 µL), which makes crucial the control of water evaporation, in order to avoid increased salt concentration and, consequently, osmolality. In addition, increased medium salt concentration affects oocyte and embryo development, independently of osmolality. Fertilization of oocytes in media with high concentration of NaCl increases polyspermy and impairs embryo development (Roh et al., 2002). Osmolality of culture media is one of the key factors affecting the success of in vitro production (IVP) of embryos (Liu and Foote, 1996). There is some evidence that osmolality of culture medium must remain stable during all the IVP process (Kruger et al., 1985).

However, recent data indicates that increased osmolality in the first two days of culture improved in vitro development and reduced apoptosis through regulation of Bax-alpha/Bcl-xl gene expression in porcine embryos (Hwang et al., 2008). Osmotic stresses can also damage DNA, affect DNA replication, DNA transcription and mRNA translation, leading to cellular damage (Burg et al., 2007).

The oil overlay avoids the water evaporation from cell culture media. However, some studies indicated that use of oil can have several disadvantages related to migration of positive lipophilic factors into the oil (Shimada et al., 2002), toxic factors from oil to media (Erbach et al., 1995) and oil storage (Otsuki et al., 2002), Moreover, deleterious factors (such as zinc) from the silicon oil can diffuse into culture media, impairing embryo development (Erbach et al., 1995). In addition, mineral oil peroxidation can release free radicals, which are transferred through the zona pelucida (Otsuki et al., 2009), affecting oocyte maturation and embryo development.

The main positive hydrophobic factors lost in oil are steroids. Shimada et al. (2002) reported a delay of oocyte maturation and a decrease in cleavage and blastocyst rates after maturation of swine oocytes in medium with an oil overlay. Since there is a strong evidence of steroids role during in vitro embryo production, mainly during oocyte maturation (Shimada and Terada, 2002; Wang et al., 2006), the addition of oil in culture systems should be avoided, in mind to prevent changes in media composition.

An alternative to the use of oil overlay to prevent changes in osmolality during culture, could be represented by the production of a humid microenvironment in four-well dishes. The placement of a reasonable water amount in the central hole of four-well plates could maintain the plate microenvironment humidity and thus prevent the medium osmolality increase. In the present study, we have tested the hypothesis that the presence of water in the central hole of four-well plates could maintain the osmolality stability during the different steps of the in vitro bovine embryo production system. Furthermore, we evaluated the osmolality changes in the washing and culture media in relation to cleavage and blastocyst rate.

Materials and methods

In all experiments, the osmolality (mOsm kg⁻¹) was assessed in 50 µL of medium from the different culture systems during oocyte washing, maturation and embryo culture in different times, using a cryoscopic osmometer (Osmomat® 030, Gonotec, Berlin, Germany). All osmolality assessments were performed in three replicates. All chemicals used were purchased from Sigma Chemical Company, St. Louis, USA, unless otherwise indicated in the text.

Experiment #1

The osmolality changes of the oocyte washing medium was assessed from 2 mL of
TCM199 (with 25 mM HEPES; pH 7.36; Gibco Labs, Grand Island, NY, USA) in 35 mm dishes with or without 2 mL of silicon oil overlay, leaved closed on a heated plate at 39°C, with a relative humidity between 60% and 80%, for different time points. The first evaluation was performed immediately after the medium preparation (hour 0) and one dish was used for each evaluation time to eliminate the effect of volume change. The osmolality was measured every 15 minutes for 4 hours.

Experiment #2

Osmolality assessments were performed during the maturation period at 0, 6, 12, 18 and 24 h of incubation. The culture systems were: i) Four-well plates containing 400 µL of maturation medium in each well, with or without 3 mL of purified water in the central hole of the plate; ii) Four-well plates with 400 µL of maturation medium in each well, with 400 µL of silicon oil covering the medium. We have as well performed osmolality assessments in the conventional microdrop system used in our laboratory (200 µL medium drops covered with silicon oil in 60 mm culture dishes).

Experiment #3

We tested the effect of osmolality change in oocyte washing medium and in different embryo culture steps, in relation to cleavage and blastocyst rate. After recovering, oocytes were washed in pre-heated TCM199 (2 mL) just added to 35 mm dishes (time 0) or 2 h after addition of medium to dishes on the heated plate at 39°C, covered or not with 2 mL of silicon oil. The oocytes remained in washing media for 10-15 min. This period was necessary to select good quality oocytes and to allocate them in different culture groups. After washing, the oocytes were cultured in four-well plates containing 400 µL of medium. Maturation, fertilization and embryo culture were performed in four-well plates, with or without water in the plate central hole. As control, we used a microdrop system (200 µL medium drops covered with silicon oil in 60 mm culture dishes). This microdrop system is a standard method used by our laboratory for in vitro production of embryos. The experiment was performed in four replicates. Osmolality was assessed before and at the end of the embryo culture period (day 7 after fertilization). The cleavage and blastocyst rates (number of blastocysts divided by the total number of oocytes) were evaluated 2 and 7 days after fertilization, respectively.

