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Baicalin improves IVM of pig oocytes and subsequent preimplantation embryo development by inhibiting apoptosis

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Abstract. Baicalin, a monomer of flavonoids extracted from dried roots of *Scutellaria baicalensis*, is used to treat female infertility. However, the effect of baicalin on oocyte maturation is unknown. In this study we investigated the effects of baicalin on the IVM of pig oocytes and subsequent embryo development following parthenogenetic activation (PA). We found that 0.1 μg mL^{-1} baicalin significantly ($P < 0.05$) increased the IVM rate of oocytes compared with the non-treatment (control) group by reducing levels of reactive oxygen species (ROS). In addition, the mRNA expression of genes related to nuclear maturation and cumulus cell expansion, mitochondrial membrane potential and ATP content was significantly ($P < 0.05$) higher in baicalin-treated than control oocytes. To determine whether baicalin treatment during IVM of pig oocytes improves subsequent development of PA embryos, we measured the cleavage and blastocyst formation rates, as well as the number of cells per blastocyst. All these parameters were significantly ($P < 0.05$) higher in the baicalin-treated than control group. In conclusion, this study demonstrates that baicalin improves pig oocyte maturation and subsequent embryo development *in vitro* by inhibiting production of ROS and reducing apoptosis in oocytes.

Additional keywords: parthenogenetic activation, reactive oxygen species.

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Introduction

Baicalin, a monomer of flavonoids extracted from dried roots of *Scutellaria baicalensis*, is a traditional Chinese medicine used to treat many female-specific conditions, including menstrual cycle problems and female infertility, as well as to prevent miscarriage (Martin and Dusek 2002; Huang *et al.* 2006; Zhong *et al.* 2008; Tian *et al.* 2009). Many studies have reported that baicalin protects the fetus. For example, baicalin protects against abortion induced by bromocriptine peptide and prevents injury of decidual cells upon lipopolysaccharide-induced abortion in mice (Ma *et al.* 2009; Wang *et al.* 2014). In addition, baicalin enhances the developmental capacity of mouse embryos *in vitro* by suppressing apoptosis, modulating DNA methyltransferase expression and promoting embryo adhesion and implantation via modulation of the Wnt/β-catenin signalling pathway (Zhang *et al.* 2015; Qi *et al.* 2016). However, the effects of baicalin on oocyte maturation have not been reported.

Oocyte quality is particularly important to improve reproductive capacity (Harvey *et al.* 2007). Many factors affect oocyte maturation, including those related to the oocyte itself and the ovary (Zeng *et al.* 2018). Oocyte maturation is a very complex process that is divided into the germinal vesicle, germinal vesicle breakdown, MI and MII stages (Holt *et al.* 2013). Errors arising at any stage lead to failure of meiosis, perturbation of oocyte development and eventually infertility (Hassold and Hunt 2001). IVM of oocytes and subsequent development can be improved by a variety of approaches, such as improving mitochondrial function (Abdulhasan *et al.* 2017), increasing antioxidant activity (Jin *et al.* 2016), regulating metabolic pathways (Jin *et al.* 2017), modulating the cell growth signalling pathway (Lee *et al.* 2017; Shi *et al.* 2018*a*) and inhibiting endoplasmic reticulum stress (Park *et al.* 2018). In this study, the IVM medium was supplemented with baicalin to improve the IVM of pig oocytes and subsequent embryo development by reducing levels of reactive oxygen species (ROS).

ROS affect the IVM of oocytes and subsequent *in vitro* development, including parthenogenetic activation (PA), IVF, developmental capacity of fertilised embryos and pregnancy in

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mammals (Agarwal *et al.* 2005). Production of ROS is associated with mitochondrial function in oocytes (Zeng *et al.* 2018). Mitochondria not only produce ATP to provide energy for cell growth and development via the redox reaction, but they also produce ROS, which perturb physiological processes in oocytes (Wallace *et al.* 2010). Thus, mitochondrial function is an indicator of oocyte quality. Therefore, treatment with baicalin during the IVM of pig oocytes may affect mitochondrial dynamics and ATP production by regulating ROS generation, thereby improving the IVM of oocytes and subsequent embryo development.

This study investigated the effects of baicalin on the IVM of pig oocytes and subsequent embryo development after PA. We determined the maturation rate, ROS levels, mRNA expression of genes related to nuclear maturation and cumulus cell expansion, mitochondrial membrane potential ($\Delta\Psi_m$) and ATP content in baicalin-treated oocytes. In addition, we determined the blastocyst formation rate, the number of cells per blastocyst and mRNA expression of pluripotency and apoptosis-related genes in blastocysts derived from baicalin-treated oocytes.

Materials and methods

Ethics approval

This study was approved by the Ethics Committee of Yanbian University (Approval ID: 20130310).

Chemicals

Baicalin was purchased from Selleck Chemicals. All other chemicals were purchased from Sigma Chemical, unless stated otherwise.

Oocyte collection and IVM

Cumulus–oocyte complexes (COCs) were collected and IVM was performed as described previously (Luo *et al.* 2018) Briefly, ovaries were obtained from a local abattoir, transported to the laboratory in a vacuum flask containing saline solution and washed three times with sterile saline. COCs were obtained from follicles with a diameter of 2–6 mm. Experiments were performed using good-quality COCs with homogeneous cytoplasm and at least three uniform layers of coherent cumulus cells. COCs were cultured in maturation medium (NCSU37) supplemented with 0.1 IU mL^{-1} pregnant mare's serum gonadotrophin, 0.1 IU mL^{-1} human chorionic gonadotrophin, 1 mM dibutyryl cAMP and various concentrations (0, 0.05, 0.1, 0.5, 1 μg mL^{-1}) of baicalin for 20–22 h, and then in NCSU37 medium lacking hormones and containing various concentrations of baicalin (0, 0.05, 0.1, 0.5, 1 μg mL^{-1}) for 18–24 h in a humidified CO_2 incubator at 38°C.

