Zygote **23** (June), pp. 327–335. © Cambridge University Press 2014
doi:10.1017/S0967199413000580 First Published Online 10 January 2014

Generation of parthenogenetic goat blastocysts: effects of different activation methods and culture media

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Date submitted: 13.5.2013. Date revised: 7.10.2013. Date accepted: 27.10.2013

Summary

The present study was carried out to investigate the effects of different activation methods and culture media on the *in vitro* development of parthenogenetic goat blastocysts. Calcium ($Ca^{2+)}$) ionophore, ethanol or a combination of the two, used as activating reagents, and embryo development medium (EDM), modified Charles Rosenkrans (mCR2a) medium and research vitro cleave (RVCL) medium were used to evaluate the developmental competence of goat blastocysts. Quantitative expression of apoptosis, stress and developmental competence-related genes were analysed in different stages of embryos. In RVCL medium, the cleavage rate of Ca^{2+} ionophore-treated oocytes (79.61 ± 0.86) was significantly ($P < 0.05$) higher than in ethanol (74.90 ± 1.51) or in the combination of both Ca^{2+} ionophore and ethanol. In mCR2a or EDM, hatched blastocyst production rate of Ca^{2+} ionophore-treated oocytes (8.33 ± 1.44) was significantly higher than in ethanol (6.46 ± 0.11) or in the combined treatment (6.70 ± 0.24). In ethanol, the cleavage, blastocyst and hatched blastocyst production rates in RVCL medium (74.90 ± 1.51, 18.30 ± 1.52 and 8.24 ± 0.15, respectively) were significantly higher than in EDM (67.81 ± 3.21, 14.59 ± 0.27 and 5.59 ± 0.42) or mCR2a medium (65.09 ± 1.57, 15.36 ± 0.52 and 6.46 ± 0.11). The expression of BAX, Oct-4 and GlUT1 transcripts increased gradually from 2-cell stage to blastocyst-stage embryos, whereas the transcript levels of Bcl-2 and MnSOD were significantly lower in blastocysts. In addition, different activation methods and culture media had little effect on the pattern of variation and relative abundance of the above genes in different stages of parthenogenetic activated goat embryos. In conclusion, Ca^{2+} ionophore as the activating agent, and RVCL as the culture medium are better than other tested options for development of parthenogenetic activated goat blastocysts.

Keywords: BAX, Bcl-2, Ca^{2+} ionophore, Ethanol, Goat, Parthenogenetic activation

Introduction

Parthenogenesis, a form of asexual reproduction in which growth and development of embryos occur without male contribution, occurs naturally in many invertebrates as well as in some vertebrates. The parthenogenetic activation of mammalian oocytes is a vital tool for investigating the roles of paternal and maternal genomes in controlling early embryonic development. The parthenogenetic activation of oocyte protocol can be used as a reference model to improve the activation process of reconstructed oocytes during somatic cell nuclear transfer studies (Kim *et al.*, 1996). Embryonic stem cells have been isolated successfully and cultured from parthenogenetically produced blastocysts in mice (Allen *et al.*, 1994; Yu *et al.*, 2011), monkeys (Cibelli *et al.*, 2002; Vrana *et al.*, 2003; Wei *et al.*, 2011), humans (Revazova *et al.*, 2007), pigs (Xu *et al.*, 2007), rabbits (Hsieh *et al.*, 2011) and cattle (Pashaias *et al.*, 2010).

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https://doi.org/10.1017/S0967199413000580 Published online by Cambridge University Press

During fertilization, the activation impulses generated by sperm penetration induce a series of calcium (Ca^{2+}) transients in mature oocytes (Miyazaki *et al.*, 1993; Carroll *et al.*, 1996). According to Miyazaki (1990), these Ca^{2+} spikes are propagated throughout the fertilized oocyte in the form of a wave, which initiates cortical granule exocytosis, resumption of second meiosis and affects later embryonic development. None of the reported experimental protocols of oocyte activation is capable of mimicking the natural sperm-induced response pattern in mammalian oocytes (Sun *et al.*, 1991). Both the amplitude and frequency of Ca^{2+} transients produced by *in vitro* stimulation affect the inactivation of maturation-promoting factor (MPF), duration of pronuclear formation, rate of blastomere compaction and blastocyst formation in parthenogenetically activated oocytes. The signalling for mammalian oocyte activation and the mechanisms that underlie these important biological events have not been fully established, although it has been confirmed that the development of a simple activation system can be capable of inducing the full series of developmental responses.

