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5-Azacytidine improves the meiotic maturation and subsequent *in vitro* development of pig oocytes

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ABSTRACT

Treatment of donor cells and/or cloned embryos with cytidine analogues, having an Aza group at its 5th carbon (5-Aza), such as 5-Azacytidine (5-Aza-C) or 5-Aza-2′-deoxycytidine (5-Aza-dC) improves the *in vitro* development of cloned embryos produced by somatic cell nuclear transfer (SCNT). *In vitro* maturation (IVM) of immature pig oocytes treated with 5-Aza-C not only results in greater ($P < 0.05$) meiotic maturation to the MII stage but also enhances the capacity of 5-Aza-C treated oocytes for early embryonic development after parthenogenetic activation (PA), *in vitro* fertilization (IVF) or SCNT in a dose-dependent manner (0–10 μM). Cloned embryos generated from 5-Aza-C (0.01 μM) treated oocytes had an increased capacity to develop to the blastocyst stage (14.1 ± 1.5% compared with 9.6 ± 1.8%), greater probability of hatching (61.8 ± 1.5% compared with 45.0 ± 3.9%) and contained a greater number of cells per blastocyst (38.5 ± 4.4 compared with 30.5 ± 3.4) than those produced from non-treated control oocytes ($P < 0.05$). Data from the present study indicate that treatment of oocytes with 5-Aza-C may be an important approach to enhance the meiotic maturation and subsequent *in vitro* development of pig embryos. Future studies should be conducted to determine the underlying mechanism of improved early embryonic development of 5-Aza-C treated oocytes.

1. Introduction

Cloned embryos produced using somatic cell nuclear transfer (SCNT) have potential application in reproductive biotechnology, restoration of extinct species and derivation of patient-specific embryonic stem cells. Use of existing SCNT technologies has limitations because of cloning inefficiencies and incomplete or aberrant nuclear reprogramming. In particular, cloned embryos have a greater amount of DNA methylation than *in vivo*-produced embryos due to incomplete reprogramming for global DNA methylation in somatic cells (Kang et al., 2001; Shen et al., 2013). Consequently, results of several studies indicate the use of cytidine analogues, having an Aza group at the 5th carbon (5-Aza) as advantageous because of the actions of this compound to demethylate the DNA of donor cells and/or cloned embryos and enhance nuclear reprogramming (Enright et al., 2003, 2005; Ding et al., 2008; Diao et al., 2013; Huan et al., 2013). The 5-Aza in compounds such as 5-Azacytidine (5-Aza-C) or 5-Aza-2′-deoxycytidine (5-Aza-dC) is incorporated into DNA which leads to rapid decreases of DNA methylation by irreversibly binding and inactivating the DNA

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methyltransferase (Dnmt) enzyme (Christman, 2002). Although both 5-Aza-C and 5-Aza-dC are potent inhibitors of Dnmt, 5-Aza-C is more toxic to cells and inhibits DNA, RNA and protein synthesis by inhibiting tRNA methyltransferases, rRNA processing and *de novo* thymidylate synthesis (Christman, 2002).

There have been previous studies in which there was investigation of the effects of treating donor cells with 5-Aza.Results have been positive, negative or there has been no effect of 5-Aza treatment on early embryonic development of SCNT embryos depending on the type, concentration or duration of treatment with the 5-Aza (Jones et al., 2001; Enright et al., 2005; Jafarpour et al., 2011; Diao et al., 2013; Ning et al., 2013). Synergistic effects of 5-Aza with Trichostatin A (TSA; a histone deacetylase inhibitor) have also been reported (Ding et al., 2008; Sangalli et al., 2012). In a previous study, Huan et al. (2013)reported that 5-Aza treatment of cloned embryos, but not donor cells, was beneficial in enhancing the developmental competence of SCNT embryos. In a few studies there has been treatment of both the donor cell and cloned embryo in combination with TSA and results indicate that there is a beneficial effect of 5-Aza (Ding et al., 2008; Wang et al., 2011a, b). Unfortunately, 5-Aza is also known to cause mutagenic changes, genome re-arrangements, micronuclei formation, altered gene expression and DNA synthesis, inhibition of cellular proliferation and induction of apoptosis in mammalian cells (Stopper et al., 1995; Jackson-Grusby et al., 1997; Enright et al., 2005; Maslov et al., 2012; Diao et al., 2013; Shen et al., 2013). Thus, treatment of donor cells and/or cloned embryos with 5-Aza may pose a long-term risk in terms of mutagenicity or teratogenicity. Indeed, results of previous studies indicate that there are DNA damaging effects of 5-Aza on cells (Stopper et al., 1995; Jackson-Grusby et al., 1997; Maslov et al., 2012; Diao et al., 2013) and embryos (Branch et al., 1996; Rosen and Chernoff, 2002; Tsuji et al., 2009; Zhao et al., 2013).