Evaluation of pig oocyte maturation

After IVM for 44 h, oocytes were pipetted in NCSU37 medium containing 0.1% hyaluronidase and HEPES buffer to remove cumulus cells. Denuded oocytes were stained with phosphate-buffered saline (PBS) containing 5 μg mL^{-1} Hoechst 33342. Stained oocytes were imaged using a fluorescence microscope (Nikon). Each experiment was repeated at least three times.

PA of oocytes

Oocytes that had extruded the first polar body were parthenogenetically activated. Briefly, oocytes were activated by a single direct current pulse of 1.5 kV cm^{-1} for 60 μs in activation medium containing 0.28 M mannitol, 0.1 mM $MgSO_4$, 0.1% polyvinyl alcohol (PVA) and 0.05 mM $CaCl_2$. Activated oocytes were washed three times with *in vitro* culture (IVC) medium, which was composed of NCSU37, 4 mg mL^{-1} bovine serum albumin (BSA), 0.074 mg mL^{-1} L-cystein and then cultured in IVC medium containing 2 mM 6-Dimethylaminopurine (6-DMAP) for 4 h. Finally, oocytes were transferred to IVC medium and incubated in a CO_2 incubator for 7 days.

Measurement of ROS levels in MII oocytes

Levels of ROS in MII oocytes were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA), as described previously (Lee *et al.* 2016). Briefly, denuded oocytes matured in the presence or absence of baicalin were placed in PBS including 0.1% polyvinyl alcohol (PVA) (PBS-PVA) and containing 10 μM DCHFDA for 30 min in the dark, washed five times with PBS-PVA and then placed in 5-μL droplets of TALP-HEPES containing 0.1% PVA. Fluorescence was detected using a fluorescence microscope equipped with ultraviolet filters. Fluorescence intensities were measured using ImageJ software (Nikon, NIS). The mean fluorescence intensity in the control group was set to 1 for comparison.

Determination of the total number of cells per blastocyst

The total number of cells was determined for blastocysts formed after PA. Blastocysts were washed with PBS-PVA a minimum of three times, stained with 20 μg mL^{-1} Hoechst 33342 for 3 min in the dark at 23°C, washed three times again with PBS-PVA and mounted on a slide and coverslipped. Stained blastocysts were imaged by fluorescence microscopy. Images were analysed using Nikon NIS-Elements software.

Immunofluorescence staining and quantitative analysis

Unless stated otherwise, all experiments were conducted at room temperature. To determine the $\Delta\Psi_m$, denuded oocytes were stained with 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen). Denuded eggs were cultured in IVC medium containing 10 μg mL^{-1} JC-1 for 20 min in 5% CO_2 at 37°C. Green and red fluorescence was examined using a microscope equipped with appropriate filters.

BODIPY FL ATP (BODIPY-ATP; A12410; Molecular Probes) was used to label ATP molecules. BODIPY FL ATP and BODIPY FL GTP (G-12411; Molecular Probes) consist of a BODIPY FL fluorophore attached to the 2′ or 3′ position of the ribose ring via a linker. Interactions between the fluorophore and the purine base are evident from the spectroscopic properties of these nucleotide analogues. The fluorescence quantum yield of BODIPY FL GTP is only approximately one-tenth that of BODIPY FL ATP. The fluorescence intensity of BODIPY-ATP was measured using Nikon Instruments software with a 550-nm excitation light filter to quantify the ATP content of oocytes. Specifically, denuded oocytes were fixed in 4% paraformaldehyde for 40 min, repeatedly washed three times with PBS-PVA,

Table 1. Primer sequences used for quantitative real-time polymerase chain reaction
BMP15, bone morphogenetic protein 15; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GDF9*, growth differentiation factor 9; *CDK1*, cyclin-dependent kinase 1; Oct4, octamer-binding transcription factor 4; *PTGS1*, prostaglandin-endoperoxide synthase 1; *PTGS2*, prostaglandin-endoperoxide synthase 2; *PTX3*, pentraxin 3; *TNFAIP6*, tumour necrosis factor alpha induced protein 6; *SHAS2*, hyaluronan synthase 2; *Sox2*, SRY (sex determining region Y)-box 2