Several chemical and physical stimuli such as Ca^{2+} ionophore, ethanol, strontium, electro-stimulus and ultrasound have been used to activate metaphase II oocytes (Loi *et al.*, 1998; Alberio *et al.*, 2001; Das *et al.*, 2003; Sato *et al.*, 2005; Varga *et al.*, 2008; De *et al.*, 2012). Ionophores, i.e. calcium ionophore and ionomycin, increase the intracellular calcium concentration in metaphase II mammalian oocytes and have been reported to activate several calcium-dependent proteolytic pathways, leading to the destruction of cyclin B, inhibition of the MPF activity and resumption of meiosis (Rinaudo *et al.*, 1997; Tomashov-Matar *et al.*, 2005; Jellerette *et al.*, 2006). Similarly, chemical activation by ethanol has been shown to increase the intracellular Ca^{2+} concentration that resulted from both extracellular entry and from mobilization of intracellular Ca^{2+} ion depots (Shiina *et al.*, 1993).

Culture medium is the most vital factor for improvements in the efficiency of *in vitro* embryo development. Commonly used media, such as TCM-199 and mSOF, have been shown to be less efficient for *in vitro* development of cloned buffalo embryos produced through hand-made cloning technology (Shah *et al.*, 2008). Commercially available sequential medium (G_1/G_2, VitroLife, Sweden) has been reported to improve the efficiency to some extent (Simon *et al.*, 2006). In the present study, three different types of media [embryo development medium (EDM), modified Charles Rosenkrans (mCR2a) medium and research vitro cleave (RVCL)] medium were evaluated for the efficiency of parthenogenetically activated goat embryo production.

The aim of the present study was to investigate the effects of different activation methods and culture media on yield of parthenogenetically activated goat blastocysts. In the first experiment, the effects of Ca^{2+} ionophore, ethanol or a combination of both ethanol and Ca^{2+} ionophore on production of blastocysts and hatched blastocysts were evaluated. In the second experiment, the effect of various culture media (EDM, mCR2a or RVCL) on goat embryo development was evaluated. In a third experiment, the quantitative expression of apoptosis, stress and developmental competence-related genes were analysed in different stages of embryos. The outcome of the experiments could help to improve the efficiency of somatic cell nuclear transfer and hand-made cloning in terms of yield and quality of blastocysts.

Materials and methods

All the present experiments comply with all relevant institutional and national animal welfare guidelines, policies and ethics committee approval. All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and disposable plasticwares were from Nunc (Roskilde, Denmark) unless specified otherwise.

In vitro maturation of cumulus–oocyte complexes

Goat ovaries were collected from a Delhi slaughter house and washed 2–3 times with normal saline (0.9%) at 35°C that contained the antibiotics penicillin (400 IU/ml) and streptomycin (50 μg/ml), trimmed with sterile scissors and washed 4–5 times with warm normal saline fortified with antibiotics. Immature goat cumulus–oocyte complexes (COCs) were punctured out from antral follicles. Grade A oocytes (those that had more than five layers of cumulus cells with evenly granulated cytoplasm) and grade B oocytes (having 3–5 layers of cumulus cells with evenly granulated cytoplasm) were collected in TCM-199. The COCs were washed twice with maturation medium [TCM-199 supplemented with 10 μg/ml luteinizing hormone (LH), 5 μg/ml follicle stimulating hormone (FSH), 1 μg/ml estradiol-17β, 50 μg/ml sodium pyruvate, 5.5 mg/ml glucose, 3.5 μg/ml L-glutamine, 50 μg/ml gentamicin, 3 mg/ml bovine serum albumin (BSA) and 10% fetal bovine serum (FBS)]. Washed COCs were incubated in *in vitro* maturation (IVM) medium and incubated at 38.5 °C in 5% CO_2 in air in an incubator with maximum humidity for 24 h (Malakar *et al.*, 2008).

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Parthenogenetic activation of matured oocytes

Matured oocytes with expanded cumulus cells were transferred into the micro-centrifuge tube that contained 0.5 mg/ml hyaluronidase in T2 (TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 μg/ml gentamicin and 2% FBS) and incubated for 1 min at 38.5 °C in 5% CO_2 in air in an incubator. COCs in hyaluronidase was vortexed for 30 s and transferred to a 35-mm Petri dish that contained T2. Completely denuded oocytes were selected and washed twice in fresh T2 to remove cumulus cells.

