Zygote

cambridge.org/zyg

Research Article

Cite this article: Singh KP *et al*. (2021) Parthenogenetic activation of buffalo (*Bubalus bubalis*) oocytes: comparison of different activation reagents and different media on their developmental competence and quantitative expression of developmentally regulated genes. *Zygote*. **29**: 49–58.
doi: 10.1017/S0967199420000519

Received: 2 December 2019
Revised: 2 June 2020
Accepted: 12 August 2020
First published online: 2 October 2020

Keywords:
Blastocyst; Buffalo; Embryo; Oocyte; Parthenogenetic activation

Author for correspondence:
M.S. Chauhan. Director, ICAR-Central Institute of Research on Goats, Makdoom, Mathura (UP), India.
E-mail: chauhanabtc@gmail.com

*Present address: Director, ICAR-National Dairy Research Institute, Karnal, Haryana, India.

CAMBRIDGE UNIVERSITY PRESS

Parthenogenetic activation of buffalo (*Bubalus bubalis*) oocytes: comparison of different activation reagents and different media on their developmental competence and quantitative expression of developmentally regulated genes

K.P. Singh[1], S.K. Mohapatra[2], R. Kaushik[1], M.K. Singh[1], P. Palta[1], S.K. Singla[1], R.S. Manik[1] and M.S. Chauhan*

[1]Animal Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana, India and [2]Department of Animal Biotechnology, College of Veterinary Science and A.H. Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India.

Summary

This study was carried out to compare the efficacy of different methods to activate buffalo A + B and C + D quality oocytes parthenogenetically and to study the *in vitro* developmental competence of oocytes and expression of some important genes at the different developmental stages of parthenotes. The percentage of A + B oocytes (62.16 ± 5.06%, range 53.8–71.3%) was significantly higher ($P < 0.001$) compared with that of C + D oocytes (37.8 ± 5.00%, range 28.6–46.1%) retrieved from slaughterhouse buffalo ovaries. Among all combinations, ethanol activation followed by culture in research vitro cleave medium gave the highest cleavage and blastocyst yields for both A + B and C + D grade oocytes. Total cell numbers, inner cell mass/trophectoderm ratio and apoptotic index of A + B group blastocysts were significantly different ($P < 0.05$) from their C + D counterpart. To determine the status of expression patterns of developmentally regulated genes, the expression of cumulus–oocyte complexes, fertilization, developmental competence and apoptotic-related genes were also studied in parthenogenetically produced buffalo embryos at different stages, and indicated that the differential expression patterns of the above genes had a role in early embryonic development.

Introduction

Buffaloes are important livestock resources that provide milk, meat and draft power in many ecologically disadvantaged agricultural systems due to their high performance in these areas. Oocytes are unique cells that alone can give rise to whole embryos. Oocyte-specific genes play important roles in regulating ovarian development and the timely progression of oogenesis and folliculogenesis. Cumulus cells play an important role in cell signalling, leading to maturation and fertilization of oocytes.

Parthenogenetic activation is a form of asexual reproduction that involves the activation of the oocyte without sperm to produce a nonviable blastocyst. Parthenogenetic embryos carry only maternal chromosomes and can be generated by inducing oocytes to resume meiosis without fertilization. Methods such as electrical stimulus, use of chemical agents such as calcium ionophores, ethanol, strontium chloride, phorbol ester, thimerosal and phospholipase zeta have been used successfully to activate bovine oocytes (Ross *et al*., 2008). The parthenogenetic activation of buffalo oocytes with zona or without zona by ethanol or calcium ionomycin followed by 6-DMAP treatment has been reported previously (Gasparrini *et al*., 2004; Shah *et al*., 2008; Singh *et al*., 2012).

Mammalian oocytes are arrested at metaphase II after ovulation or *in vitro* maturation due to high levels of maturation promoting factor (MPF). Oocytes need to resume meiosis for further development. Final maturation occurs following fertilization, due to a rise in intracellular Ca^{2+} in the form of a series of oscillations that lasts many hours, followed by inactivation of MPF (Wu *et al*., 1997). Although sperm provide the natural stimulus for oocyte activation, oocytes may also be activated parthenogenetically by a variety of physical and chemical stimuli. Several chemicals have been used to induce Ca^{2+} oscillations and increase in Ca^{2+} concentration by parthenogenetic activation of oocytes with or without treatment with 6-dimethylaminopurine (6-DMAP). These chemicals activate oocytes by a mechanism similar to that used by sperm for inducing higher intracellular Ca^{2+} levels. Ionomycin and 6-DMAP treatment has been reported for activation of zona-intact somatic cell nuclear transfer buffalo embryos (Shi *et al*., 2007) and zona-free hand-made cloned embryos (George *et al*., 2011).

Major events in mammalian preimplantation embryonic development are as follows: degradation of oocyte transcripts, transcriptional activation of the zygotic genome during cleavage, compaction, and establishment of the inner cell mass and trophectoderm lineages. The initiation of mRNA synthesis in the embryo, which is called embryonic genome activation (EGA) or maternal to embryonic transition (MET), is perhaps the most important barrier that has to be crossed for successful early embryonic development. Failure to cross this barrier, which occurs at the 8- to 16-cell stage in cattle and 2-cell stage in mice (Telford *et al.*, 1990), results in a 'developmental block'. Understanding the mechanisms and factors associated with MET are critical for improving methods and conditions for *in vitro* embryo production.

There have been very few comparative studies on the efficacy of different methods for activating buffalo A + B and C + D quality oocytes (Gasparrini *et al.*, 2004; Mishra *et al.*, 2008; Shah *et al.*, 2008; George *et al.*, 2011). This study was carried out to compare the efficacy of different methods to activate buffalo A + B and C + D quality oocytes parthenogenetically and to study the *in vitro* developmental competence of oocytes and expression of some important genes in the different developmental stages of parthenotes.

Materials and methods

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Disposable cell culture grade plasticware was purchased from Becton Dickinson and Co. (Lincoln Park, NJ, USA) or Nunc (Roskilde, Denmark). Syringes were purchased from Henke Saas Wolf GmbH (Tuttingen, Germany), whereas the 0.22-µm filters were from Millipore Corp. (Bedford, MA, USA). Fetal bovine serum (FBS) and research vitro cleave (RVCL) medium were purchased from Hyclone (Logan, Utah, USA) and Cook (Australia) respectively.

In vitro maturation, parthenogenetic activation and culture of oocytes

In vitro maturation of buffalo oocytes was carried out for parthenogenetic activation. Briefly, buffalo ovaries were transported to the laboratory within 6 h of slaughter of animals from a nearby slaughter house. Buffalo ovaries were washed 3–5 times with warm isotonic saline (32–37°C) containing 400 IU ml^{-1} penicillin and 500 µg ml^{-1} streptomycin. Follicles of 2–8 mm in diameter of oocytes were aspirated with a 18-gauge needle attached to a 10-ml syringe containing aspiration medium [TCM-199 + 0.3% bovine serum albumin (BSA) + 50 µg ml^{-1} gentamicin sulfate] and searched under a zoom stereomicroscope. After searching, the oocytes were then washed 4–6 times with washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 µg ml^{-1} gentamicin sulfate) and divided in two categories based on their cumulus cell layer. These were: (i) A + B, which had an unexpanded cumulus mass of ≥2 layers of cumulus cells and an homogenous evenly granular ooplasm; and (ii) C + D, oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm. Oocytes of both qualities were processed separately for *in vitro* maturation (IVM). After washing three times with IVM medium (TCM-199 + 10% FBS + 5 µg ml^{-1} pFSH + 1 µg ml^{-1} estradiol-17β + 0.81 mM sodium pyruvate + 50 µg ml^{-1} gentamicin sulfate), groups of 15–20 cumulus–oocyte complexes (COCs) were cultured in 100-µl droplets of IVM medium, overlaid with sterile mineral oil in 35-mm Petri dishes, and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air with 90–95% relative humidity) at 38.5°C.

