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Original article

CO_2 concentration affects *in vitro* pig embryo developmental capacity

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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 CO_2 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 CO_2 concentrations in air: 3%, 5%, 10%, or 15%. The cleavage rate of pig parthenogenetic, IVF, or ICSI embryos developed in CO_2 concentrations under 5% was the highest. There were no significant differences in the oocyte cleavage rate in ICSI embryos in CO_2 concentrations under 3% and 5% ($p>0.05$). However, as CO_2 levels increased (up to 15%) the blastocyst output on day 7, from parthenogenetic, IVF, and ICSI embryos, decreased to 0%. These findings demonstrate that CO_2 positively affects the developmental capacity of pig embryos. However, high or low CO_2 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% CO_2 concentration.

Key words: CO_2, 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 macro-molecules, 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.

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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% O_2) to develop to the 6- to 8-cell stage is reduced (Van B et al. 1997).

In vitro culture is usually done in 5% CO_2 and 95% air (20% O_2) (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% O_2 and 5% CO_2 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 CO_2 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 CO_2 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% CO_2 in air. It was hypothesized that a low or high CO_2 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% CO_2 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% CO_2 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 $CaCl_2$, 0.5 mM $MgSO_4$, 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 $CaCl_2.2H_2O$, 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 10^6 cells/mL. Oocytes were co-incubated with spermatozoa for 6 h at 39°C with 100% humidity and 3%, 5%, 10%, or 15% CO_2 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-

Table 1. Effects of CO_2 concentration on the development of pig in vitro parthenogenetic embryos.

Treatment group	Oocytes	Cleavage rate (%)	8-to-16-cell-morula (%)	Blastocyst (%)
3%	402	320 (79.6%) a	170 (53.1%) a	3 (1.8%)a
5%	396	352 (88.9%) b	218 (61.9%) b	14 (6.4%) a
10%	360	278 (77.2%) a	170 (61.2%) b	0 (0%) a
15%	424	322 (75.9%) a	182 (56.5%) a	0 (0%) a

Different superscripts within the same column denote significant difference ($p<0.05$).

Table 2. Effects of CO_2 concentration on the development of pig IVF embryos.

Treatment group	Oocytes	Cleavage rate (%)	8-to-16-cell-morula (%)	Blastocyst (%)
3%	457	365 (79.9%) a	211 (57.8%) a	21 (10.0%) a
5%	385	342 (88.8%) b	222 (64.9%) a	48 (21.6%) b
10%	290	120 (41.4%) c	73 (60.8%) a	5 (6.8%) a
15%	312	109 (34.9%) d	54 (49.5%) b	0 (0%) c

Different superscripts within the same column denote significant difference ($p<0.05$).

Table 3. Effects of CO_2 concentration on the development of pig *in vitro* ICSI embryos.

Treatment group	Oocytes	Cleavage rate (%)	8-to-16-cell-morula (%)	Blastocyst rate (%)
3%	87	68 (78.2%) a	27 (39.7%) a	3 (11.1%) a
5%	98	77 (78.6%) a	47 (61%) b	10 (21.3%) b
10%	80	30 (37.5%) b	17 (56.7%) b	0 (0%) c
15%	85	30 (35.3%) b	14 (46.7%) c	0 (0%) c

Different superscripts within the same column denote significant difference ($p<0.05$).

sion was placed in a 10 μL droplet of 8% polyvinyl-pyrrolidone (PVP) at the 3 o'clock position. The injection of the oocyte was performed in microdroplets of IVF medium under mineral oil. A single motile morphologically normal sperm that had migrated to the 9 o'clock position was selected, then tailed in microdroplets of 8% PVP, immobilized by touching its tail with the injection micropipette, and then aspirated, tail first, into the pipette. The oocyte was denuded of cumulus cells, using glass denuding pipettes, immediately before injection. The oocyte to be injected was secured with the holding pipette (9 o'clock position) adjacent to the polar body (6 o'clock position). The micropipette containing the sperm was then inserted through the zona pellucida and the oolemma into the ooplasm at the 3 o'clock position of the oocyte. Penetration of the oolemma was confirmed by aspiration of some cytoplasm into the micropipette and the sperm was then slowly injected. The pipette was withdrawn gently and the oocyte released from the holding micropipette.

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% CO_2 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 χ^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 CO_2 concentration was 3%, 5%, 10%, or 15% ($p>0.05$) (Table 1). However, the cleavage rate was significantly higher at 5% CO_2 than at 3%, 10%, or 15% CO_2 ($p<0.05$) (Table 1).

In the second experiment, the cleavage rate for oocytes fertilized at 5% CO_2 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% CO_2 ($p>0.05$) (Table 2), but it was higher compared to those incubated in 15% CO_2. The output rate for the day-7 blastocysts was significantly higher when oocytes were fertilized at 5% CO_2 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% CO_2 compared to the other 2 groups ($p>0.05$); however, the oocyte cleavage rate was not significantly different when comparing the 3% and 5% CO_2 groups to each other (Table 3). The cleavage rate was significantly higher after ICSI in the 8-to 16-cell-morulas incubated at 5% CO_2 and 10% CO_2 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% CO_2 groups to each other (Table 3). The day-7 blastocyst output was highest at 5% CO_2 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 (O_2) concentration of 5% results in superior for *in vitro* outcomes compared to those obtained under the atmospheric O_2 level of 21% (Pabon et al. 1989, Bahceci et al. 2005, Wale et al. 2010. Guo et al. 2014. Peng et al. 2015). Culturing porcine embryos at 21% O_2 has been found to generate a high quantity of reactive oxygen species, whereas 5% O_2 generates a lower level of O_2 free radicals (Kitagawa et al. 2004). Similarly, maintaining the O_2 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% O_2 (5% CO_2) dramatically decreased the viability of oocytes compared to 20% O_2 (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% O_2 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% O_2 and 5% CO_2 (Berthelot and Terqui 1996).

However, whether CO_2 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 CO_2 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 CO_2 concentration in the pig embryo culture environment *in vitro* would improve embryonic development and the 7-day blastocyst output.

The study also demonstrated that CO_2 concentration during embryo development affects the cleavage rate and kinetic development up to the blastocyst stage. Low (3%) and high (10%, 15%) CO_2 concentrations did not have a beneficial effect on *in vitro* development of pig embryos obtained from IVF and ICSI; however, 5% CO_2 was beneficial for cultured pig embryos. This suggests that culture performance may be CO_2 dependent and lowering or increasing the CO_2 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% CO_2 in air , which gave the highest blastocyst yield among all of the groups of pig embryos that were investigated.

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