Oocytes, the source of cytoplast for SCNT, store a large amount of RNA and proteins that have functions during early embryonic development until the activation of the zygotic nucleus (Gupta et al., 2009; Prather et al., 2009; Cao et al., 2014). Oocytes also contain the factors for nuclear reprogramming of the somatic nucleus during SCNT. These RNAs are believed to be transcribed during oocyte growth, as transcription ceases upon germinal vesicle breakdown (GVBD) while proteins continue to be translated during the entire period of cytoplasmic maturation (Gandolfi and Gandolfi, 2001; Ferreira et al., 2009; Prather et al., 2009). For the present study, it was hypothesized that treatment of oocytes with 5-Aza may alter the gene expression and lead to enhanced accumulation of RNA and proteins required for nuclear reprogramming and for supporting the early embryonic development of SCNT-derived embryos until the activation of zygotic nucleus (Prather et al., 2009; Zhao et al., 2013). In addition, because the oocyte nucleus is removed while conducting the SCNT procedure, treatment with 5-Aza may not increase the risks of mutagenicity or teratogenicity that are associated with 5-Aza treatment of donor cell and/or cloned embryos. To our knowledge, no previous study has been conducted to investigate the effects of treating oocytes with 5-Aza on the capacity of the oocytes to support early embryonic development after use of SCNT. The use of 5-Aza-C promotes the *in vitro* maturation of mouse oocytes (Zhao et al., 2013). Developmental capacity of 5-Aza-C treated oocytes, however, was not investigated.

The present study, therefore, was designed to investigate the *in vitro* development potential of SCNT-derived embryos produced from immature oocytes treated with 5-Aza-C during the *in vitro* maturation (IVM) period. Effects of 5-Aza-C on meiotic maturation and *in vitro* development after parthenogenetic activation (PA) or *in vitro* fertilization (IVF) were also investigated. Pigs were chosen as the animal to be studied because of the extensive experience of the research group conducting this type of research with pig oocytes/embryos and partly because of the relatively long IVM period of 42 to 44 h used to conduct experiments with pig oocytes/embryos, which is a sufficiently long experimental treatment period to evaluate any adverse effects of 5-Aza-C.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless specifically indicated.

2.1. Oocyte retrieval and in vitro maturation (IVM)

The collection and IVM of oocytes from abattoir-derived pre-pubertal pig ovaries were performed using procedures that have been previously described (Uhm et al., 2010). Briefly, cumulus-oophorus-complexes (COCs) were aspirated from medium-sized follicles (3–6 mm diameter) and were matured in groups of 50 in 500 µL of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY) supplemented with 25 mM $NaHCO_3$, 10% (v:v) pig follicular fluid, 0.57 mM cysteine, 0.22 µg/mL sodium pyruvate, 25 µg/mL gentamicin sulfate, 0.5 µg/mL FSH (Folltropin V; Vetrepharm, Canada), 1 µg/mL estradiol-17β, and 10 ng/mL epidermal growth factor under mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 in air for 42 h. The follicular fluid was prepared and used as previously described (Abeydeera and Day, 1997; Vatzias and Hagen, 1999). Briefly, follicular fluids were aspirated from medium-sized follicles (3–6 mm diameter) using 10 mL syringes fitted with 18 G hypodermic needle. The fluids were pooled and centrifuged at 3500 *g* for 30 min. The supernatant was filtered through 0.45 µ syringe filters, aliquoted and stored at -20 °C until use during a 3 to 4 week period. To evaluate the effect of 5-Aza-C on meiotic progression and subsequent *in vitro* development of oocytes, COCs were cultured in IVM medium supplemented with or without 5-Aza-C (0.001, 0.01, 0.1, 1.0 or 10.0 µM) for 42 h, as per the experimental design. Meiotic progression of oocytes was assessed at the end of IVM period by staining with fluorescent Hoechst 33342 stain, as previously described (Uhm et al., 2010). Briefly, oocytes were denuded of cumulus cells using 0.1% (v:v) hyaluronidase solution, fixed for 5 min in fixative solution containing 2% (v:v) formalin and 0.25% (v:v) gluteraldehyde, mounted on clean glass slides and stained with glycerol based Hoechst 33342 (12.5 µg/mL) solution for 10 min. Stained nuclei, which appeared blue when visualized using UV illumination with an epifluorescent microscope fitted with blue filter (excitation: 330–385 nm; emission: 420 nm; dichromatic: 400 nm), were then classified as GV, GVBD, MI, or MII based on their chromosomal configuration (Park et al., 2011).