Gene	Primer sequence (5′–3′)	Product size (bp)
GAPDH	Forward: GCTTGCCTCCAGTGTCCTC	179
	Reverse: GGCGTTGGCGATTTCAT	
GDF-9	Forward: CCTCTACAACACTGTCCGGC	91
	Reverse: GTCCCCTGATGGAAGGGTTC	
CDK1	Forward: TAATAAGCTGGGATCTACCACATC	130
	Reverse: TGGCTACCACTTGACCTGTA	
Cyclin B	Forward: AGCTAGTGGTGGCTTCAAGG	101
	Reverse: GCGCCATGACTTCCTCTGTA	
Mos	Forward: GGTGGTGGCCTACAATCTCC	165
	Reverse: TCAGCTTGTAGAGCGCGAAG	
PTGS1	Forward: CAACACGGCACACGACTACA	121
	Reverse: CTGCTTCTTCCCTTTGGTCC	
PTGS 2	Forward: ACAGGGCCATGGGGTGGACT	194
	Reverse: CCACGGCAAAGCGGAGGTGT	
TNFAIP6	Forward: AGAAGCGAAAGATGGGATGCT	106
	Reverse: CATTTGGGAAGCCTGGAGATT	
SHAS2	Forward: TCCTCCTGGGTGGTGTGATT	309
	Reverse: TGTCTCCTTGGGTGGCATTATC	
PTX3	Forward: GCAGGTTGTGAAACAGCGAT	114
	Reverse: TTTGACCCAAATGCAGGCAC	
Bax	Forward: GAAACCCCTAGTGCCATCAA	189
	Reverse: GGGACGTCAGGTCACTGAAT	
Bcl2	Forward: CGGGACACGGAGGAGGTTT	196
	Reverse: CGAGTCGTATCGTCGGTTG	
Nanog	Forward: TTCCTTCCTCCATGGATCTG	214
	Reverse: ATCTGCTGGAGGCTGAGGTA	
Sox2	Forward: CGCAGACCTACATGAACG	103
	Reverse: TCGGACTTGACCACTGAG	
Oct4	Forward: AAGCAGTGACTATTCGCAAC	136
	Reverse: CAGGGTGGTGAAGTGAGG	

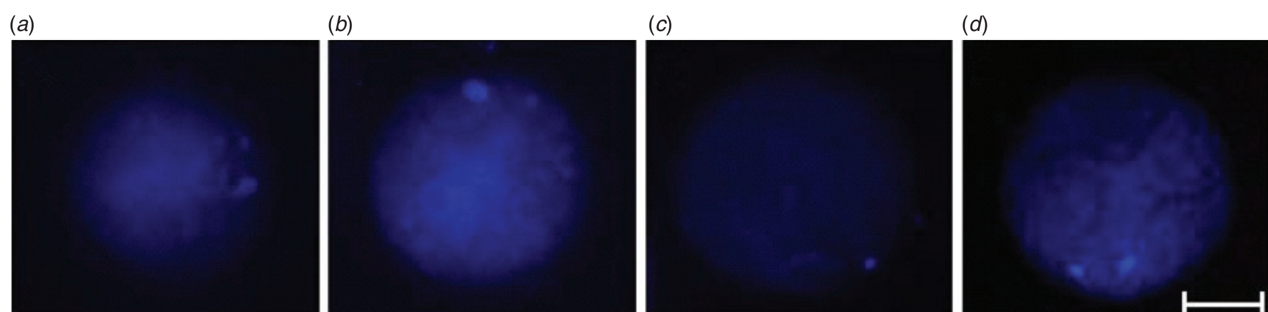

Fig. 1. Staining of chromatin with Hoechst 33342 during IVM of pig oocytes. (*a*) Germinal vesicle (GV) stage, (*b*) GV breakdown stage, (*c*) MI stage and (*d*) MII stage. Scale bar = 100 μm.

treated with 1 μM BODIPY FL ATP (BODIPY-ATP; A12410; Molecular Probes, Eugene, OR, USA) for 40 min in the dark, washed five times with PBS-PVA and then mounted onto glass slides. Images were acquired using a Nikon fluorescence microscope.

Terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling assay

The terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) assay was performed as described previously (Guo *et al.* 2018). Briefly,

blastocysts were washed three times with PBS-PVA, fixed with 4% (v/v) paraformaldehyde for 1 h and permeabilised with 1% (v/v) Triton X-100 for 40 min at 23°C. After repeating the washing step, blastocysts were blocked with PBS-BSA for 1 h and stained using a fluorescein-conjugated TUNEL assay kit (Roche, Mannheim, Germany) at 37°C for 50 min in the dark. Thereafter, blastocysts were washed with PBS-PVA, stained with Hoechst 33342, washed again with PBS-PVA and mounted onto glass slides. Images were acquired using an epi-fluorescence microscope and analysed using Nikon NIS-Elements software.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from 150 mature oocytes, cumulus cells obtained from 500 COCs and 40 porcine PA blastocysts using a Dynabeads mRNA DIRECT kit (Life Technologies) according to the manufacturer's instructions, and analysed qualitatively using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). cDNA was synthesised using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using an Agilent Mx3005P Real-Time PCR System (Stratagene). Each PCR mixture contained 1 μL cDNA, 0.5 μL (10 μM) forward primer, 0.5 μL (10 μM) reverse primer, 10 μL SYBR Premix Ex Taq (RR420B; Takara) and 8 μL nuclease-free water. The PCR was conducted using the following conditions: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s and 72°C for 1 min. The primer sequences are given in Table 1. Relative expression of each gene was quantified using the $2^{-\Delta\Delta CT}$ method, using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control. Each sample was analysed in triplicate.

Statistical analysis

Each experiment was repeated at least three times. Data were analysed using the Chi-squared test in SPSS 16.0 (SPSS Inc.). Two-sided $P < 0.05$ was considered significant.