Experiment 1: Effect of different chemical activation methods on blastocyst development

For chemical activation, the denuded oocytes were incubated in primary embryo development medium (EDM) [TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 100 μl/ml non-essential amino acid (100×), 50 μl/ml essential amino acid (50×), 10 mg/ml BSA and 50 μg/ml gentamicin] in three groups. In group A, denuded oocytes were incubated in EDM that contained 5 μM Ca^{2+} ionophore A23187 for 5 min and in group B, oocytes were kept in EDM that contained 7% cell culture-tested ethanol for 8 min at 38.5 °C in 5% CO_2 in air in an incubator. Similarly, in group C, oocytes were incubated in EDM that contained 2.5 M Ca^{2+} ionophore and 3.5% cell culture-tested ethanol for 7 min at 38.5 °C in 5% CO_2 in air in an incubator. Activated oocytes were washed in EDM for complete removal of Ca^{2+} ionophore and ethanol followed by incubation in EDM that contained 2 mM 6-dimethylaminopurine (6-DMAP), at 38.5 °C in 5% CO_2 in air in an incubator for 4 h.

Experiment 2: Effect of different culture media on development of parthenogenetic activated goat blastocyst

In order to evaluate the developmental competence of parthenogenetically produced goat embryos, the activated oocytes from Experiment 1 were washed with EDM to remove 6-DMAP and kept in three different culture media: (i) EDM; (ii) RVCL supplemented with 1% BSA; and (iii) modified mCR2a medium that contained 108.3 mM NaCl, 24.9 mM $NaHCO_3$, 1 mM L-glutamine, 2.9 mM KCl, 2.5 mM hemicalcium lactate, 0.5 mM sodium pyruvate, 0.5 mM glycine, 0.5 mM alanine, 1 mM glucose, 100 μl/ml non-essential amino acid (100×), 50 μl/ml essential amino acid (50×), 10 mg/ml BSA and 50 μg/ml gentamycin). Uncleaved embryos were removed after 72 h of culture, and 50% of the medium was taken out from the droplet and replaced with replacement medium (EDM, mCR2a or RVCL medium supplemented with 10, 10 or 4% FBS respectively). Afterwards, the medium was replaced on alternate days for 6–8 days. Blastocysts and hatched blastocysts were observed after 7–8 days of culture.

Experiment 3: Gene expression analysis by real-time polymerase chain reaction (PCR)

Expression of apoptosis, stress and developmental competence-related genes were analysed in different stages of embryonic development. Embryos at 2–4-cell, 8–16-cell, morulae and blastocysts stage, produced by different activation methods and culture media were separately selected for the gene expression study. Total RNA was isolated using the TRIzol method (Invitrogen Corporation, Carlsbad, CA, USA) and quantified by measurement of the absorbance ratio at 260:280 nm. First-strand complementary DNA (cDNA) was synthesized from 500 ng of total RNA by reverse transcriptase polymerase chain reaction (RT-PCR) using RevertAid™ First Strand cDNA synthesis kit (Fermentas). The transcript abundance of genes related to developmental competence of embryos was quantified by real-time PCR (qRT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene and the expression of each target gene was normalized to GAPDH. The sequences of the primer sets used for qRT-PCR analysis are shown in Table 1. The primers were amplified on a LightCycler® 480 instrument with software version 1.5 (Roche Diagnostics, Mannheim, Germany). The 'crossing point' or Cp values were determined by a 'second-derivative max method' in the software. All qRT-PCR runs were performed in triplicate and each reaction mixture was prepared in a total volume of 10 μl. The reaction mixture consisted of 2 μl of cDNA as template, 5 μl of 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) that contained 0.5 μM of gene-specific primer. The following cycling conditions were employed for all the genes: preincubation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 60°C for 30s and extension at 72°C for 30s. The relative abundance of transcripts was calculated using the equation, $2^{-(\Delta Cp\ sample - \Delta Cp\ control)}$ developed by Livak & Schmittgen (2001).

Statistical analysis

Data pertaining to the developmental rates were analysed using SigmaPlot (Version 11) after the arcsine transformation of the percentage values. The differences between means were analysed by one-way analysis of variance (ANOVA). Significance was determined at P-values < 0.05.

Results

In vitro maturation of COCs

In the present study, only grades A and B immature oocytes were selected for *in vitro* maturation (Fig.1*A*).