2.2. Parthenogenetic activation (PA) of oocytes

Presumptive diploid parthenotes were produced by electro-activation using similar procedures as those previously described (Gupta et al., 2007a). Briefly, *in vitro* matured oocytes were denuded of cumulus cells in TL-HEPES medium (Parrish et al., 1988) supplemented with 0.1% (v:v) hyaluronidase. Oocytes having first polar body (PB1) in the perivitelline space were selected and electro-activated in activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, and 1.0 mM $CaCl_2$) using a single DC pulse of 1.0 kV/cm for 30 μs delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA). Activated oocytes were then cultured at 39 °C in North Carolina State University 23 (NCSU-23) medium supplemented with 7.5 μg/mL cytochalasin B (CB) for 4 h under mineral oil in a humidified atmosphere of 5% CO_2 in air to prevent the extrusion of second polar body (PB2). Presumptive diploid parthenotes were subsequently washed in CB-free NCSU-23 medium and cultured in *in vitro* culture (IVC) medium for 6 days.

2.3. In vitro fertilization (IVF)

In vitro fertilization of matured oocytes was performed using similar procedures as those previously described (Gupta et al., 2010). Briefly, *in vitro* matured oocytes were denuded of cumulus cells using 0.1% (v:v) hyaluronidase solution. Denuded oocytes having PB1 in the perivitelline space were selected, washed three times with fertilization medium [modified Tris-buffered medium containing 1 mM caffeine sodium benzoate and 0.1% (v:v) BSA] and were placed in groups of 10 to 15 oocytes per 50 μL droplets of the fertilization medium under mineral oil. Boar caudal epididymis sperm were obtained from an abattoir and placed in TL-HEPES and subjected to swim-up procedures for 10 min in Tyrode's albumin lactate pyruvate medium for sperm (Sp-TALP; (Parrish et al., 1988)). The sperm cells were collected after the swim-up procedures were imposed and were used for insemination of oocytes at a final sperm concentration of 5×10^5 sperm/mL. Sperm and oocytes were co-incubated at 39 °C in a humidified atmosphere of 5% CO_2 in air for 6 h. At the end of the co-incubation, presumptive zygotes were washed to remove the adhering sperm and subsequently cultured in IVC medium for 6 days.

2.4. Somatic cell nuclear transfer (SCNT)

Nuclear transfer was performed using similar procedures as those previously described (Gupta et al., 2007b). Briefly, *in vitro* matured oocytes were denuded of cumulus cells in TL-HEPES supplemented with 0.1% (v:v) hyaluronidase and enucleated by the aspirating PB1 and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (25 μm internal diameter). Enucleated oocytes were subsequently reconstructed by inserting a small sized (˜15 μm in diameter), smooth bordered pig fibroblast cell into the perivitelline space of each enucleated oocyte utilizing the same pipette used for enucleation. Donor cells for SCNT were prepared using similar procedures as those previously described (Uhm et al., 2009). Membrane fusion of donor cell with cytoplast and simultaneous activation was induced by a single DC pulse of 2.1 kV/cm for 30 μs, delivered by a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA). Fused couplets were cultured in NCSU23 medium supplemented with 7.5 μg/mL CB for 4 h at 39 °C under mineral oil in a humidified atmosphere of 5% CO_2 in air to prevent the extrusion of second polar body. Presumptive diploid clones were subsequently washed in CB-free NCSU-23 medium and cultured in IVC medium for 7 days.

2.5. In vitro culture (IVC) of embryos

Presumptive zygotes, produced using PA, IVF or SCNT, were cultured in NCSU-23 medium supplemented with 0.4% (v:v) essentially fatty acid free bovine serum albumin (BSA) for 6–7 d using similar procedures as previously described(Gupta et al., 2008a). The embryos were evaluated for occurrence of first cleavage (2–4 cell embryos) and blastocyst formation on Day 2 and Day 6 of IVC, respectively. The rates of cleavage and blastocyst formation were expressed as the percentage of 2–4 cell embryos (Day 2) or blastocyst (Day 6) out of the total number of mature oocytes used in respective experimental group. The SCNT embryos were further cultured for as long as 7 days to evaluate the capacity to expand and hatch. The blastocysts were considered as hatched, when there was a complete shedding of the zona pellucida on Day 7 of IVC. The hatching capacity of blastocyst was expressed as the percentage of total number of blastocysts that hatched completely (Gupta et al., 2007b; Sugimura et al., 2010). The blastocysts (Day 6 for PA and IVF embryos and, Day 7 for SCNT embryos) were subsequently stained with Hoechst 33342 for counting of total cell number as described previously in Section 2.1 of this manuscript.

2.6. Design of experiments

2.6.1. Experiment 1: effect of 5-Aza-C on meiotic maturation of pig oocytes

The first group of experiments was designed to evaluate the effects of 5-Aza-C on the meiotic maturation of pig oocytes. A total of five replicates were performed and in each of the replications, immature oocytes from the same collection of ovaries were randomly allotted to six different groups. Immature oocytes (n = 1523) were cultured in IVM media containing 0 (Control; n = 252), 0.001 (n = 243), 0.01 (n = 248), 0.1 (n = 249), 1.0 (n = 262) and 10 μM (n = 269) of 5-Aza-C. At the end of IVM period (42 h), the oocytes were denuded and stained with Hoechst 33342 for examining the nuclear morphology.