Table 2. Effects of different concentrations of baicalin on oocyte maturation during IVM of porcine oocytes

Within columns, different superscript letters indicate significant differences ($P < 0.05$)

Baicalin (μg mL^{-1})	No. cultured embryos	No. (%) oocytes reaching MII
0	320	191 (59.7)^{a}
0.05	311	205 (65.9)^{ab}
0.1	311	210 (68.1)^{b}
0.5	319	224 (70.2)^{b}
1	324	189 (58.3)^{a}

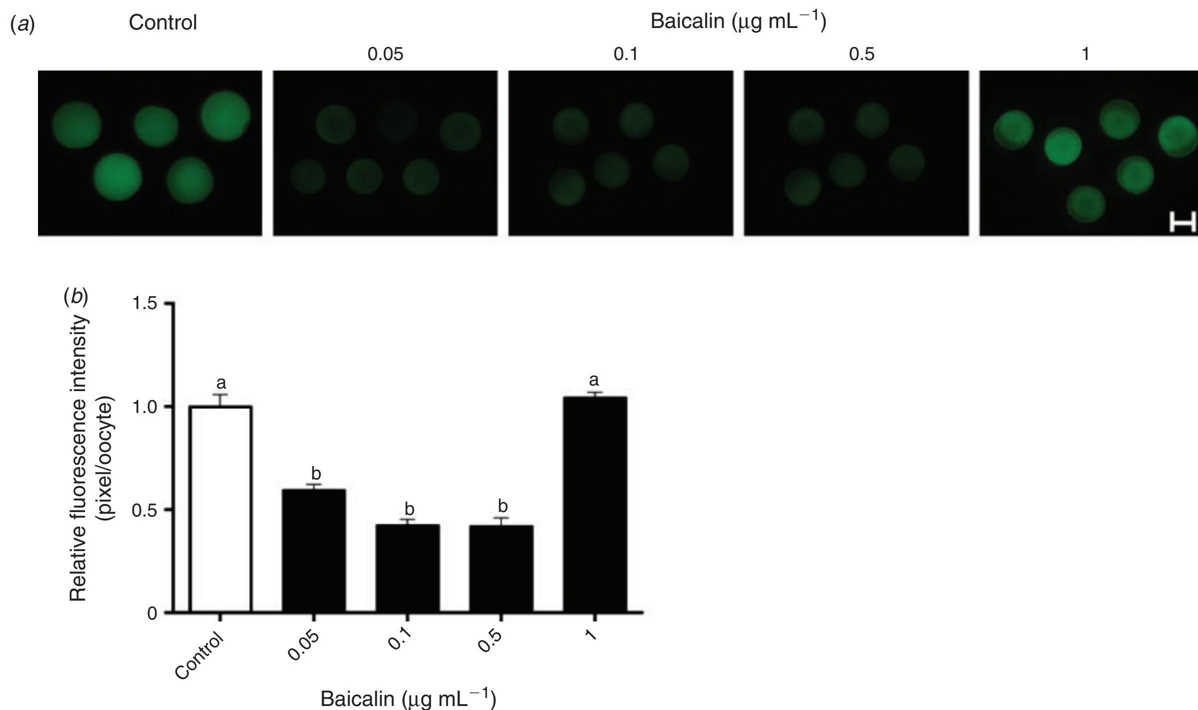

Fig. 2. Effects of baicalin on reactive oxygen species (ROS) production in pig MII oocytes. (*a*) Epifluorescence of ROS in pig MII oocytes. Oocytes were treated with different concentrations of baicalin, as indicated, and stained with 2′,7′-dichlorodihydrofluorescein diacetate to detect ROS. Scale bar = 100 μm. (*b*) Quantification of the fluorescence intensity of ROS staining, determined using Image-Pro Plus 6.0 (Media Cybernetics). Data are the mean ± s.e.m. Different letters above columns indicate significant differences ($P < 0.05$).

Results

Effects of different baicalin concentrations on the IVM of pig oocytes

The effects of different concentrations of baicalin (0, 0.05, 0.1, 0.5 and 1 μg mL^{-1}) on the IVM of pig oocytes were examined. The maturation rate of oocytes was determined by monitoring extrusion of the first polar body (Fig. 1). The maturation rate of oocytes treated with 0.1 and 0.5 μg mL^{-1} baicalin was significantly higher than that of control oocytes (68.1% and 70.2% vs 59.7% respectively; $P < 0.05$; Table 2).

Effects of baicalin on ROS levels in pig MII oocytes and on Bax *and* Bcl2 *expression in mature oocytes and cumulus cells*

Next, we investigated the effects of the different baicalin concentrations on ROS production in pig oocytes. ROS levels were lower in oocytes treated with 0.05, 0.1 and 0.5 μg mL^{-1} baicalin than in control oocytes and oocytes treated with other concentrations of baicalin (Fig. 2*a*, *b*). We decided to treat oocytes with 0.1 μg mL^{-1} baicalin in subsequent experiments. mRNA expression of the proapoptotic *Bax* gene was significantly lower in mature oocytes and cumulus cells in the baicalin-treated than control group, whereas mRNA expression of the antiapoptotic *Bcl-2* gene did not differ between the baicalin-treated and control groups (Fig. 3*a*, *b*).