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Table 1 List of primers

Name of genes	Sense primer sequence (5′→3′)	Anti-sense primer sequence (5′→3′)
MnSOD	cgtgactttggttcctttg	ggataagacctgttgttcctgg
Oct-4	gactatctgccgttttgagg	tactcgtccgctttctcttt
BAX	tttctctttggaaaggtgtt	acactcggaccacgtctttc
Bcl-2	gcaggtattggtgagtg	attgttcccgtagagttcc
GLUT1	ctgatcctgggtcgcttc	ggatacctcccccacgtaca

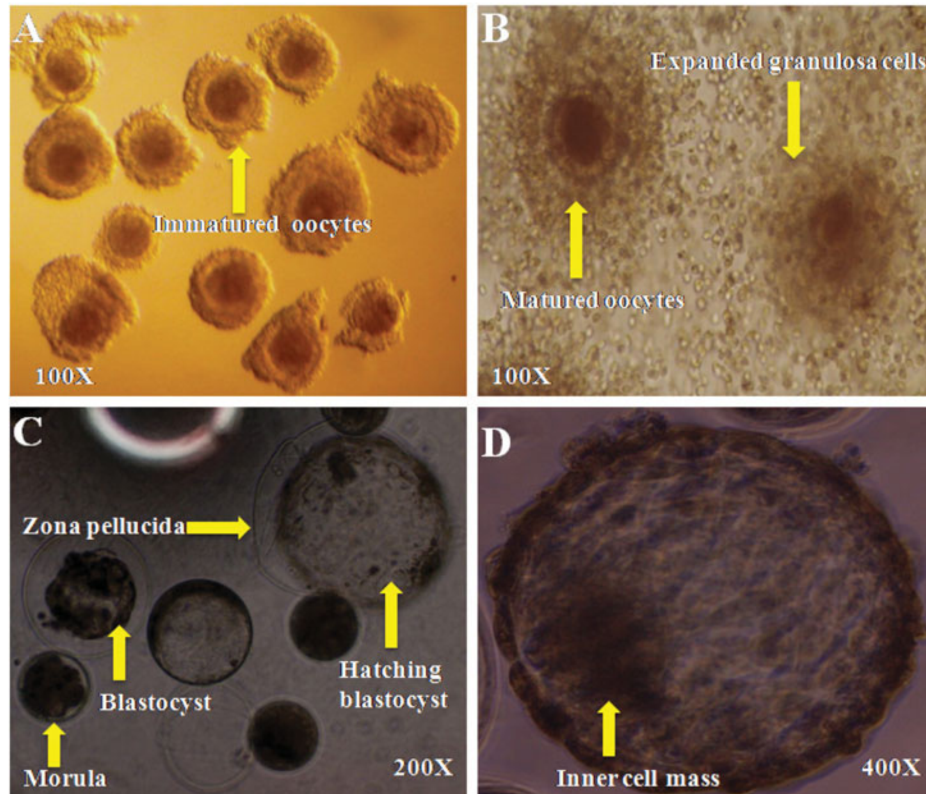

Figure 1 Development of parthenogenetically activated goat embryos. (*A*) Immature goat oocytes. (*B*) Matured oocytes. (*C*) Morulae and blastocysts. (*D*) Hatched blastocyst. (*A*,*B*) ×100 magnification; (*C*) ×200 magnification; (*D*) ×400 magnification.

Generally, oocytes from different species reach metaphase II stage (maturation) at different times when cultured *in vitro*. Bovine and ovine oocytes mature *in vitro* in 18–24 h, but goat oocytes require 27 h for maturation. *In vitro* maturation of COCs was assessed by the expansion of their cumulus cells (Fig. 1*B*).

Experiment 1: Effect of different chemical activation methods on parthenogenetically activated embryo production

The results of Experiment 1 are shown in Table 2. When RVCL was used as a culture medium, cleavage rate in Ca^{2+} ionophore-mediated activation (79.61 ± 0.86) was significantly higher than for ethanol (74.90 ± 1.51) or the combination of Ca^{2+} ionophore and ethanol (73.06 ± 0.58) (Table 2). No significant difference was observed in terms of blastocysts or hatched blastocyst production rate in the above condition (Table 2; Fig. 1*C*,*D*). In mCR2a and EDM culture media, hatched blastocyst production rate in Ca^{2+} ionophore-treated oocytes (8.33 ± 1.44) was significantly higher than for ethanol (6.46 ± 0.11) or the Ca^{2+} ionophore with ethanol combination (6.70 ± 0.24), and no significant difference was found in terms of cleavage and blastocyst production rate (Table 2).