2.6.2. Experiment 2: post-activation in vitro development ability of 5-Aza-C treated oocytes

This group of experiments was designed to evaluate the *in vitro* developmental capacity of 5-Aza-C treated oocytes after PA. A

total of five replicates were performed and in each of the replication, immature oocytes from the same collection of ovaries were randomly allocated to six different groups as described for Experiment 1. At the end of the IVM period (42 h), the matured oocytes (n = 3741) were selected based on the presence of PB1 and were electro-activated. The embryos were cultured for 6 days and were evaluated for cleavage on Day 2 and blastocyst formation on Day 6 of IVC. The number of matured oocytes in each group were 615 (Control), 587 (0.001 μM), 659 (0.01 μM), 615 (0.1 μM), 626 (1.0 μM) and 639 (10 μM), respectively. The Day 6 blastocysts were subsequently stained with Hoechst 33342 for counting the nuclear number per blastocyst.

2.6.3. Experiment 3: post-fertilization in vitro developmental capacity of 5-Aza-C treated oocytes

This group of experiments was designed to evaluate the *in vitro* developmental capacity of 5-Aza-C treated oocytes after IVF. A total of five replicates were performed and with each of the replications, immature oocytes from the same collection of ovaries were randomly allotted to five different groups as described for Experiment 1. At the end of the IVM period (42 h), the matured oocytes (n = 1491) were selected by the presence of PB1 and were fertilized using boar sperm. In each of the replicates, the same boar sperm sample was used. The fertilized embryos were cultured for 6 days and were evaluated for cleavage on Day 2 and blastocyst formation on Day 6 of IVC. The number of matured oocytes in each group were 302 (Control), 304 (0.001 μM), 301 (0.01 μM), 284 (0.1 μM) and, 300 (1.0 μM), respectively. The Day 6 blastocysts were subsequently stained with Hoechst 33342 for counting the nuclear number per blastocyst.

2.6.4. Experiment 4: in vitro development ability of SCNT embryos produced from pig oocytes matured in the presence of 5-Aza-C

This group of experiments was designed to evaluate the *in vitro* developmental capacity of 5-Aza-C treated oocytes after SCNT. A total of five replicates were performed and with each of the replications, immature oocytes from the same collection of ovaries were randomly allocated to a control or 5-Aza-C (Conc.: 0.01 μM) group using the procedures described for Experiment 1. At the end of the IVM period (42 h), the matured oocytes were selected by the presence of PB1, enucleated (n = 513) and were reconstructed with the somatic cell nucleus of pig fibroblast (passage no: 3-5). In each of the replicates, the same passage fibroblast was used. The SCNT-derived embryos were cultured for 7 days and were evaluated for cleavage on Day 2, blastocyst formation on Day 6 and hatching of blastocysts on Day 7 of IVC. The number of enucleated cytoplast used in each group were 251 (Control) and 262 (0.01 μM), respectively. On Day 7 of IVC, all blastocysts (including non-hatched) were stained with Hoechst 33342 for counting the nuclear number per blastocyst.

2.7. Statistical analyses

Statistical analyses were performed using SPSS software (Version: 18.0, SPSS Inc, Chicago, IL, USA). ANOVA with Games-Howel *post-hoc* test was used as the test of significance to compare the rates of oocyte maturation, cleavage (2–4 cells), blastocyst formation and total cell number per blastocyst in PA and IVF embryos. The rates of cleavage, blastocyst formation and hatching capacity of SCNT-derived embryos were tested for significance using the Chi-square test. Percentage data were subjected to Arc sine transformation before analysis. Shapiro-Wilk test and Lavene's test were used to test the Normal distribution and variance of data, respectively, before assessing the data using an ANOVA. Data are presented as mean ± SD. Differences at $P \leq 0.05$ were considered significant.

3. Results

3.1. Experiment 1: effect of 5-Aza-C on meiotic maturation of pig oocytes

In the first group of experiments, there was investigation of the dose-dependent effect of 5-Aza-C on meiotic maturation of pig oocytes. Immature oocytes, collected from middle sized (3–6 mm diameter) follicles of pre-pubertal pig ovaries, were matured in the absence (Control) or presence of 5-Aza-C at different concentrations for 42 h and were evaluated for meiotic changes at the end of the IVM period. At the tested dose range, 5-Aza-C had a dose-dependent beneficial effect on the proportion of oocytes developing to the MII-stage (Figs. 1 and 2). The greatest extent of meiotic maturation was observed with use of a concentration of 0.01 μM where the percentage of oocytes developing to the MII stage was 85.9 ± 4.7% as compared with 75.2 ± 3.7% in the control group ($P < 0.05$). At the concentration of 1.0 μM or less, treatment with 5-Aza-C did not result in any increase in the morphological degeneration of oocytes.