For production of embryos, both (A + B) and (C + D) grade oocytes were activated with three artificial activation reagents: (i) 7% ethanol; (ii) CaI; or (iii) electrical pulse. These activated oocytes were cultured in three types of medium: (i) modified Charles–Rosenkrans + amino acids-2 (mCR2); (ii) modified synthetic oviductal fluid (mSOF); and (iii) RVCL medium (RVCL®, Cook, Australia) and were compared in this study. Briefly, oocytes were denuded of cumulus cells by incubation in 0.2% hyaluronidase in Dulbecco's phosphate-buffered saline (PBS) for 2 min after 24 h IVM. The denuded oocytes with a prominent polar body were activated by subjecting them to one of the three following treatments: (i) 7% ethanol for 7 min; (ii) 5 µM CaI A23187 for 5 min; or (iii) electrical pulse (double DC pulse of 1.2 kV cm^{-1} or 2.1 kV/cm or 3.3 kV cm^{-1} for 20 µs each) using a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA), followed by incubation with 2 mM 6-dimethyl aminopurine in respective culture media for 4 h in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) at 38.5°C.

At the end of oocyte incubation with DMAP, the activated oocytes were washed separately several times with mCR2, mSOF or RVCL medium, respectively. The presumptive parthenotes were shifted to *in vitro* culture (IVC) medium [mCR2aa + 0.6% BSA + 10% FBS, mSOF with 1% fatty acid-free (FAF) BSA and RVCL supplemented with 1% FAF-BSA] and cultured in 100-µl droplets of these media on original beds of granulosa cells for up to 8 days post activation in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C. The medium was replaced with 50% of fresh IVC medium every 48 h.

Assessment of blastocyst quality

Cleavage and blastocyst rate were recorded on day 8 of *in vitro* culture and were taken as a measure of embryo development. To examine the health of the embryos produced from both grade oocytes, total cell numbers of trophectoderm (TE) and inner cell mass (ICM) of day 7 blastocysts were determined by differential staining, as described by Thouas *et al.* (2001), with few modifications. Briefly the blastocysts were washed with DPBS for 10 to 15 s and transferred to 500 µl of solution I (5 µg/ml Hoechst 33342) and incubated for 40 min at 37°C. Then blastocysts were washed with DPBS and immediately transferred to 500 µl of solution II (0.04% Triton X-100) for 1 min, after that blastocysts were washed again with DPBS, followed by incubation in 25 µg/ml propidium iodide for 40 s. The number of nuclei was counted using an inverted microscope (Nikon Diaphot) fitted with a UV lamp and excitation filters (excitation wavelength: 330–380 nm; barrier filter: 420 nm). ICM cells were stained blue while troph ectodermal cells stained a red colour.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

To examine the quality of the embryos, the total cell number and the number of apoptotic nuclei in the blastocyst produced from A + B and C + D grade oocytes were determined using TUNEL staining. TUNEL was carried out using an *in situ* cell death detection kit (cat. no. 11 684 795 910; Roche, Mannheim, Germany) as described previously by Mohapatra *et al.* (2015a). Briefly, day 8 blastocysts were collected, washed three times with PBS + 0.3% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde for

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

1 h at room temperature. Fixed blastocysts were permeabilized by incubation with 0.5% Triton X-100 for 1 h. The blastocysts were then incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h at 37°C in the dark. The treated blastocysts were added to RNase (50 µg/ml) and were stained with Hochst 33342 (5 µg/ml) for 5 min at 37°C in the dark. For the positive control, blastocysts were treated with DNase solution (100 U/ml) for 20 min at 37°C prior to incubation with FITC-conjugated dUTP and TdT. The stained blastocysts were washed with DPBS (Ca^{2+}- and Mg^{2+}-free) and were mounted on glass slides in 3 µl droplets of antifade solution and were flattened with a cover slip. Cell counting was performed from the digital images obtained on an inverted Nikon fluorescence microscope. Apoptotic index = (number of TUNEL-positive nuclei/total number of nuclei in blastocyst) × 100.

Quantitative real-time polymerase chain reaction (PCR)

Relative mRNA abundance of developmental genes was determined by quantitative real-time PCR (RT-qPCR) as described previously (Singh *et al.*, 2014; Mohapatra *et al.*, 2015b). Briefly, total RNA was isolated from immature or *in vitro* matured oocytes, embryos at 2-, 4- and 8- to 16-cell, morula and blastocyst stages ($n = 10$ each), using the RNAqueous Micro Kit (Ambion Inc., The RNA Company, Austin, TX, USA) in accordance with the manufacturer's instructions, but with some modifications. Briefly, the oocytes/embryos were lysed with lysis buffer, after which RNA was eluted from the column after several washes with the wash solution 1 and wash solution-2/3. Genomic DNA contamination was removed by DNase treatment at 37°C for 20 min. Before cDNA synthesis, RNA concentration was measured and set at 20 ng/µl. cDNA was synthesized using first-strand cDNAs Superscript III, cDNA synthesis kit (Invitrogen) in accordance with the manufacturer's instructions. For cDNA synthesis, 2 µl total RNA (40 ng), 1 µl dNTP mix (10 mM) and 1 µl oligodT (50 µM) were heated at 65°C for 5 min, after which 2 µl RT-buffer (10×), 4 µl $MgCl_2$ (25 mM), 2 µl DTT (0.1 M), 1 µl RNase Out (40 U/µl) and 1 µl RT enzyme (200 U/µl) were added and the mixture was incubated at 50°C for 50 min and then at 85°C for 5 min. After that, 1 µl RNase H (2 u/µl) was added and the mixture incubated at 37°C for 20 min. Nuclease-free water was then added to make the volume up to 20 µl in a 200 µl tube. The synthesized cDNA was stored at −80°C until use for RT-qPCR.

Quantification of mRNA was carried out using the CFX96 real-time system (Bio-Rad, Hercules, CA, USA). For RT-qPCR, a relative quantification method was used. For this, cDNA from all samples were diluted at a 1:3 ratio. Genes were quantified using a CFX 96 thermocycler (Bio-Rad) and detected using the Maxima_ SYBR Green/ROX qPCR Master Mix (2×) (Fermentas). All reactions were run in triplicate, and three biological replicates were carried out. Relative levels of expression were determined using the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (target gene) − Ct (internal reference), and ΔΔCt = ΔCt sample − ΔCt calibrator. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal reference gene. Initially the expression study was performed with housekeeping genes such as beta-actin, H2A, 18S rRNA and GAPDH in buffalo oocytes/embryos. Some reference genes were not stable in different stages but GAPDH was found to be stable in all embryo stages. The 2-cell stage for each gene served as the calibrator in developmental stages. RT-qPCR reactions were performed using the PCR conditions used for all genes, and were as follows: initial denaturation at 95°C for 3 min, 40 cycles (denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s), and melting cycle starting from 65°C up to 95°C with a 0.5°C s^{-1} transition rate. The annealing temperatures for all genes are listed in Table S1. Relative mRNA expression was expressed as n-fold mRNA expression relative to the calibrator. The specificity and integrity of PCR products was ensured through melt curve analysis. No PCR products were obtained when reverse transcriptase was omitted from cDNA synthesis or when DNA templates were omitted from the PCR reaction.

Statistical analysis

Statistical analysis was carried out using Sigma Stat version 3.1 software (Aspire Software International, VA, USA). Datasets were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Holm–Sidak test. Differences were considered to be statistically significant at $P < 0.05$. Data were presented as the mean ± standard error of the mean.