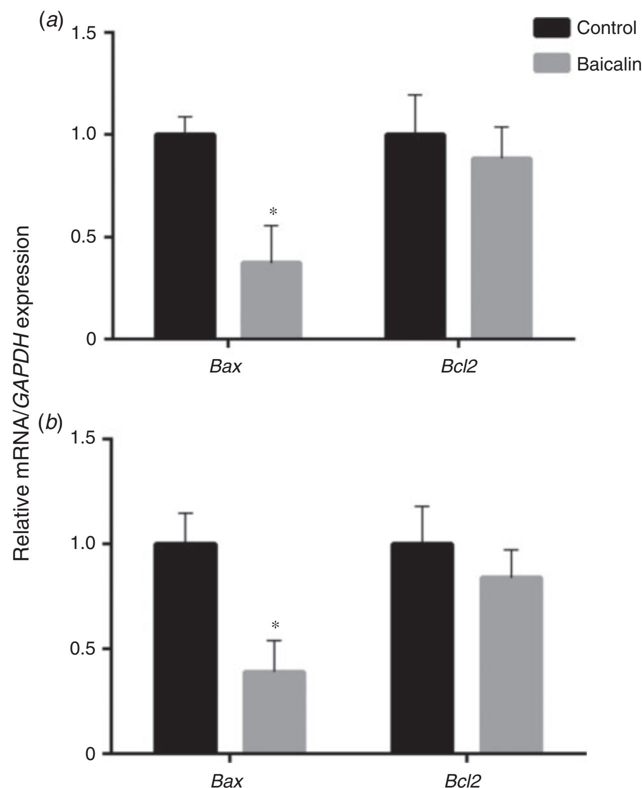

Fig. 3. Mean (± s.e.m.) levels of the apoptosis gene *Bax* and the anti-apoptosis gene *Bcl2* in pig (*a*) MII pig oocytes and (*b*) corresponding cumulus cells in control and baicalin (0.1 μg mL^{-1})-treated groups. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Baicalin significantly decreased *Bax* expression in both pig MII oocytes and the corresponding cumulus cells. *$P < 0.05$ compared with the control group.

Effects of baicalin on the expression of genes related to oocyte maturation and cumulus cell expansion

We investigated the effects of baicalin treatment on the mRNA expression of the nuclear maturation-related genes bone morphogenetic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), cyclin B, cyclin-dependent kinase 1 (*CDK1*; also known as *p34cdc2*) and MOS Proto-Oncogene, Serine/Threonine Kinase (*Mos*) in oocytes. Expression of *BMP15*, *GDF9*, *cyclin B* and *CDK1* mRNA was significantly higher in baicalin-treated than control oocytes ($P < 0.05$; Fig. 4*a*). In addition, we measured mRNA expression of the cumulus cell expansion-related genes prostaglandin-endoperoxide synthase 1 (*PTGS1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), pentraxin 3 (*PTX3*), tumour necrosis factor alpha induced protein 6 (*TNFAIP6*) and hyaluronan synthase 2 (*SHAS2*). Expression of

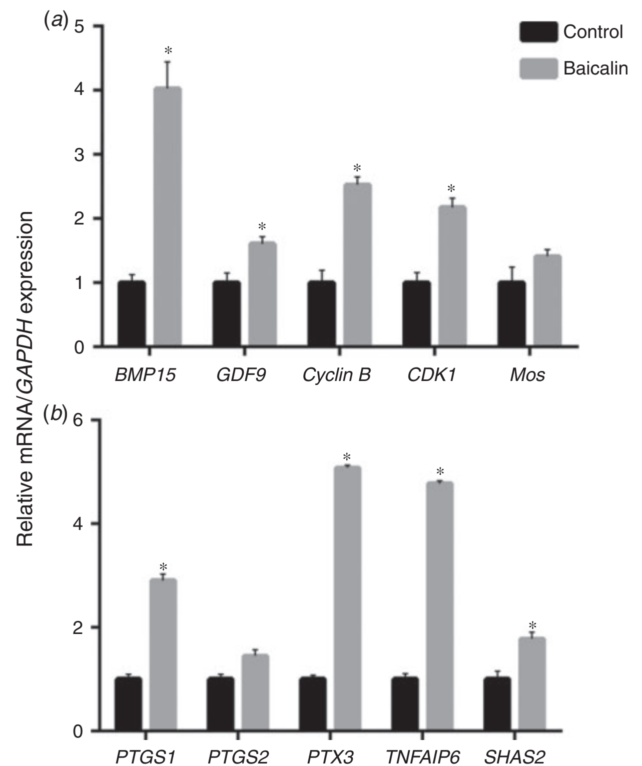

Fig. 4. Effects of 0.1 μg mL^{-1} baicalin on the expression of genes related to (*a*) nuclear maturation and (*b*) cumulus cell expansion in pig MII oocytes. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Data are the mean ± s.e.m. *$P < 0.05$ compared with the control group. *BMP15*, bone morphogenetic protein 15; *GDF9*, growth differentiation factor 9; cyclin B; *CDK1*, cyclin-dependent kinase 1; *MOS*, MOS Proto-Oncogene, Serine/Threonine Kinase; *PTGS1*, prostaglandin-endoperoxide synthase 1; *PTGS2*, prostaglandin-endoperoxide synthase 2; *PTX3*, pentraxin 3; *TNFAIP6*, tumour necrosis factor alpha induced protein 6; *SHAS2*, hyaluronan synthase 2.