In vitro embryo production

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100IU mL⁻¹ penicillin and 50 µg mL⁻¹ streptomycin sulphate. Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 mL minute⁻¹). The COCs were recovered and selected according to Leibfried and First (1979) under a stereomicroscope. Grade 1 and 2 COCs (n=20-30) were washed in TCM199 (Gibco Labs, Grand Island, NY, USA) in different systems according to each group and randomly distributed into 400 µL of maturation medium and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO₂ and 95% air, for 24 h. The maturation medium used was TCM199 (Gibco Labs, Grand Island, NY, USA); it contained Earle’s salts and L-glutamine supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg mL⁻¹ sodium bicarbonate, 5.0 µg mL⁻¹ LH (Bioniche, Belleville, ON, Canada), 0.5 µg mL⁻¹ FSH (Bioniche, Belleville, ON, Canada), 10% (v/v) bovine calf serum (Gibco Labs, Grand Island, NY, USA), 100IU mL⁻¹ penicillin and 50 µg mL⁻¹ streptomycin sulphate. The initial osmolality of maturation medium used in all experiments varied between 284-287 mOsm kg⁻¹. After in vitro maturation, oocytes were fertilized with tested frozen semen from a Jersey bull. Semen was fractionated on discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradients, as described by Parrish et al., (1986). The sperm were diluted to a final concentration of 2x10⁶ sperm mL⁻¹ in Fert-TALP medium containing 10 µg mL⁻¹ heparin (Parrish et al., 1988). In vitro fertilization was carried out by co-culture of sperm and oocytes for 18 h in four-well plates in the same atmospheric conditions as the ones used for maturation.

After gamete co-incubation period, the cumulus cells were removed by 2 min of vortex. Presumptive zygotes were cultured at 39°C in an incubator culture chamber (CBS Scientific, Del Mar, CA, USA) with saturated humidity atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ for 7 days in 400 µL synthetic oviduct fluid (SOF) medium in four-well plates (Nunc, Roskilde, Denmark).

Statistical analysis

Osmolality assessments of maturation and washing media were performed as repeated measures data and analyzed using the MIXED procedure with a repeated measure statement. Main effects of treatment group, hour and their interaction were determined. Differences between osmolality at a specific time point were compared between groups using estimates. Data from osmolality of SOF media, cleavage and blastocyst rates were submitted to ANOVA using the General Linear Models (GLM) and multi-comparison between groups was evaluated by least square means. The data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. The percentage data were submitted to arcsine transformation. All analyses were performed using SAS software package (SAS Institute Inc., Cary, NC, USA). The results are presented as means ± standard error of the mean; a P<0.05 was considered statistically significant.

Results and discussion

In embryo production system, oocytes are harvested and transferred to a washing dish with culture medium. Generally, the osmolality in washing dish is disregarded because of the large amount of medium (~2 mL). Addressing this question, we observed that the osmolality of oocyte washing medium changed a great deal over time after 60 minutes on a 39°C heated plate (291 mOsm kg⁻¹), which was not detected when the medium was overlaid with silicon oil (280 mOsm kg⁻¹; P<0.05; Figure 1). These results suggest that oil overlay addition is only necessary when the washing medium remains more than 45 minutes on the heated plate.

After washing procedure, oocytes are usually matured in microdrops of medium covered by oil or in four-well plates. We investigated the need of an oil overlay or purified water in the central hole of the four-well plate in order to minimize evaporation and changes in oocyte maturation medium osmolality. The presence of water in the central hole of four-well plates maintained the osmolality in the same pattern as the oil overlay (294±1.8 mOsm kg⁻¹ for Nunc water vs 293 mOsm kg⁻¹ for Nunc oil; P>0.05; Table 1), being the medium final osmolality similar to that observed in the conventional microdrop culture system (294 mOsm kg⁻¹). However, the osmolality after 12 hours of culture was higher when medium was disposed in dishes without water or oil (P<0.05).

These results show that purified water in the four-well plates central hole can be used to replace oil overlay, in order to maintain the osmolality of oocyte maturation and embryo development media. This oil-free system can
be a great alternative to maintain the osmolality when using co-culture systems of oocytes and follicular cells to study the physiology of nuclear maturation in oocytes (Stefanello et al., 2006; Barreta et al., 2008). It is advisable to avoid the oil in co-culture systems, as the follicular cells produce steroids during the culture and the oil can sequester these steroids and impair the results (Reinsberg et al., 2004).

In order to evaluate the effect of changing osmolality on embryo development, we tested the high and low water evaporation systems in relation to cleavage and blastocyst rates. The cleavage rates were not affected by different washing or culture systems varying between 75.1 and 90.4% (P>0.05; Figure 2A). Kim et al. (2002) tested the effect of different osmolalities on bovine pronuclear formation and polyspermic fertilization rate and concluded that oocytes are more sensitive to osmotic stress than spermatozoa. Nevertheless, these authors did not test the effect of osmolality changes on the cleavage rates.