Results

The morphology of A + B grade oocytes and different stages of parthenotes generated by ethanol activation and cultured on RVCL medium are shown in Fig. 1. Blastocysts generated by CaI and electric pulse and cultured on mCR2 and mSOF media are also shown in Fig. 1(*H*, *I*). The morphology of C + D grade oocytes and different stages of parthenotes generated by ethanol activation and cultured on RVCL medium are shown in Fig. 2(*H*, *I*). Blastocysts generated by CaI and electric pulse and cultured on mCR2 and mSOF media are also shown in Fig. 2. In the present study, the percentage of A + B oocytes (62.16 ± 5.06%, range 53.8–71.3%) was significantly higher ($P < 0.001$) compared with that of C + D oocytes (37.8 ± 5.00%, range 28.6–46.1%).

Parthenogenetic activation of buffalo oocytes

The developmental competence of A + B and C + D grade oocytes after parthenogenetic activation with three chemicals and subsequent culture in three media was examined in the present work. Table 1 summarizes the developmental competence of A + B grade oocytes. After ethanol or CaI or electrical pulse activation, a significant increase ($P < 0.05$) in cleavage rate and blastocyst rate was found in all three treatment groups when activated oocytes were cultured in mCR2 and RVCL media compared with that of culture in mSOF medium. Similar results were found for C + D grade oocytes after activation with three chemicals and subsequent culture in three media (Table 2). Among all combinations, ethanol activation followed by RVCL culture gave the highest cleavage and blastocyst yield for both A + B and C + D grade oocytes.

In the present study, the developmental competence of blastocysts derived from A + B grade oocytes activated with ethanol and cultured in RVCL were compared with blastocysts derived from C + D grade oocytes activated with ethanol and cultured in RVCL. The quality assessment of blastocyst produced through parthenogenetic activation of both grade oocytes was carried out on the basis of total cell number (TCN) and ICM/TE ratio using differential staining and apoptotic index using TUNEL assay. TCN of the blastocysts produced from A + B grade oocytes was observed to be 171.32 ± 2.64, while ICM to trophectoderm ratio was found to be 0.16 ± 0.006 (Fig. 1 G) and the TCN of

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

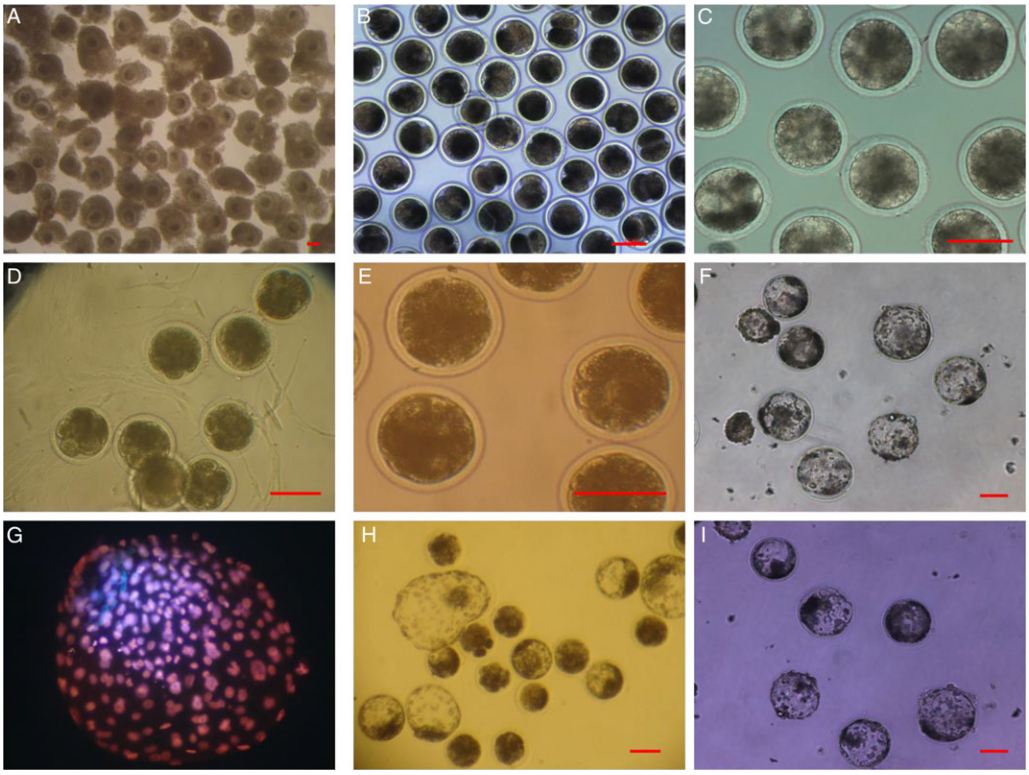

Figure 1. Morphology of buffalo A + B grade oocytes and embryos. (*A*) A + B grade oocytes, (*B*) 2-cell stage, (*C*) 4-cell stage, (*D*) 8–16-cell stage, (*E*) morula, (*F*) blastocysts, (*G*) differential staining of blastocyst generated by ethanol activation and cultured on RVCL medium, (*H*) blastocysts generated from CaI and cultured on RVCL medium, (*I*) blastocysts generated from electric pulse and cultured on RVCL medium. Scale bars, 100 µm.

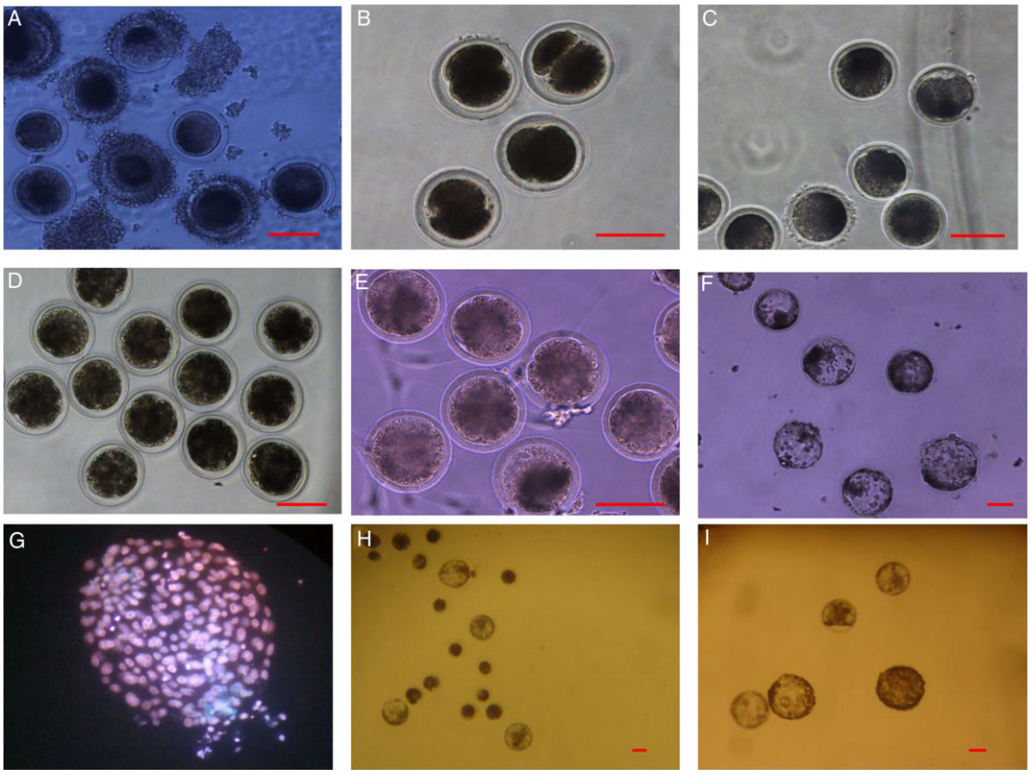

Figure 2. Morphology of buffalo C + D grade oocytes and embryos. (*A*) C + D grade oocytes, (*B*) 2-cell stage, (*C*) 4-cell stage, (*D*) 8–16-cell stage, (*E*) morula, (*F*) blastocysts. (*G*) Differential staining of blastocyst generated by ethanol activation and cultured on RVCL medium. (*H*) Blastocysts generated from CaI and cultured on RVCL medium. (*I*) Blastocysts generated from electric pulse and cultured on RVCL medium. Scale bars, 100 µm.

the blastocysts produced through C + D grade oocytes was observed to be 163.04 ± 3.83, while ICM to trophectoderm ratio was found to be 0.10 ± 0.086 (Fig. 2 G). TCN, ICM/TE and apoptotic index of A + B group blastocysts were significantly different ($P < 0.05$) from that of their C + D counterpart (Table 3).