Experiment 2: Effect of different culture media on parthenogenetic activated embryo production

The outcome of Experiment 2 is shown in Table 3. In ethanol-activated parthenogenetic embryos, cleavage,

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Table 2 Effect of different chemical activation methods on development of parthenogenetic activated embryos (data from four trials)

Culture medium	Activation methods	No. of oocytes	Cleavage % (n)	Blastocyst % (n)	Hatched blastocyst % (n)
mCR2a	Ethanol	570	371 (65.09 ± 1.57)	57 (15.36 ± 0.52)	24 (6.46 ± 0.11)^a
	Ca^{2+} ionophore	561	396 (70.59 ± 1.31)	68 (17.17 ± 0.70)	33 (8.33 ± 1.44)^b
	Ca^{2+} ionophore + ethanol	519	358 (68.97 ± 1.23)	55 (15.36 ± 0.40)	24 (6.70 ± 0.24)^a
EDM	Ethanol	606	411 (67.81 ± 3.21)	60 (14.59 ± 0.27)	23 (5.59 ± 0.42)^a
	Ca^{2+} ionophore	569	382 (67.14 ± 0.86)	62 (16.23 ± 1.10)	30 (7.85 ± 1.39)^b
	Ca^{2+} ionophore + ethanol	491	338 (68.83 ± 0.96)	48 (14.20 ± 0.46)	18 (5.32 ± 0.57)^a
RVCL	Ethanol	518	388 (74.90 ± 1.51)^a	71 (18.30 ± 1.52)	32 (8.24 ± 0.15)
	Ca^{2+} ionophore	564	449 (79.61 ± 0.86)^b	92 (20.49 ± 0.70)	39 (8.68 ± 1.46)
	Ca^{2+} ionophore + ethanol	568	415 (73.06 ± 0.58)^a	73 (17.59 ± 0.32)	33 (7.95 ± 0.18)

^{a,b,c}Values having different superscripts in the same column differ significantly ($P < 0.05$).
Results are expressed as mean ± standard error of the mean (SEM).
EDM, embryo development medium; mCR2a, modified Charles Rosenkrans; RVCL, research vitro cleave.

Table 3 Effect of different culture media on development of parthenogenetic activated embryo (data from four trials)

Activation methods	Culture medium	No. of oocytes	Cleavage % (n)	Blastocyst % (n)	Hatched blastocyst % (n)
Ethanol	mCR2a	570	371 (65.09 ± 1.57)^a	57 (15.36 ± 0.52)^a	24 (6.46 ± 0.11)^a
	EDM	606	411 (67.81 ± 3.21)^a	60 (14.59 ± 0.27)^a	23 (5.59 ± 0.42)^a
	RVCL	518	388 (74.90 ± 1.51)^b	71 (18.30 ± 1.52)^b	32 (8.24 ± 0.15)^b
Ca^{2+} ionophore	mCR2a	561	396 (70.59 ± 1.31)^a	68 (17.17 ± 0.70)^a	33 (8.33 ± 1.44)
	EDM	569	382 (67.14 ± 0.86)^b	62 16.23 ± 1.10)^a	30 (7.85 ± 1.39)
	RVCL	564	449 (79.61 ± 0.86)^c	92 (20.49 ± 0.70)^b	39 (8.68 ± 1.46)
Ca^{2+} ionophore + ethanol	mCR2a	519	358 (68.97 ± 1.23)^a	55 (15.36 ± 0.40)^a	24 (6.70 ± 0.24)^a
	EDM	491	338 (68.83 ± 0.96)^a	48 (14.20 ± 0.46)^a	18 (5.32 ± 0.57)^b

^{a,b,c}Values having different superscripts in the same column differ significantly ($P < 0.05$).
Results are expressed as mean ± standard error of the mean (SEM).
EDM, embryo development medium; mCR2a, modified Charles Rosenkrans; RVCL, research vitro cleave.