3.2. Experiment 2: post-activation in vitro developmental capacity of 5-Aza-C treated oocytes

In Experiment 2, there was investigation of *in vitro* development of 5-Aza-C treated oocytes after PA. The PA system was chosen for the study because it avoids the possible confounding variation in results due to the male/sperm factor such as polyspermy which are common in pigs. Oocytes, matured in the absence (Control) or presence of 5-Aza-C at different concentrations were selected for the presence of PB1 and activated using electro-activation to produce diploid parthenotes. The 5-Aza-C treatment did not affect the capacity of oocytes to cleave and form blastocyst after imposing PA (Figs. 1 and 3). On the contrary, there was a dose- dependent increase in the capacity of 5-Aza-C treated oocytes to develop to the blastocyst stage (Table 2) and the most desirable result was at a concentration of 0.01 μM where the percentage of oocytes developing to the blastocyst stage was 22.6 ± 3.6% compared with 12.2 ± 2.2% in the control group ($P < 0.05$). There was no significant effect of the 5-Aza-C on the total cell number per blastocyst

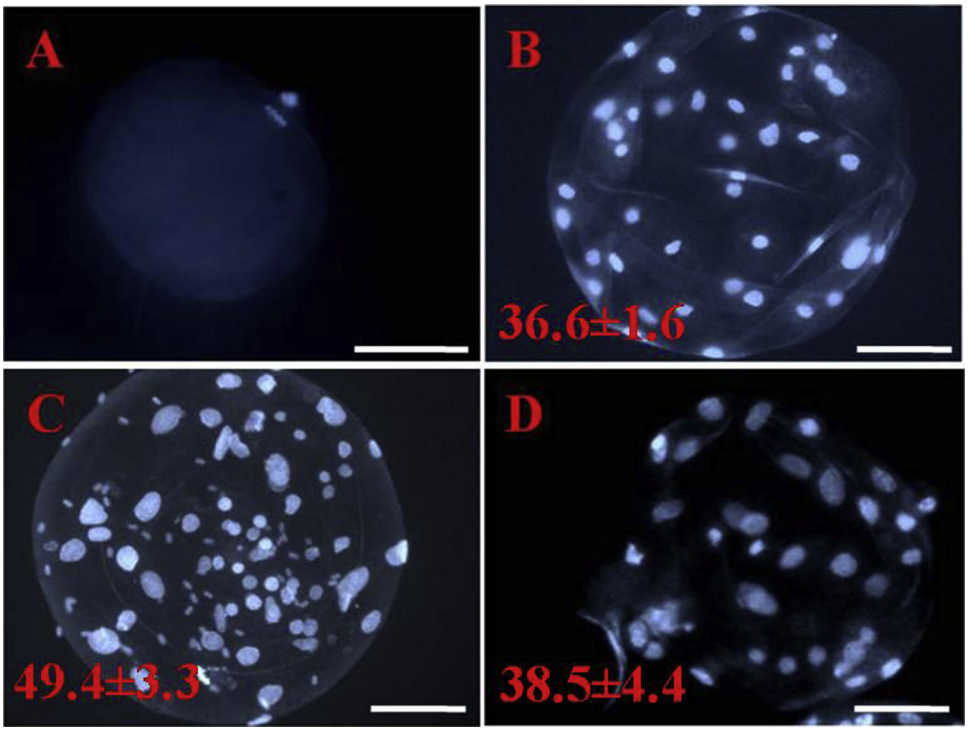

Fig. 1. Representative mature oocyte (A) and blastocysts (B–D) produced from pig oocytes matured after treatment with 5-Aza-C; Oocyte/blastocysts were stained with Hoechst 33342 fluorescent stain to visualize the nucleus; A: MII stage oocyte; B-D: Pig blastocysts produced by parthenogenetic activation (B; Day 6), *in vitro* fertilization (C; Day 6) or somatic cell nuclear transfer (D; Day 7); Numerical values within images represent the mean (± SD) number of cells per blastocyst; Scale bar: 50 μm.

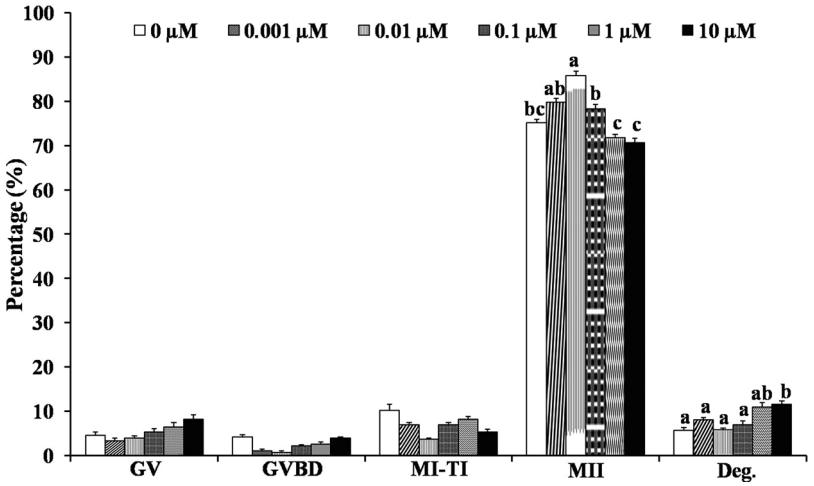

Fig. 2. *In vitro* maturation of pig oocyte matured in the absence or presence of 5-Aza-C at different concentrations; Percentage values were calculated from the total number of oocytes used in respective groups; Bars with different superscripts (a, b, c) among groups differ ($P < 0.05$); GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; MI-TI: Metaphase I – Telophase – I; MII: Metaphase II; Deg.: Degenerated oocytes.