Expression level of the cumulus–oocyte complex, fertilization, developmental competence and apoptosis-related genes in parthenogenetically produce embryos

The expression of developmentally regulated genes was studied in the parthenogenetically produced buffalo embryo at different stages. The relative mRNA abundances of *CX37*, *GDF9*, and

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

Table 1. Effect of culture medium and activation methods on cleavage rate and blastocysts rate of parthenogenetically activated embryos derived from (A + B) quality oocytes*

Activation methods (reagents)	Culture medium	No. of oocytes (A + B grade)	Cleavage rate % (*n*)	Blastocyst rate % (*n*)
Ethanol	mCR2	320	77.80 ± 1.47 (249)	25.93 ± 0.90 (83)
	mSOF	308	48.64 ± 3.74^{a} (150)	7.01 ± 1.02^{a} (22)
	RVCL	301	75.34 ± 0.71 (227)	33.93 ± 0.39^{b} (102)
Calcium ionophore	mCR2	297	62.76 ± 0.98 (187)	16.63 ± 1.10 (50)
	mSOF	302	45.95 ± 0.94^{a} (138)	6.15 ± 0.45^{a} (19)
	RVCL	316	68.37 ± 0.51 (216)	18.16 ± 0.93^{b} (58)
Electrical pulse	mCR2	280	46.89 ± 1.87 (131)	8.17 ± 0.66 (23)
	mSOF	290	30.20 ± 0.65^{a} (88)	3.36 ± 0.31^{a} (10)
	RVCL	269	56.97 ± 2.37^{b} (154)	13.30 ± 0.29^{b} (36)

*Data from five trials.
^{a,b}Values having different superscripts along columns, differ significantly ($P < 0.05$).

Table 2. Effect of culture medium and activation methods on cleavage rate and blastocysts rate of parthenogenetically activated embryos derived from (C + D) quality oocytes*

Activation methods (reagents)	Culture medium	No. of oocytes (C + D grade)	Cleavage rate % (*n*)	Blastocyst rate % (*n*)
Ethanol	mCR2	472	24.95 ± 2.29 (117)	1.23 ± 0.54 (6)
	SOF	490	19.39 ± 0.63 (95)	0.39 ± 0.24 (2)
	RVCL	468	38.57 ± 2.57^{a} (179)	3.81 ± 0.40^{a} (18)
Calcium ionophore	mCR2	432	23.60 ± 0.27 (102)	1.58 ± 0.19 (7)
	SOF	449	22.35 ± 0.72 (101)	0.61 ± 0.25^{a} (3)
	RVCL	465	34.36 ± 1.02^{a} (160)	4.06 ± 0.15^{b} (19)
Electrical pulse	mCR2	470	20.18 ± 1.37 (96)	0.98 ± 0.43 (5)
	SOF	457	18.95 ± 0.55 (86)	0.59 ± 0.24 (3)
	RVCL	466	31.11 ± 0.48^{a} (145)	2.10 ± 0.23^{a} (10)

*Data from five trials.
^{a,b}Values having different superscripts along columns, differ significantly ($P < 0.05$).

Table 3. Comparison of TCN, ICM/TE ratio and apoptotic index between blastocysts derived from A + B and C + D grade oocytes

Blastocyst type	TCN	ICM/TE	Apoptotic index
A + B	171.32 ± 2.64^{a}	0.16 ± 0.006^{a}	5.74 ± 0.40^{a}
C + D	163.04 ± 3.83^{b}	0.10 ± 0.010^{b}	8.42 ± 0.37^{b}

^{a,b}Values having different superscripts along columns, differ significantly ($P < 0.05$).

BMP15 genes were significantly ($P < 0.05$) higher in 4-cell stage embryos and relatively lower in the other stages such as 2-cell, 8–16-cell, morula and blastocysts (Fig. 3). *CX43* expression was significantly ($P < 0.05$) higher in the morula and 8–16-cell stage embryos compared with that in 4-cell stage and with embryos at all the stages examined (Fig. 3).

Among the genes that played a significant role in fertilization, expression of zona pellucida (ZP) *ZP2* and *ZP3* genes was analyzed. Relative mRNA abundance of *ZP2* was significantly ($P < 0.05$) higher in 4-cell stage embryos compared with the 2-cell stage; expression was found to be lower in 8–16-cell stage embryos, morula and blastocysts (Fig. 3). *ZP3* was found to be significantly ($P < 0.05$) higher in 2-cell stage embryos and its expression was found to be the same in 4-cell and 8–16-cell stages, and morula and blastocysts (Fig. 2).

The mRNA abundance of the *ZAR1* gene was found to be significantly ($P < 0.05$) higher in 4-cell and 2-cell stage embryos and lower in 8–16-cell stage, morula and blastocysts (Fig. 4). The expression of the *HSF1* gene was the same in all stages; therefore there was no significant difference ($P < 0.05$) among any developmental stage (Fig. 4). *GLUT1* expression was found to be significantly ($P < 0.05$) higher in morula and blastocysts and lower in abundance in 2-cell, 4-cell and 8–16-cell stage embryos (Fig. 4). *bFGF* was expressed more in the morula and its expression was the same in other examined stages (Fig. 4).

The pro-apoptotic gene *BAX* showed relatively higher expression in blastocysts and its expression was the same in all examined stages (Fig. 4). Expression of the anti-apoptotic gene *MCL1* was higher in the 8–16-cell stage compared with that 2-cell and 4-cell stages and morula. Very low expression was observed in blastocysts (Fig. 4).

Discussion

Parthenogenetic activation has been used as a tool to study mechanisms involved in the initiation of embryonic development. To