blastocyst and hatched blastocyst production rates in RVCL medium (74.90 ± 1.51, 18.30 ± 1.52 and 8.24 ± 0.15, respectively) were significantly higher than in EDM (67.81 ± 3.21, 14.59 ± 0.27 and 5.59 ± 0.42) and mCR2a (65.09 ± 1.57, 15.36 ± 0.52 and 6.46 ± 0.11) (Table 3). No significant difference was found in terms of hatched blastocyst production rate among these three media with calcium ionophore-mediated activation. But cleavage and blastocyst development rates in RVCL medium (79.61 ± 0.86 and 20.49 ± 0.70) were significantly higher than in mCR2a medium (70.59 ± 1.31 and 17.17 ± 0.70) and EDM (67.14 ± 0.86 and 16.23 ± 1.10) (Table 3). Similarly, when both calcium ionophore and ethanol were used as an activating agent cleavage, blastocyst and hatched blastocyst production rates in RVCL medium (73.06 ± 0.58, 17.59 ± 0.32 and 7.95 ± 0.18) were significantly higher than for EDM (68.83 ± 0.96, 14.20 ± 0.46 and 5.32 ± 0.57) and mCR2a (68.97 ± 1.23, 15.36 ± 0.40 and 6.70 ± 0.24) (Table 3). The RVCL medium, in comparison with mCR2a and EDM, significantly affected the development of parthenogenetically activated goat embryos.

Experiment 3: Gene expression analysis by qRT-PCR

Expression of apoptosis, stress and developmental competence-related genes were analysed in different stages of embryonic development. The relative abundance of BAX, Bcl-2, MnSOD, Oct-4 and GLUT1 transcripts were evaluated in 2–4-cell, 8–16-cell, morula- and blastocyst-stage embryos (Fig. 2). The effects of different embryo culture media and activation methods were categorically examined for relative abundance of above transcripts. Irrespective of different media and activation methods used, most genes were not expressed or expressed at a greatly reduced rate in 2–4-cell stage embryos (Fig. 2). Variation in expression pattern was observed in subsequent developmental stages vis-à-vis 8–16-cell, morula and blastocyst-stage embryos. The BAX transcript level was significantly higher in 8–16-cell, morula- and blastocyst-stage, whereas Bcl-2 and MnSOD were found to be expressed at much higher levels in 8–16-cell and morula stages but decreased expression was noticed in blastocyst-stage embryos (Fig. 2). The expression of developmental

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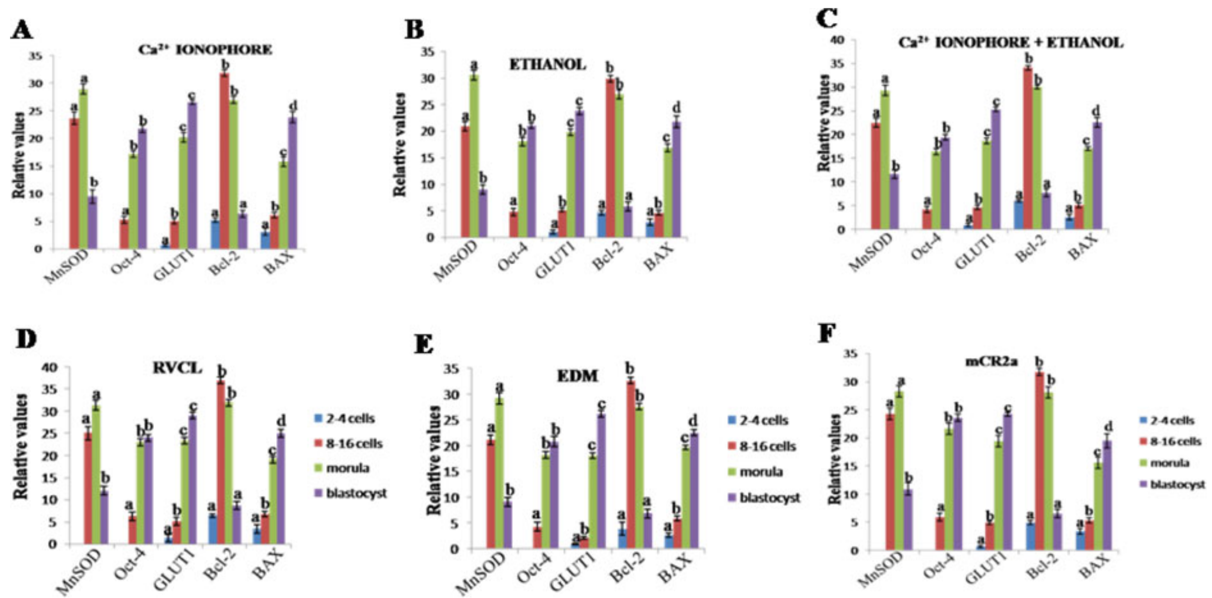