(Table 2).

3.3. Experiment 3: post-fertilization in vitro developmental capacity of 5-Aza-C treated oocytes

In Experiment 3, there was assessment of the *in vitro* development of 5-Aza-C treated oocytes after IVF. Oocytes matured in the absence (Control) or presence of 5-Aza-C at different concentrations were selected for the presence of PB1 and subjected to IVF. There was a dose-dependent beneficial effect of 5-Aza-C treatment on the developmental capacity of oocytes to cleave and form blastocyst

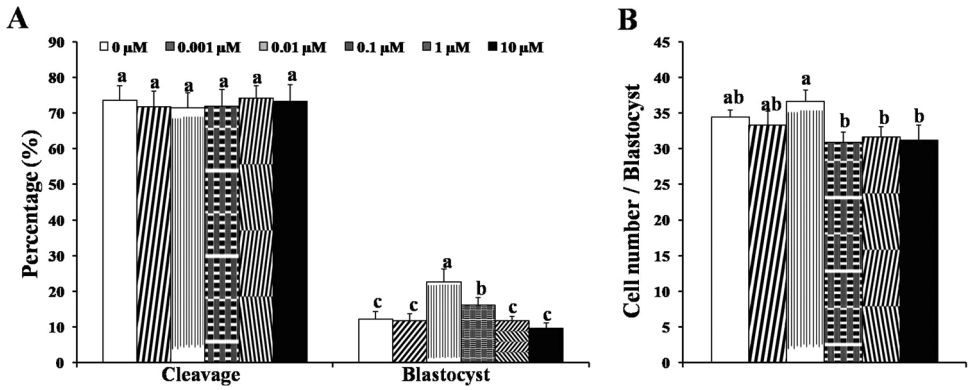

Fig. 3. Post-activation *in vitro* development of pig oocytes matured treated with 5-Aza-C at different concentrations or not treated with 5-Aza-C; A: *In vitro* development; Rates of cleavage and blastocyst formation are expressed as the percentage of 2–4 cell embryos (Day 2) or blastocysts (Day 6) of the total number of mature oocytes used in respective experimental group; B: Total cell numbers per blastocyst; Bars with different superscripts (a, b, c) among groups differ ($P < 0.05$).

with IVF (Figs. 1 and 4). At a concentration of 0.01 μM, the capacity of 5-Aza-C treated oocytes to cleave (68.8 ± 4.6%compared with 56.6 ± 4.8%; $P < 0.05$) and develop to the blastocyst stage (22.4 ± 2.2 compared with 11.8 ± 1.1%; $P < 0.05$) was the greatest in the treated compared with the control group. The blastocysts generated from pig oocytes treated with 0.01 μM 5-Aza-C also contained a greater number of cells than those developing from non-treated control oocytes (49.4 ± 3.3Tcompared with 39.3 ± 3.3; $P < 0.05$).

3.4. Experiment 4: in vitro developmental capacity of SCNT embryos produced from pig oocytes matured in the presence of 5-Aza-C

In Experiment 4, there was assessment of the capacity of 5-Aza-C treated oocytes to reprogram the somatic cell nucleus and support early embryonic development after performing SCNT. Based on the results of the previous experiments, only the 0.01 μM concentration of 5-Aza-C was selected to be used in Experiment 4. Oocytes matured in the presence of 0.01 μM 5-Aza-C were enucleated and reconstructed with the somatic cell nucleus. It was observed that 5-Aza-C treated oocytes did not differ ($P > 0.05$) from those of non-treated oocytes in the capacity to fuse and cleave (Figs. 1 and 5). The proportion of fused couplets developing to the blastocyst stage, however, was greater ($P < 0.05$) in 5-Aza-C treated than non-treated control group (14.1 ± 1.5%compared with 9.6 ± 1.8%). The blastocysts generated from 5-Aza-C treated oocytes also had a greater ($P < 0.05$) capacity to expand and hatch (61.8 ± 1.5%compared with 45.0 ± 3.9%) and contained a greater ($P < 0.05$) number of cells (38.5 ± 4.4 compared with 30.5 ± 3.4) than those generated from non-treated control oocytes (Table 4).