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

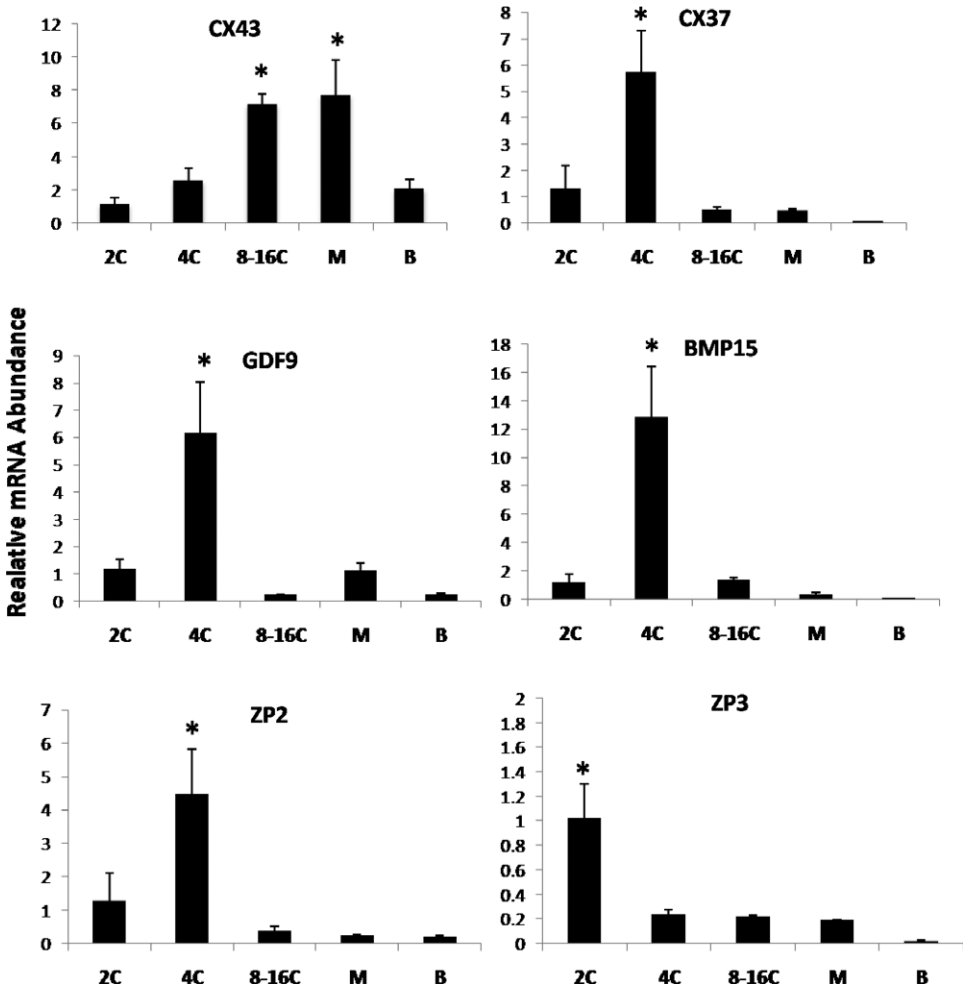

Figure 3. Relative mRNA abundance of some cumulus–oocyte complex and fertilization related important genes in different developmental stages (2C, 2-cell stage; 4C, 4-cell stage; 8–16C, 8–16 cell stage; M, morula stage; B, blastocyst stage) of parthenogenetically activated buffalo embryos. Bars with different superscripts differ significantly ($P < 0.05$). Ten embryos at each stage were used and the experiment was repeated three times with each run in triplicates.

our knowledge, this is the first report on parthenogenetic activation of buffalo A + B and C + D grade oocytes. In the present study, buffalo oocytes of both grades could be activated successfully and developed parthenogenetically up to the blastocyst stage by treatment with CaI, ethanol or electrical activation followed by 6-DMAP treatment and cultured in three different media. Some studies on parthenogenetic activation of buffalo oocytes by ethanol or ionomycin followed by 6-DMAP treatment have been reported previously (Gasparrini *et al.*, 2004; George *et al.*, 2011; Singh *et al.*, 2012, 2014) but no report was available to compare different culture media in two different groups of oocytes.

The use of chemical agents and electrical stimuli led to an increase in cytoplasmic Ca^{2+} levels, but most of these failed to generate Ca^{2+} oscillations, which are the hallmark of sperm-mediated oocyte activation. Instead, they caused just a single, long rise in Ca^{2+} (Swann and Ozil, 1994), which was not sufficient to completely downregulate MPF activity. Thimerosal and strontium ions are the only known chemical agents capable of causing sustained Ca^{2+} oscillations (Swann, 1991; Kline and Kline, 1992). However, thimerosal caused the destruction of meiotic spindle in oocytes (Cheek *et al.*, 1993). In the view of their inability to mimic Ca^{2+} oscillations, chemical agents are used in combination with a protein synthesis inhibitor such as cycloheximide or a protein kinase inhibitor such as 6-DMAP that prevent MPF re-accumulation (Alberio *et al.*, 2001), yet these agents also contribute to reduction in the developmental competence of parthenogenetically activated oocytes. Whereas exposure to 6-DMAP caused a high frequency of chromosomal abnormalities in parthenogenetic cattle embryos (De La Fuente and King, 1998), cycloheximide seemed to prolong the completion of the first cell cycle, resulting in lower cleavage rates (Holm *et al.*, 2003).

The present study has clearly shown that high efficiency of oocyte activation in the buffalo can be achieved by sequential treatment with either CaI or ethanol followed by incubation in 6-DMAP. Our results are in agreement with previous findings on a combination treatment of chemical agents (Gasparrini *et al.*, 2004). We compared two groups of oocytes (A + B and C + D grade) activated with three different reagents and cultured in three different media. The A + B grade oocytes activated with ethanol and CaI had significantly ($P > 0.05$) higher cleavage rates and blastocyst production when these activated oocytes were cultured with RVCL and mCR2 media respectively. Oocytes activated with electric pulse also had higher cleavage and blastocyst development rates in RVCL and mCR2 media, respectively, which were lower than that of ethanol and CaI. The oocytes activated with ethanol, CaI and electric pulse were comparatively lower in terms of cleavage and blastocyst production rate when cultured in mSOF medium. Previous studies have also reported that the parthenogenetic activation of buffalo oocytes (with and without zona) with ethanol and CaI produced a better cleavage and blastocyst development rate when cultured in RVCL medium (George *et al.*, 2011). Shah *et al.* (2008) found the compaction of buffalo zona-free

