L. Zhang, Z. Lin, Y. Bi, X. Zheng, H. Xiao, Z. Hua
Hubei Key Laboratory of Animal Embryo Engineering and Molecular Breeding, Hubei Institute of Animal Science and Veterinary Medicine, Hubei Academy of AgroSciences, Wuhan 430064 China

Abstract

Culture gas atmosphere is one of the most important factors affecting embryo development in vitro. The main objective of this study was to compare the effects of CO2 concentration on the subsequent pre-implantation developmental capacity of pig embryos in vitro, including embryos obtained via parthenogenesis, in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). Pig embryos were developed in four different CO2 concentrations in air: 3%, 5%, 10%, or 15%. The cleavage rate of pig parthenogenetic, IVF, or ICSI embryos developed in CO2 concentrations under 5% was the highest. There were no significant differences in the oocyte cleavage rate in ICSI embryos in CO2 concentrations under 3% and 5% (p>0.05). However, as CO2 levels increased (up to 15%) the blastocyst output on day 7, from parthenogenetic, IVF, and ICSI embryos, decreased to 0%. These findings demonstrate that CO2 positively affects the developmental capacity of pig embryos. However, high or low CO2 levels do not significantly improve the developmental capacity of pig embryos. The best results were obtained for all of the pig embryos at a 5% CO2 concentration.

Key words: CO2, developmental capacity, pig embryos, in vitro

Introduction

A large number of immature oocytes collected from mammalian ovaries fail to develop to the preimplantation stage after in vitro maturation, fertilization, and culture (Galli et al. 2001, Cognie’ et al. 2004). Pre-implantation development is a time of dynamic change and reprogramming, involving extensive modifications of the genome, proteome, metabolome, and epigenome; hence, the zygotes and embryos are extremely sensitive to the external environment (Marcho et al. 2015). Several factors have been implicated in these failures, including oocyte quality, culture conditions, media, peptide growth factors, amino acids or macromolecules, and culture gas atmosphere (Kane 2003, Merton et al. 2003). Among the factors that affect in vitro embryo development, culture gas atmosphere is considered to one that is very important. A high grade of follicular vascularity has been shown to be correlated with a higher rate of pregnancy and live births following embryo transfer in women (Chui et al. 1997), suggesting that a threshold oxygen supply is important.
The dissolved oxygen content of human follicular fluid ranges between 1.3 and 5.5%, with studies showing that the oxygen content is not associated with frequency of oocyte meiotic maturation or fertilization, or embryo cleavage or morphology (Van et al. 1997, Huey et al. 1999). However, following fertilization, the ability of oocytes from low oxygen follicles (<1.5% O₂) to develop to the 6- to 8-cell stage is reduced (Van B et al. 1997).

In vitro culture is usually done in 5% CO₂ and 95% air (20% O₂) (Kitagawa et al. 2004). Similarly, in vitro development of pig embryos obtained from slaughtered females after artificial insemination was found to be optimal under an atmosphere of 5% O₂ and 5% CO₂ in air (Berthelot and Terqui 1996). To the best of our knowledge, there have been no studies examining the long-term developmental effects of different CO₂ concentrations on pig embryos obtained from in vitro maturation (IVM) of oocytes and then activated or after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). In the present study, the effects of CO₂ concentration on the subsequent pre-implantation developmental capacity of pig embryos in vitro was examined, including embryos obtained via parthenogenesis, IVF, and ICSI. The concentrations studied were 3%, 5%, 10%, and 15% CO₂ in air. It was hypothesized that a low or high CO₂ concentration after activation, IVF, or ICSI would improve the day-7 blastocyst output rate.

Materials and Methods

Oocytes collection

Porcine ovaries were obtained from an abattoir affiliated with COFCO (China Oil and Food Import and Export Corporation) in Wuhan and transported to the laboratory in 0.9% saline (w/v) supplemented with 100 IU/mL penicillin G and 100 IU/mL streptomycin sulphate at 30°C–35°C. Cumulus-oocyte complexes (COCs) were collected immediately from follicles with a diameter of 3–8 mm using a 10-gauge needle attached to a disposable 10-mL syringe and were stored in Dulbecco’s Phosphate Buffered Saline (DPBS) supplemented with 5% fetal bovine serum (FBS). COCs with uniform cytoplasm and several layers of cumulus cells were used for maturation.