4. Discussion

In the present study, treatment of immature pig oocytes with a small concentration (0.01 μM) of5-Aza-C resulted in increased meiotic maturation and, these oocytes had a greater capacity to support early embryonic development after PA, IVF or SCNT.

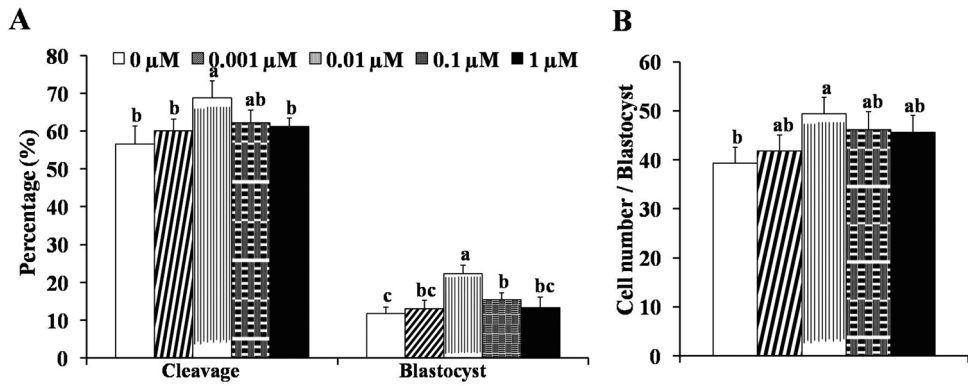

Fig. 4. Post-fertilization *in vitro* development of pig oocytes matured after treatment with 5-Aza-C at different concentrations or not treated with 5-Aza-C; A: *In vitro* development; Rates of cleavage and blastocyst formation are expressed as the percentage of 2–4 cell embryos (Day 2) or blastocysts (Day 6) of the total number of mature oocytes used in respective experimental group; B: Total cell number per blastocyst; Bars with different superscripts (a, b, c) among groups differ ($P < 0.05$).

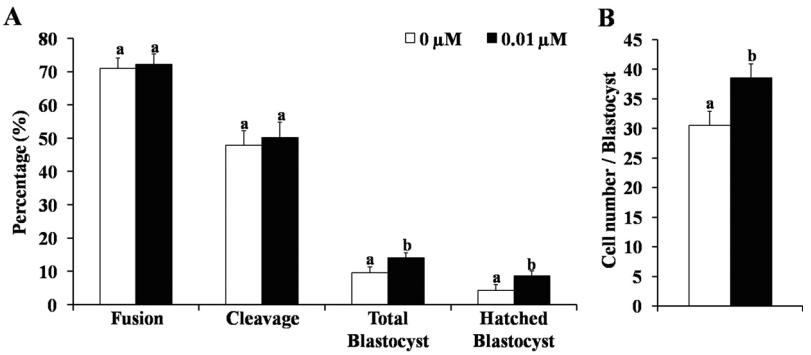

Fig. 5. *In vitro* developmental capacity of cloned embryos produced from pig oocytes matured in the absence or presence of 5-Aza-C; A: *In vitro* development; Rates of cleavage and blastocyst formation were expressed as the percentage of 2 to 4 cell embryos (Day 2) or blastocyst (Day 6) of the total number of enucleated oocytes used in respective experimental groups. Blastocysts were considered as hatched, when there was complete shedding of the zona pellucida on Day 7 of IVC; Hatching capacity of blastocysts was expressed as the percentage of total number of blastocysts that hatched completely; B: Total cell number per blastocyst (Day 7); Bars with different superscripts (a, b) among groups differ ($P < 0.05$).