After the end of the culture period, the osmolality and blastocyst rates were assessed. The osmolality of SOF medium disposed in plates with water in the central hole was lower (282-284 mOsm kg\(^{-1}\)) than the one observed when the hole was kept empty for 7 days of culture (295-298 mOsm kg\(^{-1}\); P<0.05; Figure 2C). In our system, there was no difference in culturing oocyte under oil (33% blastocyst from a total of 60 oocytes) when compared with oocyte cultured in a four-well plate with purified water in the central hole (29.7 and 29.9% blastocyst from a total of 107 and 109 oocytes; P>0.05). However, oil is a limitation in IVF system, as lipophilic compound cannot be present in the culture medium. Therefore, it is suitable to avoid oil, when even possible, because toxic effect, breakdown and soluble compounds in oil are factors that may interfere in the cell culture. Shimada et al. (2002), reported that the presence of oil in the swine oocyte maturation system resulted in less progesterone concentration in the medium, delayed swine oocyte maturation and reduced cleavage and blastocyst rate. The evaporation increased osmolality and salt concentration, which is harmful to oocyte and embryo viability. Regardless of the evidence that high osmolality during early embryonic stages decreases the number of apoptotic cells and improves blastocyst formation, the increase in osmolality is highly detrimental for later stages of embryo development (Nguyen et al., 2003; Hwang et al., 2008). Bovine embryos require a low constant osmolality, near 270 mOsm kg\(^{-1}\) (reviewed by Wright and Bondioli, 1981). Liu and Foote (1996) showed a drastic reduction in bovine embryo

### Table 1. Maturation media osmolality (mOsm kg\(^{-1}\)) in different culture systems during 24 hours of incubation at 39°C in a saturated humidity atmosphere containing 5% CO\(_2\) and 95% air.

| Culture system | Maturation media osmolality (mOsm kg\(^{-1}\)) |
|---------------|---------------------------------------------|
| 0 h           | Nunc 287±0.7a 291±0.9b 294±1.5b 297±1.2b 305±2.4b |
|               | Nunc oil 285±1.9a 280±0.9b 291±1.5b 291±1.5b 293±0b |
|               | Nunc water 284±0.7a 287±1.2b 290±0.7b 292±1b 294±1.8b |

Data presented as mean ± standard error of the mean. Different superscript letters (a, b) in the same column indicate statistical difference among groups (P<0.05).

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**Figure 1.** Washing media osmolality in 35 mm dishes on a heated plate (39°C) with or without oil overlay. The measurements were performed each 15 minutes during 4 hours. The experiment was carried out in three replicates. Data presented as mean ± standard error of the mean. Asterisk (*) represents difference among groups at specific time points (P<0.05).

**Figure 2.** Effect of controlling water evaporation on cleavage rate (Panel A), blastocyst rate (Panel B) and osmolality of synthetic oviduct fluid medium (Panel C). Oocytes were washed in medium that remained 0 or 2 h on a heated plate with or without oil overlay. The presumptive embryos were cultured in 400 μL of medium in four-well plates with or without water in its central hole. The total number of oocytes cultured in each treatment is shown at the base of cleavage bars (Panel A). The SOF medium osmolality was measured at the end of the culture system (7 days after fertilization; Panel C). Data presented as mean ± standard error of the mean. Different letters indicate statistical difference among groups (P<0.05).
production when medium osmolality was 300 mOsm kg⁻¹. In addition, high levels of NaCl impair fertilization, increase polyspermy, decrease blastocyst formation and decrease number of blastomeres (Liu and Foote, 1996; Roh et al., 2002). Therefore, the evaporation probably affected oocyte viability, due to both increase in salt concentration and osmolality.

Interestingly, blastocyst rate decreased significantly when oocytes were washed in medium without oil overlay maintained on the heated plate for 2 h, independently of culture system (15.5 and 15.1% blastocyst; P<0.05; Figure 2B). In experiment #1, we demonstrated that the osmolality increased to near 310 mOsm kg⁻¹, when the washing medium remained for 2 h on the heated plate without oil. The short-term oocytes exposure to a hyperosmotic medium before maturation did not affect the cleavage rate, but the blastocyst rate was impaired. These results indicate that the hyperosmotic media in a short-term exposure can affect the oocyte viability. Bovine oocyte seems to be very susceptible to injury by osmotic stress, which may end to cytoplasm and molecular impair, resulting in cellular death by apoptosis (Burg et al., 2007).

Moreover, the blastocyst rates were affected when the plate was without water during embryonic culture, independent of the washing medium treatment (16.2, 15.5 and 16.8% blastocyst; P=0.05). The blastocyst rate was higher in groups cultured with water in the four-well plate central hole (29.7 and 29.9% blastocyst; P=0.05; Figure 2B). The percentage of blastocyst (~30%) obtained when osmolality was controlled (control and treatment with water and oil) evidenced that the oocyte maturation and embryo culture system were suitable (Rizos et al., 2002; Roh et al., 2002; Stefanello et al., 2006).

Conclusions

The maintenance of osmotic equilibrium and the embryo development rate confirm that four-well plate with water in the central hole can be a feasible alternative to oil for bovine embryo culture. The oil overlay is essential if the oocytes remain in the washing medium more than 45 minutes on the heated plate, to obtain an acceptable embryo development rate.

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