In vitro maturation of oocytes

Selected COCs were washed three times in DPBS supplemented with 5% FBS and three times in maturation medium (Medium 199; Gibco) supplemented with 10% (v/v) pig follicular fluid (PFF), 0.1% (w/v) polyvinyl alcohol, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 100 IU/mL streptomycin sulphate (Gibco), 100 IU/mL penicillin G (Gibco), 10 IU/mL PMSG (Ningbo Second Hormone Factory), and10 IU/mL hCG (Ningbo Second Hormone Factory) (Abeydeera. 2000). COCs were incubated for 42-44 h in a 5-well dish at 39°C in a Submarine Incubation System with 100% humidity and 5% CO₂ in air.

The maturation medium was incubated for at least 3 h at 39°C in the Submarine Incubation System with 100% humidity and 5% CO₂ in air.

Activation of oocytes

After 42–44 h in culture, oocytes were denuded from cumulus cells by gentle pipetting in DPBS with hyaluronidase (1 mg/mL). Oocytes with polar body I (pb I) were selected, washed three times with activation fluid supplemented with 0.3 M mannitol, 1 mM CaCl₂, 0.5 mM MgSO₄, and 0.05 mg/mL bovine serum albumin (BSA), and then activated by a single DC pulse of 1.5 kV/cm for 30 μ sec using a BTX Electro-Cell Manipulator 2001 (BTX Inc, San Diego, CA, USA).

In vitro fertilization

After 42-44 h in culture, oocytes were denuded from cumulus cells as above. Then the denuded oocytes were washed three times in IVF medium and 20 oocytes were placed in 50 uL drops of IVF medium that had been covered with warm mineral oil in a 35 x 10 mm dish. The dishes were kept in the incubator for 30 min and then spermatozoa were added for IVF. A semen pellet was thawed and washed three times by centrifugation at 600 x g for 6 min in IVF medium consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris, 11 mM glucose, 5 mM pyruvate sodium, 100 IU/mL streptomycin sulphate (Gibco), and 100 IU/mL penicillin G (Gibco). After washing, the sperm pellet was resuspended in IVF medium (same as above). After appropriate dilution, 50 uL of the sperm suspension was added to 50 uL of the medium that contained oocytes to give a final sperm concentration of 1.0 x 10⁶ cells/mL. Oocytes were co-incubated with spermatozoa for 6 h at 39°C with 100% humidity and 3%, 5%, 10%, or 15% CO₂ in air.

Intracytoplasmic sperm injection

The ICSI procedure was performed using commercially available holding and injection pipettes (Sunlight Medical, Inc., Florida USA), Leica micromanipulators (Leica, Heidelberg, Germany), and Eppendorf TransferMan 4r.

Just before the ICSI procedure, the sperm suspen-
**Embryo culture**

For *in vitro* development, the embryos after activation, IVF, or ICSI were washed three times with NCSU-23 containing 4 mg/ml BSA (Petters.1993), then cultured in the same media, which had been previously covered with mineral oil, in a polystyrene culture dish. The embryos were then incubated at 39°C in the Submarine Incubation System with 100% humidity and 3%, 5%, 10%, or 15% CO₂ in air. The cleavage rate and blastocyst output rate were assessed on days 2 and 7, respectively.

**Statistical analysis**

The data were collected from at least three replicates with each treatment represented in all replicates. Data were analyzed with the $\chi^2$ test. A probability of p<0.05 was considered to be significant.
Results

In the first experiment, the day-7 blastocyst output was not significantly different among groups whether the CO₂ concentration was 3%, 5%, 10%, or 15% (p>0.05) (Table 1). However, the cleavage rate was significantly higher at 5% CO₂ than at 3%, 10%, or 15% CO₂ (p<0.05) (Table 1).

In the second experiment, the cleavage rate for oocytes fertilized at 5% CO₂ was significantly higher compared with the other groups (p<0.05) (Table 2). The cleavage rate was not significantly different among the 8- to 16-cell-morulas incubated in 3%, 5%, and 10% CO₂ (p>0.05) (Table 2), but it was higher compared to those incubated in 15% CO₂. The output rate for the day-7 blastocysts was significantly higher when oocytes were fertilized at 5% CO₂ compared to the other groups (p<0.05) (Table 2).