Because there has been no previous study in which there was investigation of the effect on 5-Aza-C on pig oocytes, there was an initial evaluation of the dose-dependent effect of 5-Aza-C on meiotic maturation of pig oocytes. The 5-Aza-C had a positive effect on the meiotic maturation of pig oocytes and, at an optimal dose of0.01 µM, there was an improvement in generation of MII oocytes from 75.2 ± 3.7% to 85.9 ± 4.7% ($P < 0.05$). The cause of improvement in meiotic maturation is however, unknown. Results of previous studies indicate mammalian oocytes have both CG and non-CG methylation, of which non-CG methylation constitutes nearly two-thirds of all methylcytosines in GV oocytes (Shirane et al., 2013). During oocyte maturation, the amount of CG methylation remains similar but the non-CG methylation increases and is enriched in the coding region of genes notably at maternally methylated imprint control regions and some CG-rich island regions (Yu et al., 2017). The non-CG methylation is catalyzed by DNA methyltransferases (Dnmt) 3a, and 3 L but can also be increased as a result of inhibition of Dnmt1 due to compensatory up-regulation of Dnmt3a (Lucifero et al., 2007; Shirane et al., 2013). The 5-Aza-C is a potent inhibitor of Dnmt1 and thereby may have increased the non-CG methylation of the coding region of genes to result in improved maturation potential (Yu et al., 2017). Conversely, inhibition of Dnmt1 by treatment with 5-Aza-C may have inhibited *de novo* CG-methylation (Shirane et al., 2013)with the result being expression and accumulation of those genes for which the encoded proteins may have functions during oocyte maturation and subsequent embryonic development until zygotic genome activation. Indeed, hyper-methylation of the oocyte genome, through knocking-out of the *Stella* gene, impairs zygotic genome activation in mice(Li et al., 2018).Results from *in vitro* studies in mice also indicate the treatment of GV oocytes with 5-Aza-C increased the expression of *Gdf9* and *Bmp15* genes (Zhao et al., 2013) that are known to be involved in the regulation of meiotic maturation and zygotic genome activation(Lin et al., 2014; Sudiman et al., 2014). Results of previous studies also indicate DNA methylation is a prerequisite for chromosome condensation during metaphase (Schmid et al., 1985, 1986). Thus, inhibition of DNA methylation as a result of 5-Aza-C treatment may result in chromosome de-condensation, which may result in genes becoming accessible to transcriptional factors to bind and initiate transcription (Zhao et al., 2013). Because oocytes are enucleated prior to SCNT, there was not evaluation of the chromosomal configuration and DNA methylation of 5-Aza-C treated oocytes in the present study.The SCNT-derived embryos produced from 5-Aza-C treated oocytes had an increased capacity to develop up to blastocyst stage. Further, the SCNT-derived blastocysts were of greater quality in terms of total cell number per blastocyst and the capacity to expand and hatch, which are considered reliable criteria for evaluation of embryo quality *in vitro* (Kidson et al., 2004; Gupta et al., 2007b; Jiang et al., 2011; Li et al., 2014). The increased developmental capacity of SCNT-derived embryos, generated from 5-Aza-C treated oocytes, is not likely due to altered DNA methylation because nuclear DNA of oocytes does not contribute to the genome of the cloned embryos. On the contrary, it may be due to altered mRNA and protein profiles of the oocytes as a result of 5-Aza-C induced changes in DNA methylation, chromosomal decondensation and/or inhibition of DNA, RNA and protein synthesis resulting from inhibition of tRNA methyltransferases, rRNA processing and *de novo* thymidylate synthesis (Schmid et al., 1985, 1986; Christman, 2002; Zhao et al., 2013). During oocyte maturation, transcription ceases at GVBD whereas protein translation continues to occur to accumulate RNA and proteins (Gandolfi and Gandolfi, 2001; Ferreira et al., 2009; Prather et al., 2009). These RNA and proteins function in regulation of the first two cellular divisions in pig embryos and establishment of the correct chromatin configuration so that the cascade of actions of gene expression can begin at the time of zygotic genome activation at the 4-cell stage (Prather et al., 2009). Thus, any alteration in RNA and protein profiles of the oocyte as a result of treatment with 5-Aza-C can affect post-SCNT developmental potential of the oocytes. This is quite likely because pig oocytes treated with 5-Aza-C during IVM and subjected to PA or IVF also had a greater capacity to form blastocyst than non-treated oocytes. Given that 5-Aza-C can also affect RNA function and stability and inhibit protein translation (Christman, 2002; Paczkowski et al., 2011; Aimiuwu et al., 2012), its other pleiotropic effects on improving the early embryonic development are also likely. Results from experiments with a cell free-extract system indicate that the nuclear reprogramming was improved when somatic cells were reprogrammed with the oocyte extracts and treatment with epigenetic modifiers (Yang et al., 2012). It, therefore, is likely that 5-Aza-C induced alterations in the cytoplasm may have some role in improving the developmental potential of the embryos.

It is also interesting to note that 5-Aza-C treatment of oocytes can be used to improve the *in vitro* development capacity of pig

oocytes following IVF and, therefore, may have an important implication in assisted reproduction in both animals and human. The improvement in post-fertilization early embryonic development was not due to a male/sperm factor such as polyspermy because the beneficial effect of 5-Aza-C on early embryonic development was also observed with PA embryos, where *in vitro* development characteristics resemble those of IVF embryos but there is avoidance of possible confounding variations due to male/sperm factors such as polyspermy (Kure-bayashi et al., 2000; Gupta et al., 2008b, 2009). Future studies should be conducted to determine whether IVF embryos generated from 5-Aza-C treated oocytes are chromosomally normal to support full-term development. This is particularly important for IVF embryos because in a previous study results indicated that there was an induction of apoptosis and chromosomal instability in mouse oocytes treated with 5-Aza-C (Zhao et al., 2013). Nonetheless, it remains to be a lesser concern for SCNT-derived embryos where the oocyte nucleus is removed prior to transplanting the donor nucleus.

5. Conclusions

In conclusion, results of the present study indicate that treatment of pig oocytes with 5-Aza-C enhances the meiotic maturation and subsequent *in vitro* development of pig embryos. Future studies should be conducted to determine the underlying mechanism of improved meiotic maturation and subsequent early embryonic development of 5-Aza-C treated oocytes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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