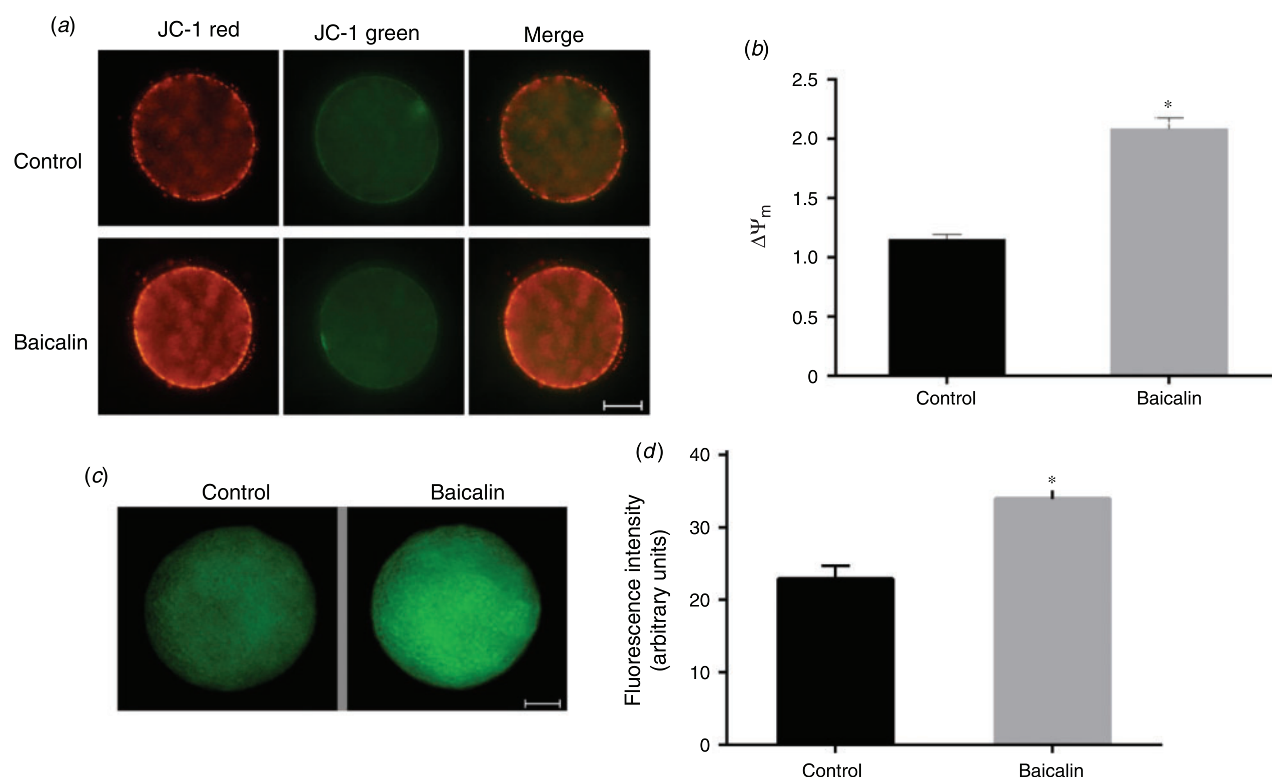

Fig. 5. Effects of 0.1 μg mL^{-1} baicalin on mitochondrial membrane potential ($\Delta\Psi_m$) and the ATP content in pig MII oocytes. (*a*) Staining of MII oocytes with JC-1; $\Delta\Psi_m$ is determined as the ratio of red to green fluorescence. Scale bar = 100 μm. (*b*) Quantification of relative $\Delta\Psi_m$ using Image-Pro Plus 6.0 (Media Cybernetics). (*c*) Labelling of ATP with BODIPY-ATP. Scale bar = 100 μm. (*d*) Quantification of the fluorescence intensity of BODIPY-ATP. Data are the mean ± s.e.m. *$P < 0.05$ compared with the control group.

Table 3. Effects of baicalin during IVM on subsequent embryo development after parthenogenetic activation

Within columns, different superscript letters indicate significant differences ($P < 0.05$)

Baicalin (μg mL^{-1})	No. cultured embryos	No. (%) embryos reaching 2- to 4-cell stage	No. (%) embryos reaching blastocyst stage
0	191	145 (75.9)^{a}	52 (27.2)^{a}
0.05	205	168 (82.0)^{ab}	70 (34.1)^{a}
0.1	210	188 (85.5)^{b}	104 (49.5)^{b}
0.5	224	191 (86.0)^{b}	102 (45.5)^{b}
1	189	143 (75.6)^{a}	53 (28.0)^{a}

PTGS1, *SHAS2*, *PTX3* and *TNFAIP6* mRNA was significantly higher in baicalin-treated than control oocytes; however, the mRNA expression of *PTGS2* did not significantly differ between the two groups (Fig. 4*b*).

Effects of baicalin on $\Delta\Psi_m$ and ATP activity in pig oocytes

Mitochondrial dynamics and the ATP content play an important role in the IVM of mammalian oocytes. Thus, we examined mitochondrial function and the ATP content in mature oocytes using JC-1 and BODIPY-ATP staining respectively. The $\Delta\Psi_m$ is an indicator of mitochondrial function and can be measured using the fluorescent dye JC-1, which changes from green to red as $\Delta\Psi_m$ increases. Mitochondria in baicalin-treated oocytes exhibited intense red fluorescence, indicative of a high $\Delta\Psi_m$, whereas mitochondria in control oocytes had a lower $\Delta\Psi_m$ (Fig. 5*a*). Quantitative analysis revealed that the red : green fluorescence ratio was significantly higher in baicalin-treated than control oocytes (Fig. 5*b*). Moreover, the ATP content was significantly higher in baicalin-treated than control oocytes (Fig. 5*c*, *d*). These results indicate that baicalin treatment during IVM improves the $\Delta\Psi_m$ and ATP content in pig oocytes.