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

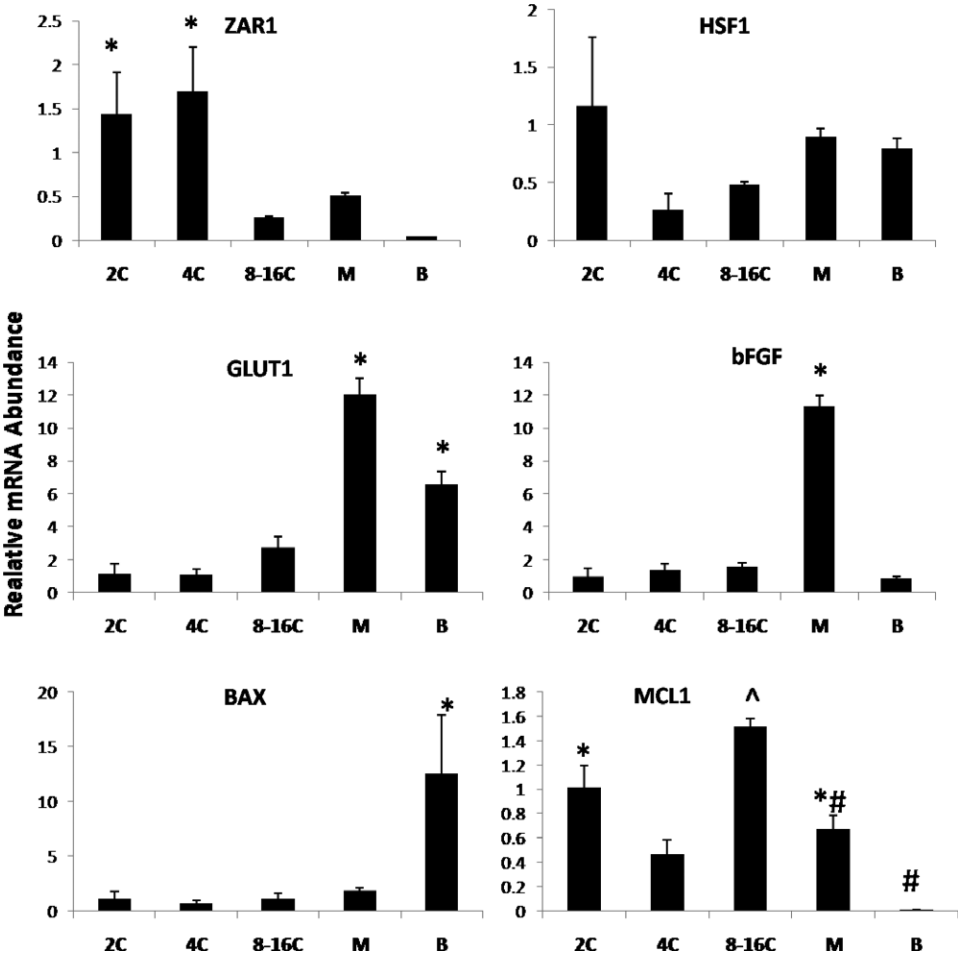

Figure 4. Relative mRNA abundance of some apoptosis and developmental competence related important genes in different developmental stages (2C, 2-cell stage; 4C, 4-cell stage; 8–16C, 8–16 cell stage; M, morula stage; B, blastocyst stage) of parthenogenetically activated embryos. Bars with different superscripts differ significantly ($P < 0.05$). Ten embryos at each stage were used and the experiment was repeated three times with each run in triplicates.

embryos and their subsequent development to the blastocyst stage in RVCL and mSOF media but not in mCR2 media. Mishra *et al.* (2008) reported that the combination of 7% ethanol + DMAP gave better cleavage and blastocyst production rates in buffalo. Saikhun *et al.* (2004), found that ethanol and CaI with DMAP had significantly ($P < 0.05$) higher cleavage and blastocysts rates compared with those activated by electric pulse + DMAP. The parthenogenetic activation of sheep oocytes also had superior cleavage and blastocyst development rates in CaI and ethanol (Loi *et al.*, 1998; Nandedkar *et al.*, 2009). The reason for this contradictory result may be the quality of oocytes obtained from slaughter house ovaries and optimal culture condition. A similar result was obtained in C + D grade buffalo oocytes. Ethanol, CaI and electric pulse-activated C + D grade buffalo oocytes were found to give better cleavage and blastocysts development rates in RVCL and mCR2 media respectively, but there were very poor cleavage and blastocysts development rates in mSOF medium. There is no report available describing the culture of these oocytes separately. Mishra *et al.* (2008) reported the recovery of A, B, C and D grade buffalo oocytes from slaughter ovaries, but they did not reported separate culture of these oocytes. Regarding low embryo development from C, D grade oocytes, possible reasons may be that these oocytes were either denuded without a cumulus layer or had one layer of cumulus cells, as cumulus cells play important roles in embryo development.

In the present study, the temporal mRNA expression pattern of developmentally important genes in buffalo embryos produced parthenogenetically was analysed using RT-qPCR. These transcripts are involved in several areas such as compaction and as growth factors (*CX37*, *CX47*, *GDF9*, *BMP15*), zona pellucida (*ZP2*, *ZP3*), metabolism (*GLUT1*), stress (*HSF1)* and maternal (*ZAR1*).

During murine preimplantation development, functional gap junctions were first observed at compaction at the 8-cell stage and mRNA encoding the *CX43* gene (Connexin-43) and Cx43 protein were detected from the 4-cell stage onwards (Nishi *et al.*, 1991). *CX43* and *CX37*, also known as Gap junction protein α1 (*GJA1*) and Gap junction protein α4 (*GJA4*), respectively, have been found within the developing ovarian follicle (Kidder and Mhawi, 2002). These are the major gap junctional proteins expressed in the cumulus–oocyte complex (COC) and granulosa cells and predominantly mediate cell-to-cell communication in ovarian follicles (Granot and Dekel, 2002). Wrenzycki *et al.* (1998) investigated the expression of *CX43* in bovine *in vitro* produced embryos up to the morula stage; *CX43* expression was found in *in vivo* produced bovine blastocysts (Wrenzycki *et al.*, 1996). Gómez *et al.* (2009) reported that *CX43* was upregulated in bovine IVF blastocysts but downregulated in parthenogenetic blastocysts. Nemcova *et al.* (2006) found expression of *CX43* in bovine day 7 and day 8 blastocysts. For buffalo, both *CX43* and *CX37* transcripts were present in all developmental stages of parthenotes at different levels.

Growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) are two well characterized oocyte-derived growth factors that play crucial roles in follicle growth

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press

and ovulation in all mammalian species. *GDF9* was originally identified in mouse ovary (McGrath *et al.*, 1995). *GDF9* plays a critical function as a growth and differentiation factor during early folliculogenesis and as a key regulator of several granulosa cell proteins involved in cumulus expansion and maintenance of an optimal oocyte microenvironment. *GDF9* and *BMP15* expression was reported in buffalo oocytes (Nath *et al.*, 2013; Murlidharan *et al.*, 2015), bovine oocytes (Donnison and Pfeffer, 2004), sheep oocytes (Galloway *et al.*, 2000), mouse oocytes (Dong *et al.*, 1996) and human (McGrath *et al.*, 1995). *In vitro* produced bovine embryos for 2-cell, 4-cell and 8-cell stage embryos expressed these genes, but expression was not significantly detected in 16-cell stage, morula or blastocysts (Donnison and Pfeffer, 2004). McGrath *et al.* (1995) also reported that *GDF9* mRNA expression was lost rapidly after fertilization in human. In our results both genes were present in all the developmental stage including blastocysts, but variable expression patterns and higher mRNA expression was found for 4-cell stage embryos.

Zygote arrest 1 is an evolutionarily conserved, ovary-specific maternal factor that plays essential roles during oocyte-to-embryo transition. In human and mouse, *Zar1* mRNA was present in oocytes and 1-cell embryos, but decreased significantly at the 2-cell stage and was not detected at further stages of development or in tissues other than the ovary (Wu *et al.*, 2003). In pig and bovine, the *ZAR1* transcript was present in all embryonic development stages. Very low expression was found in morula and blastocysts (Uzbekova *et al.*, 2006). Brevini *et al.* (2004) reported *ZAR1* transcript expression present in all developmental stages of bovine embryos up to blastocyst formation. In our results, we found a similar pattern, but *ZAR1* expression was higher in 4-cell and 2-cell stage embryos.

The induction of heat shock genes in eukaryotes by heat or other forms of stress is mediated by a transcription factor known as heat shock factor 1 (*HSF1*), which is a maternal effect gene. Heat shock transcription factor 1 is the main regulator of the stress response that triggers the transcription of several genes encoding heat shock proteins (HSPs). Metchat *et al.* (2009) reported that *HSF1*$^{-/-}$ females produced oocytes but no viable embryos. Christians *et al.* (2000) reported that *HSF1* is one of the few maternal factors in mammals required for normal embryonic development beyond the 1-cell or 2-cell stages and is necessary for female reproductive success. Eunju *et al.* (2014), found expression of the *HSF1* gene in all the embryonic stages in mouse, but very low expression was found in blastocysts. Similar results were found in this study, the *HSF1* transcript was expressed in all embryonic developmental stages of buffalo parthenotes.

GLUT1 is a ubiquitous, facilitative glucose transporter and a major source of metabolic energy for most mammalian cells, but it cannot support embryonic development before compaction (Brinster, 1965). However *in vitro* produced embryos from different developmental stages of mouse (Hogan *et al.*, 1991; Pantaleon *et al.*, 2001), bovine (Balasubramanian *et al.*, 2007) and buffalo (Kumar *et al.*, 2015; Sadeesh *et al.*, 2014) expressed the *GLUT1* transcript. This result agreed with previous reports; the *GLUT1* transcript was expressed in all developmental stages of buffalo embryos but with higher expression levels in morula and blastocysts.