Figure 2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of stress, apoptotic and developmental competence-related genes at different stages of parthenogenetically activated goat embryos developed in (*A*–*C*) respective activation methods; and (*D*–*F*) culture media. EDM, embryo development medium; mCR2a, modified Charles Rosenkrans; RVCL, research vitro cleave. ^{a,b,c,d}Values indicate .

competence-related genes (Oct-4 and GLUT1) increased gradually with the progression of embryonic development and attained maximum at the morula and blastocyst stages (Fig. 2). With relevance to different activation methods and embryo culture media used, a homogenous expression pattern of the above genes was observed in different developmental stages of parthenogenetically activated goat embryos (Fig. 2). Different activation methods and culture media imparted very small influence on variation pattern of transcripts abundance of same genes.

Discussion

During fertilization, sperm entry provides a natural stimulus for oocyte activation, which influences early embryonic development (Collas *et al.*, 1993). According to Bootman & Berridge (1995), the sperm–oocyte interaction leads to release of intracellular Ca^{2+} from endoplasmic reticulum, which interacts to produce a series of repetitive waves that are responsible for meiotic progression and early embryo development. In a study by Kline & Kline (1992), the released Ca^{2+} ion binds to both inositol 1,4,5-triphosphate receptors (InsP3R) and ryanodine receptors (RyR). The InsP3R channel has been shown to be involved in propagation; maintenance of the Ca^{2+} waves and RyR channels has been linked to the conversion of the zona glycoprotein (ZP2) to its post-fertilization form ZP2f (Ayabe *et al.*, 1995). The continuous release of calcium in a pulsatile manner enables the oocyte to produce strong ionic signals, while, at the same time, the harmful toxic effects associated with prolonged exposure to high cytoplasmic Ca^{2+} concentrations should be avoided. It is, therefore, important to consider that artificial methods of activation provide a non-toxic calcium signal to the metaphase II-arrested oocyte. Each of the methods of activation tested in the present study utilized a different mechanism for the elevation of intracellular calcium ion. Ethanol induced a single Ca^{2+} rise that resulted from both extracellular entry and some mobilization of intracellular stores (Shiina *et al.*, 1993). However, according to Hoth & Penner (1992), ionomycin exclusively mobilizes intracellular Ca^{2+} stores when used as an activating agent. A combination of both calcium and ionomycin has been observed to induce the release of calcium from extracellular entry and from some mobilization of intracellular stores. Each of the artificial activators induced the release of only a single wave rather than a series of repetitive calcium waves, generated during fertilization. A single rise in calcium levels from either source (extracellular calcium influx or by mobilization of intracellular stores) has been shown in most oocytes to enable progression from metaphase II arrest to pronuclear formation (Loi *et al.*, 1998). These multiple and periodic oscillations in intracellular calcium concentrations were responsible for suppression of MPF and mitogen activation promoter factor (MAPK), and led to activation of the oocyte (White & Yue, 1996).

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Calcium ionophore A2187 increased intracellular Ca^{2+} concentration, which in turn destroyed the existing calcium-sensitive cytostatic factor (CSF) and resulted in the reduction of MPF activity (Swann & Ozil 1994). The combination of calcium ionophores with 6-dimethylaminopurine (6- DMAP) induces high rates of activation, pronucleus formation and development to the blastocyst-stage in sheep (Loi *et al.*, 1998), cattle (Liu *et al.*, 1998) and goat (De *et al.*, 2012). The outcome of the present study revealed that the Ca^{2+} ionophore significantly increased the production rate of hatched blastocysts, but had no effect on cleavage and blastocysts production rate, when activated oocytes were cultured in mCR2a and EDM. Calcium ionophore increased the cleavage rate in RVCL medium but exerted no difference on production of blastocyst and hatched blastocysts in same medium.

Similarly, when oocytes were activated by ethanol followed by kinase inhibitor treatment (6-DMAP), the formation of a second pronucleus was suppressed. It was postulated that 6-DMAP inhibits MAP kinase and leads to a disruption of spindle organization in metaphase II oocytes and irregularities in the gross morphology of the oocyte (Szollosi *et al.*, 1993; Moore *et al.*, 1995). Although 6-DMAP treatment enhanced the speed of pronuclear formation and suppressed polar body extrusion, this treatment has no measurable effect on the levels of MPF kinase in ovine oocytes after chemical activation (Bogliolo *et al.*, 1996). Ethanol was reported to induce oocyte activation in sheep (Loi *et al.*, 1998), cattle (Simone *et al.*, 2004), domestic cat (Grabiec *et al.*, 2007), pronucleus formation and development into blastocyst stage in cattle, either alone or in combination with strontium (Simone *et al.*, 2004). The current study found that, compared with Ca^{2+} ionophore, either ethanol alone or in combination with Ca^{2+} ionophore did not significantly increase the development of caprine embryos. The combination of ethanol and Ca^{2+} ionophore may be relatively detrimental for oocyte architecture, and result in a lower cleavage rate and blastocyst production rate in goat.