In the third experiment, the oocyte cleavage rate was significantly higher after ICSI at 3% and 5% CO₂ compared to the other 2 groups (p>0.05); however, the oocyte cleavage rate was not significantly different when comparing the 3% and 5% CO₂ groups to each other (Table 3). The cleavage rate was significantly higher after ICSI in the 8- to 16-cell-morulas incubated at 5% CO₂ and 10% CO₂ compared to the other 2 groups (p<0.05) (Table 3); however, the cleavage rate was not significantly different when comparing the 5% and 10% CO₂ groups to each other (Table 3). The day-7 blastocyst output was highest at 5% CO₂ in air (Table 3).

Discussion

Pre-implantation embryos are known to be highly sensitive to the external environment as they undergo a series of dynamic changes and reprogramming in preparation for implantation (Marcho et al. 2015). The importance of the pre-implantation embryonic environment has become better understood in recent years. (Ka et al. 2018). Several studies have attempted to reproduce the in vivo environment, hoping to improve in vitro culture conditions and IVF outcomes (Mohamed et al. 2017). Among the factors that affect in vitro embryo development, culture gas atmosphere is considered to be one that is vitally important. Several studies have demonstrated that the greater physiological oxygen (O₂) concentration of 5% results in superior for in vitro outcomes compared to those obtained under the atmospheric O₂ level of 21% (Pabon et al. 1989, Bahcecci et al. 2005, Wale et al. 2010. Guo et al. 2014, Peng et al. 2015). Culturing porcine embryos at 21% O₂ has been found to generate a high quantity of reactive oxygen species, whereas 5% O₂ generates a lower level of O₂-free radicals (Kitagawa et al. 2004). Similarly, maintaining the O₂ level at 5% during preimplantation embryo cultivation (Gardner et al. 2016, Rebecca et al. 2016), was found to enhance early cleavage, blastocyst formation, cell number, and clinical outcome (Gardner et al. 1996, Lane et al. 1997. Kovacic et al. 2010, Kirkegaard et al. 2013, Kim et al. 2017).

In several studies involving the bovine model of IVM, 5% O₂ (5% CO₂) dramatically decreased the viability of oocytes compared to 20% O₂ (Pinyopummintr et al. 1995. Hashimoto et al. 2000), whereas oxygen levels appeared to have no effect on pig oocytes (Park et al. 2005). Furthermore, blastocyst development rates have been shown to both improve (Park et al. 2005) or remain unchanged when porcine oocytes were matured in 20% oxygen (Kikuchi et al. 2002). Media composition may play a role in these discrepancies, as Hashimoto (2000) also reported that 5% O₂ during IVM supports bovine embryo development if the concentration of glucose is increased. During embryo culture, a low oxygen atmosphere can modify embryonic cell allocation, thus increasing (Fischer-Brown et al. 2002) or decreasing (Van et al. 1997) the proportion of inner cell mass (ICM). In pig embryos obtained from slaughtered females after artificial insemination, in vitro development was found to be optimal at an atmosphere of 5% O₂ and 5% CO₂ (Berthelot and Terqui 1996).

However, whether CO₂ in air affects in vitro embryo development over the long-term has not been established. To the best of our knowledge, the present study is the first to examine the influence of CO₂ concentration on pig embryos from oocyte IVM and activation, IVF, or ICSI developmental competence up to the blastocyst stage. This study examined whether lowering or increasing the CO₂ concentration in the pig embryo culture environment in vitro would improve embryonic development and the 7-day blastocyst output.

The study also demonstrated that CO₂ concentration during embryo development affects the cleavage rate and kinetic development up to the blastocyst stage. Low (3%) and high (10%, 15%) CO₂ concentrations did not have a beneficial effect on in vitro development of pig embryos obtained from IVF and ICSI; however, 5% CO₂ was beneficial for cultured pig embryos. This suggests that culture performance may be CO₂ dependent and lowering or increasing the CO₂ level will affect pig embryo development and the day-7 blastocyst output. Our findings are similar to those obtained by Berthelot (1996); however, the source of embryos was different. In that study, in vivo embryos were derived from slaughtered females after artificial insemination.
In conclusion, the in vitro development of porcine parthenogenetic, IVF, and ICSI embryos was optimal in an atmosphere of 5% CO₂ in air, which gave the highest blastocyst yield among all of the groups of pig embryos that were investigated.

Acknowledgements

This study was supported by the China Major Program of Genetically Modified Organism New Species Cultivation (2016ZX08006001-005), the Innovation Center for Agricultural Sciences and Technologies of Hubei Province (2018-620-004-001), and the Top Talent Project of Hubei Academy of Agricultural Sciences.

We would like to thank LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

References