The fibroblast growth factor (FGF) family is emerging as a group of factors that are involved in the regulation of ovarian function. Studies in mammals have located bFGF and its receptors to preantral ovarian follicles in the rat (Nilsson *et al.*, 2001). Expression of the bFGF transcript was reported in bovine blastocysts (Gilbert *et al.*, 1996). For buffalo, the transcript was expressed in all developmental stages including blastocysts. bFGF promotes early bovine embryonic development *in vitro* during the fourth cell cycle, synergistically with transforming growth factor β (Larson *et al.*, 1992).

Apoptosis is dependent upon the actions and interactions of at least 100 gene products, the most widely studied of which are members of the BCL-2 family (Adams and Cory, 1998). These can be subdivided into two groups: anti-apoptotic cell death suppressors (*BCL-2*, *BCL-XL*, *BCL-W* and *MCL-1*) and pro-apoptotic cell death inducers (*BAX*, *BAK* and *MTD*). Sadeesh *et al.* (2014) reported that exposure of buffalo oocytes to summer heat stress increased the relative mRNA abundance of pro-apoptotic gene *BAX* at the 8- to 16-cell stages. In our case, *BAX* expression was found to be higher at the blastocyst stage and *MCL-1* expression was higher in 8- to 16-cell and 2-cell stages. Elamaran *et al.* (2012) found that oxygen concentration and cysteamine supplementation during *in vitro* production of buffalo embryos affected mRNA expression of *BCL-2*, *BCL-XL*, *MCL-1*, *BAX* and *BID*. More recently, one study reported on gene expression in different stages of buffalo parthenotes, and showed differential gene expression of various developmental genes in different embryonic developmental stages (Fumei *et al.*, 2020).

In conclusion, the percentage of A + B grade oocytes was significantly higher compared with that of C + D grade oocytes retrieved from slaughterhouse buffalo ovaries. Different methods employed to activate buffalo A + B and C + D quality oocytes parthenogenetically, *in vitro* developmental competence of oocytes, and relative mRNA abundance were studied. Among all combinations, ethanol activation followed by RVCL culture gave the highest cleavage and blastocyst yield for both A + B and C + D grade oocytes. *In vitro* developmental competence of the A + B group was better compared with its C + D counterpart. The expression profiles of *CX37*, *CX47*, *GDF9*, *BMP15*, *ZP2*, *ZP3*, *GLUT1*, *HSF1*, *ZAR1*, *BAX* and *MCL1* were also studied for different stages of parthenogenetically produced buffalo embryos, and indicated that the dynamics of the above genes had a role in early embryonic development.

Embryos produced through IVF are a very precious way to produce a life; parthenotes could be alternate sources for production of embryos by different activation reagents that could be used for future culture of embryonic stem cells, genomics and proteomics studies in embryology.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199420000519

Authors' contribution. KPS designed and carried out the work, SKM and RK analyzed data, KP, MK, PP, RS drafted the manuscript, MSC and SKS provided funds for study.

Financial support. We are grateful for National Agriculture Innovative Project (NAIP) grants to SKS (C2-1-(5)/2007) and MSC (C-2067 and 075).

Conflict of interest. The authors declare that there are no competing interests.

Ethical standards. Not applicable.

References

Adams JM and Cory S (1998). The Bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1322–6.

Alberio R, Zakhartchenko V, Motlik J and Wolf E (2001). Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. *Int J Dev Biol* **45**, 797–809.

Balasubramanian S, Son WJ, Kumar BM, Ock SA, Yoo JG, Im GS, Choe SY and Rho GJ (2007). Expression pattern of oxygen and stress-responsive gene transcripts at various developmental stages of *in vitro* and *in vivo* preimplantation bovine embryos. *Theriogenology* **68**, 265–75.

Brevini TA, Cillo F, Colleoni S, Lazzari G, Galli C and Gandolfi F (2004). Expression pattern of the maternal factor zygote arrest 1 (*Zar1*) in bovine tissues, oocytes, and embryos. *Mol Reprod Dev* **69**, 375–80.

Brinster RL (1965). Studies on the development of mouse embryos *in vitro*. *J Reprod Fertil* **10**, 227–40.

Cheek TR, McGuinness OM, Vincent C, Moreton RB, Berridge MJ and Johnson MH (1993). Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms. *Development* **119**, 179–89.

Christians E, Davis AA, Thomas ST and Benjamin IJ (2000). Maternal effect of Hsf1 on reproductive success. *Nature* **407**, 693–94.

De La Fuente R and King WA (1998). Developmental consequences of karyokinesis without cytokinesis during the first mitotic cell cycle of bovine parthenotes. *Biol Reprod* **58**, 952–62.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N and Matzuk MM (1996). Growth differentiation factor-9 is required early ovarian folliculogenesis. *Nature* **383**, 531–5.

Donnison M and Pfeffer PL (2004). Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol Reprod* **71**, 1813–21.

Elamaran G, Singh KP, Singh MK, Singla SK, Chauhan MS, Manik RS and Palta P (2012). Oxygen concentration and cysteamine supplementation during *in vitro* production of buffalo (*Bubalus bubalis*) embryos affect mRNA expression of *BCL-2*, *BCL-XL*, *MCL-1*, *BAX* and *BID*. *Reprod Domest Anim* **47**, 1027–36.

Eunju K, Guangming W, Hong M, Ying L, Rebecca TH, Masahito T, Michelle S, Don PW, Hans RS and Shoukhrat M (2014). Nuclear reprogramming by interphase cytoplasm of two-cell mouse embryos. *Nature* **509**, 101–4.

Fumei C, Qiang F, Liping P, Zhen H, Zhuangzhuang X, Pengfei Z, Tingxian D, Chunying P, Xianwei L, Yangqing L and Ming Z (2020). Maternal transcription profiles at different stages for the development of early embryo in buffalo. *Reprod Domest Anim* **55**, 503–14.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juenge JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH and Ritvos O (2000). Mutation in an oocyte-derived growth factor gene (*BMP15*) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet* **25**, 279–83.

Gasparrini B, Boccia L, Rosa AD, Palo R D, Campanile G and Zicarelli L (2004). Chemical activation of buffalo (*Bubalus bubalis*) oocytes by different methods: effects of aging on post-parthenogenetic development. *Theriogenology* **62**, 1627–37.

George A, Shah RA, Sharma R, Palta P, Singla SK, Manik RS and Chauhan MS (2011). Activation of zona-free buffalo (*Bubalus bubalis*) oocytes by chemical or electrical stimulation, and subsequent parthenogenetic embryo development. *Reprod Domest Anim* **46**, 444–7.

Gilbert AS, Mark BH, Andrew JW, Mayi YAP, Karen J and Mark EW (1996). Regulation of early embryonic development by growth factors: growth factor gene expression in cloned bovine embryos. *J Anim Sci* **74**, 50–7.

Gómez E, Gutiérrez-Adán A, Díez C, Bermejo-Alvarez P, Muñoz M, Rodriguez A, Otero J, Alvarez-Viejo M, Martín D, Carrocera S and Caamaño JN (2009). Biological differences between *in vitro* produced bovine embryos and parthenotes. *Reproduction* **137**, 285–95.

Granot I and Dekel N (2002). The ovarian gap junction protein connexin43: regulation by gonadotropins. *Trends Endrocrinol Metab* **13**, 310–13.

Hogan A, Heyner S, Charron MJ, Copeland NG, Gilbert DJ, Jenkins NA, Thorens B and Schultz GA (1991). Glucose transporter gene expression in early mouse embryos. *Development* **113**, 363–72.