Effects of baicalin treatment during IVM on subsequent development of PA embryos

To determine whether baicalin treatment during IVM of pig oocytes improves subsequent embryo development after PA,

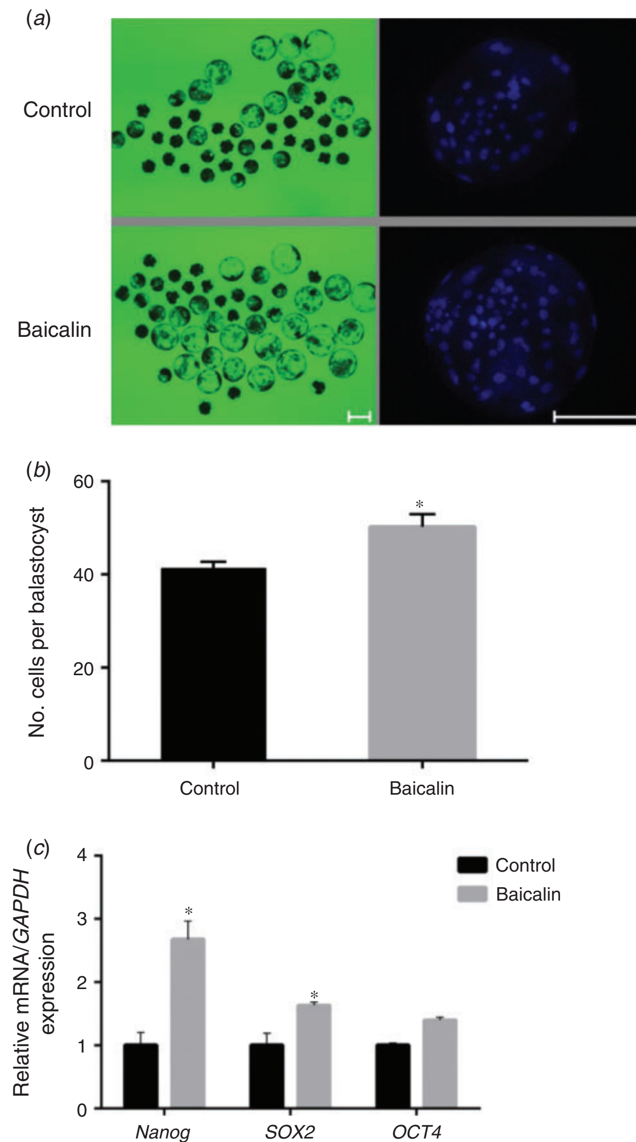

Fig. 6. Effects of 0.1 μg mL^{-1} baicalin on subsequent embryo development of pig oocytes. (*a*) Blastocyst development and Hoechst 33342 staining following parthenogenetic activation (PA) of oocytes. Scale bars = 100 μm. (*b*) The mean (± s.e.m.) number of cells per blastocyst was greater in the baicalin-treated than control group. (*c*) Levels level of pluripotency-related genes in blastocysts following PA of oocytes in the baicalin-treated and control groups. Expression of *Nanog* and SRY (sex determining region Y)-box 2 (*SOX2*) increased significantly in the baicalin-treated group. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Data are the mean ± s.e.m. **P* < 0.05 compared with the control group.

we determined the cleavage and blastocyst formation rates and the number of cells per blastocyst. The cleavage and blastocyst formation rates were significantly higher in the 0.1 μg mL^{-1} baicalin-treated than control group (Table 3). In addition, the number of cells per blastocyst (Fig. 6*a*, *b*) and mRNA expression of the pluripotency-related genes SRY (sex determining region Y)-box 2 (*Sox2*) and *Nanog* were significantly higher in the baicalin-treated than control group (Fig. 6*c*). Expression of *Bax* and *Bcl2* mRNA was significantly lower and higher respectively in the baicalin-treated than control group (Fig. 7*c*). The percentage of apoptotic cells in blastocysts was significantly lower in the baicalin-treated than control group (Fig. 7*a*, *b*).

Discussion

IVF and somatic cell nuclear transfer (SCNT) are important means to save endangered species (Williams *et al.* 2006). Nevertheless, oocyte maturation is essential for IVF and SCNT, as well as subsequent embryo development in assisted reproductive technologies (Agarwal *et al.* 2005; Wu *et al.* 2011; Jin *et al.* 2018). Thus, elucidation of the effects of baicalin on the IVM of oocytes has implications for animal breeding. The present study investigated the effects of baicalin on the IVM of pig oocytes, including nuclear maturation, cumulus cell expansion, $\Delta\Psi_m$ and the ATP content, and subsequent *in vitro* embryo development.

Baicalin is a traditional Chinese medicine that is widely used in the medical field. This monomer has many pharmacological functions, including antioxidative, antiapoptotic and anti-inflammatory effects (Hou *et al.* 2012; Yang *et al.* 2012; Wen *et al.* 2013; Guo *et al.* 2014; Shi *et al.* 2018*b*). Wang *et al.* (2016) reported that, in rats, baicalin reduces alcohol-induced liver damage by reducing oxidative stress and inflammation. Many factors affect the IVM of oocytes. For example, oxidative stress perturbs the IVM of oocytes and subsequent embryo development. We hypothesised that baicalin would improve the IVM of pig oocytes through its antioxidative and antiapoptotic effects. As predicted, treatment with 0.1 μg mL^{-1} baicalin significantly reduced ROS levels in oocytes and *Bax* mRNA expression in oocytes and cumulus cells. Moreover, treatment of oocytes with a higher concentration (1 μg mL^{-1}) of baicalin resulted in higher ROS levels in oocytes compared with other concentrations of baicalin tested, but did not differ significantly compared with control. This is consistent with a previous study that showed that high concentrations (50 μM) of baicalin could be used to treat cancer because they promote apoptosis of tumour cells, but had no effect on normal cells (Dou *et al.* 2018). Thus, baicalin may significantly improve the IVM of pig oocytes by inhibiting ROS production and thereby reducing apoptosis in oocytes and cumulus cells.