Different culture media were employed for successful development of mammalian embryos. Modified synthetic oviduct fluid (mSOF) medium supplemented with essential and non-essential amino acids, sodium citrate, myoinositol and FBS has been used in cattle (Booth *et al.*, 2001), horses (Lagutina *et al.*, 2007) and goats (Jena *et al.*, 2012) for culture of hand-made cloned embryos and parthenogenetic activated caprine embryos (De *et al.*, 2012). Several media have been used for porcine embryos, i.e. Whitten's medium (Wright, 1977); North Carolina State University (NCSU)-23 and NCSU-37 medium (Petters & Wells, 1993); Beltsville Embryo Culture Medium (BECM)-3 (Dobrinsky *et al.*, 1996); and porcine zygote medium (PZM)-3 and PZM-4 (Du *et al.*, 2007; Yoshioka *et al.*, 2002). Among these media, NCSU-23 has been used widely as the most successful medium for culture of porcine embryos after *in vitro* fertilization. mCR2a medium has been used in buffalo (Shah *et al.*, 2008) and goats (Zhang *et al.*, 2007) for culture of hand-made cloned embryos. RVCL medium significantly increases cleavage, morulae and blastocyst production in cloned embryo of buffalo (Shah *et al.*, 2008) and parthenogenetic activated embryos of goat (De *et al.*, 2012). The present study showed that, compared with mCR2a medium or EDM, RVCL medium significantly affects the development of parthenogenetic activated goat blastocysts.

Apoptosis plays an important role in cellular differentiation and embryonic development. Environmental stressors, such as those created by *in vitro* culture, induce unplanned apoptosis in cultured embryos, which lead to abnormal development and lower embryo viability (Jurisicova *et al.*, 1998; Byrne *et al.*, 1999). In the present study, expression analysis of pro-apoptotic (BAX) and anti-apoptotic genes (Bcl-2) indicated that the incidence of apoptosis reached its peak at the 8–16 cells stage of embryonic development. Hence, it can be speculated that apoptosis may play a rule in the developmental arrest of 8–16 cells stage goat embryos. MnSOD is an oxidative stress response gene and has been implicated in cellular differentiation process. The expression analysis of MnSOD shows that transcripts are not present at the 2–4-cell stage. The abundance of MnSOD was first observed at the 8–16-cell stage, attained maximum levels at the morula stage and decreased subsequently. This pattern may be due to the onset of differentiation procedure at the morula stage and to combat a gradual increase in oxidative stress with the advancement of culture process. The expression of glucose transporter (GLUT1) increased gradually starting from the 2–4-cell stage to the blastocyst-stage embryo. The metabolic activities of embryos increased with the advancement of its cell number and uptake of glucose also enhanced with it. Therefore the gradual rise in expression of the *GLUT1* gene supported the notion that, to meet the increasing demand of glucose, the expanding cell number of embryonic cells required additional amounts of glucose transporter protein. Various activation methods and embryo culture media did not affect significantly on rate of compaction of blastomeres and cell number of morula or blastocyst-stage parthenogenetic activated goat embryos. So they imparted a small effect on relative expression of these stress, apoptosis and developmental related genes.

Conclusion

In conclusion, based on overall embryonic growth and development rates, activating agents Ca^{2+} ionophore

https://doi.org/10.1017/S0967199413000580 Published online by Cambridge University Press

was found to have the most beneficial impact on goat oocyte activation without compromising embryonic quality. For supporting growth and development of parthenogenetic goat blastocysts, RVCL medium was found to be better than EDM and mCR2a. The expression of BAX (pro-apoptotic), Oct-4 and GLUT1 (development related) transcripts increases gradually from the 2-cell stage to blastocyst-stage embryos, whereas the transcript level of Bcl-2 (anti-apoptotic), MnSOD (stress related) was significantly lower in parthenogenetic activated blastocyst-stage goat embryos. Importantly, different activation methods and embryo culture media imparted a small variation in transcript abundance pattern of these genes.

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