Holm P, Booth PJ and Callesen H (2003). Developmental kinetics of bovine nuclear transfer and parthenogenetic embryos. *Cloning Stem Cells* **5**, 133–42.

Kidder GM and Mhawi AA (2002). Gap junctions and ovarian folliculogenesis. *Reproduction* **123**, 613–20.

Kline D and Kline JT (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* **149**, 80–9.

Kumar P, Verma A, Kumar M, De S, Kumar R and Datta TK (2015). Expression pattern of glucose metabolism genes correlate with development rate of buffalo oocytes and embryos *in vitro* under low oxygen condition. *J Assist Reprod Genet* **32**, 471–8.

Larson RC, Ignotz GG and Currie WB (1992). Transforming growth factor β and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. *Mol Reprod Dev* **33**, 432–5.

Loi P, Ledda S, Fulka J, Cappai P and Moor RM (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol Reprod* **58**, 1177–87.

McGrath SA, Aurora F, Esquela and Lee SJ (1995). Oocyte-specific expression of growth differentiation factor-9. *Mol Endocrinol* **9**, 131–6.

Metchat A, Malin Å, Christiane B, Virginie D, Lea S, Henri A and Elisabeth SC (2009). Mammalian heat shock factor1 is essential for oocyte meiosis and directly regulates Hsp90 expression. *J Biol Chem* **284**, 9521–8.

Mishra V, Misra AK and Sharma R (2008). A comparative study of parthenogenic activation and *in vitro* fertilization of bubaline oocytes. *Anim Reprod Sci* **103**, 249–59.

Mohapatra SK, Sandhu A, Singh KP, Singla SK, Chauhan MS, Manik RS and Palta P (2015a). Establishment of trophectoderm cell lines from buffalo (*Bubalus bubalis*) embryos of different sources and examination of *in vitro* developmental competence, quality, epigenetic status and gene expression in cloned embryos derived from them. *PLoS One* **10**, e0129235.

Mohapatra SK, Sandhu A, Neerukattu VS, Singh KP, Selokar NL, Singla SK, Chauhan MS, Manik RS and Palta P (2015b). Buffalo embryos produced by hand-made cloning from oocytes selected using brilliant cresyl blue staining have better developmental competence and quality and are closer to embryos produced by *in vitro* fertilization in terms of their epigenetic status and gene expression pattern. *Cell Reprogram* **17**, 141–50.

Murlidharan K, Eswari S and Vijayarani K (2015). Expression profile of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (*BMP15*) genes in buffalo oocytes. *Anim Sci Rep* **9**, 10–15.

Nandedkar P, Chohan P, Patwardhan A, Gaikwad S, Bhartiya D (2009). Parthenogenesis and somatic cell nuclear transfer in sheep oocytes using PolScope. *Ind J Exp Biol* **47**, 550–8.

Nath A, Sharma V, Dubey PK, Pratheesh MD, Gade NE, Saikumar G and Sharma GT (2013). Impact of gonadotropin supplementation on the expression of germ cell marker genes (*MATER*, *ZAR1*, *GDF9* and *BMP15*) during *in vitro* maturation of buffalo (*Bubalus bubalis*) oocyte. *In Vitro Cell Dev Biol Anim* **49**, 34–41.

Nemcova L, Machatkova M, Hanzalova K, Horakova J and Kanka J (2006). Gene expression in bovine embryos derived from oocytes with different developmental competence collected at the defined follicular developmental stage. *Theriogenology* **65**, 1254–64.

Nilsson E, Parrott JA and Skinner MK (2001). Basic fibroblast growth factor induces primordial Follicle development and initiates folliculogenesis. *Mol Cell Endocrinol* **175**, 123–30.

Nishi M, Kumar NM and Gilula NB (1991). Developmental regulation of gap junction gene expression during mouse embryonic development. *Dev Biol* **146**, 117–30

Pantaleon M, Ryan JP, Gil M and Kaye P (2001). An unusual subcellular localization of GLUT1 and link with metabolism in oocytes and preimplantation mouse embryos. *Biol Reprod* **64**, 1247–54.

Ross PJ, Beyhan Z, Lager AE, Yoo SY, Malcuit C, Schellander K, Fissore RA and Cibelli JB (2008). Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. *BMC Dev Biol* **19**, 8–16.

Sadeesh EM, Meena K, Balhara S and Yadav PS (2014). Expression profile of developmentally important genes between hand-made cloned buffalo embryos produced from reprogramming of donor cell with oocytes extract and selection of recipients to blast through brilliant cresyl blue staining and *in vitro* fertilized embryos. *J Assist Reprod Genet* **31**, 1541–52.

Saikhun J, Kitiyanant N, Songtaveesin C, Pavasuthipaisit K and Kitiyanant Y (2004). Development of swamp buffalo (*Bubalus bubalis*) embryos after parthenogenetic activation and nuclear transfer using serum fed or starved fetal fibroblasts. *Reprod Nutr Dev* **44**, 65–78.

Shah RA, George A, Singh MK, Kumar D, Chauhan MS, Manik R, Palta P and Singla SK (2008). Hand-made cloned buffalo (*Bubalus bubalis*) embryos: comparison of different media and culture systems. *Cloning Stem Cells* **10**, 435–42.

Shi D, Lu F, Wei Y, Cui K, Yang S, Wei J and Liu Q (2007). Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. *Biol Reprod* **77**, 285–91.

Singh KP, Kaushik R, Garg V, Sharma R, George A, Singh MK, Manik RS, Palta P, Singla SK and Chauhan MS (2012). Expression pattern of pluripotent markers in different embryonic developmental stages of buffalo (*Bubalus bubalis*) embryos and putative embryonic stem cells generated by parthenogenetic activation. *Cell Reprogram* **14**, 530–8.

Singh KP, Kaushik R, Mohapatra SK, Garg V, Ameshbabu KR, Singh MK, Palta P, Manik RS, Singla SK and Chauhan MS (2014). Quantitative expression of pluripotency-related genes in parthenogenetically produced buffalo (*Bubalus bubalis*) embryos and in putative embryonic stem cells derived from them. *Gene Exp Patterns* **16**, 23–30.

Swann K (1991). Thimerosal causes calcium oscillations and sensitizes calcium induced calcium release in unfertilized hamster eggs. *FEBS Lett* **278**, 175–8.

Swann K and Ozil JP (1994). Dynamics of the calcium signal that triggers mammalian egg activation. *Int Rev Cytol* **152**, 183–222.

Telford NA, Watson AJ and Schultz GA (1990). Transition of maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* **26**, 90–100.

Thouas GA, Korfiatis NA, French AJ, Jones GM and Trounson AO (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reprod Biomed Online* **3**, 25–9.

Uzbekova S, Roy-Sabau M, Dalbiès-Tran R, Perreau C, Pappillier P, Mompart F, Thelie A, Pennetier S, Cognie J, Cadoret V, Royere D, Monget P and Mermillod P (2006). Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod Biol Endocrinol* **4**, 12.

Wrenzycki C, Herrmann D, Carnwath JW and Niemann H (1996). Expression of the gap junction gene connex in 43 (Cx43) in preimplantation bovine embryos derived *in vitro* or *in vivo*. *J Reprod Fertil* **108**, 17–24.

Wrenzycki C, Herrmann D, Carnwath JW and Niemann H (1998). Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA. *J Reprod Fertil* **112**, 387–98.

Wu B, Ignotz G, Currie B and Yang X (1997). Dynamics of maturation-promoting factor and its constituent proteins during *in vitro* maturation of bovine oocytes. *Biol Reprod* **56**, 253–9.

Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick S and Matzuk MM (2003). Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat Genet* **33**, 187–91.

https://doi.org/10.1017/S0967199420000519 Published online by Cambridge University Press