The activity of maturation-promoting factor (composed of *CDK1* and *cyclin B*) is important for nuclear maturation during IVM of mammalian oocytes (Xia and Zhang 2007). Meanwhile, *BMP15* and *GDF9* are key factors in granulosa cell development and oocyte fertilisation in most mammals (Su *et al.* 2004). In addition, cumulus cell expansion is a marker of the IVM capacity of oocytes and of subsequent embryo developmental capacity (Wigglesworth *et al.* 2013).

In this study, we investigated whether baicalin improves the IVM of oocytes by affecting the expression of genes related to nuclear maturation (*CDK1*, *cyclin B*, *BMP15*, *GDF9* and *Mos*) and cumulus cell expansion (*PTGS1*, *PTGS2*, *TNFAIP6*, *PTX3* and *SHAS2*). Baicalin significantly increased expression of *CDK1*, *cyclin B*, *BMP15*, *GDF9*, *PTGS1*, *TNFAIP6*, *PTX3* and *SHAS2* mRNA when added to the IVM medium of pig

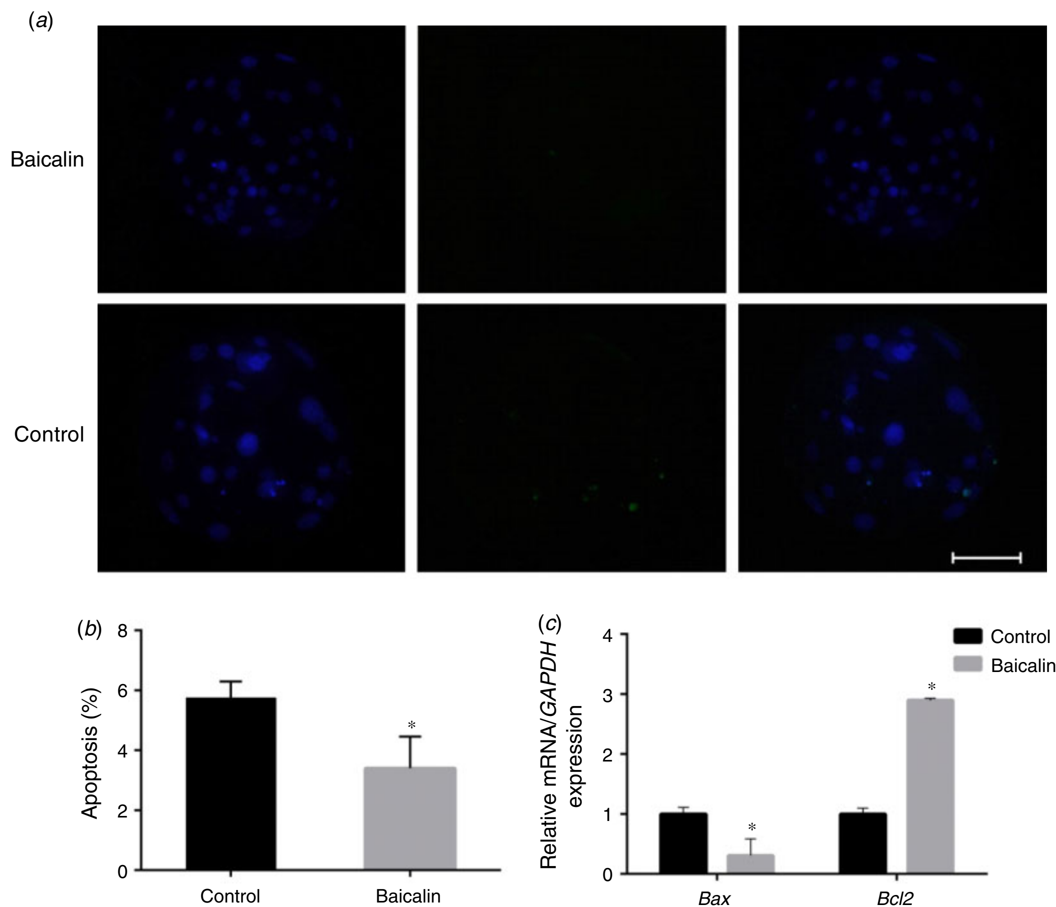

Fig. 7. Effects of baicalin on terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) and relative mRNA expression of apoptosis-related genes in pig blastocysts. (*a*) TUNEL (green) and DNA staining (blue) in blastocysts. Scale bar = 100 μm. (*b*) Percentage of apoptotic cells in blastocysts. (c) Expression of *Bax* and *Bcl2* mRNA, relative to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), in blastocysts. Data are the mean ± s.e.m. $*P < 0.05$ compared with the control group.

oocytes. These results indicate that baicalin improves the IVM of oocytes by increasing the mRNA expression of genes related to nuclear maturation and cumulus cell expansion.

Mitochondrial function is an important indicator of oocyte quality (Zeng *et al.* 2018). Oocyte maturation is often accompanied by changes in the distribution of mitochondria, which provide energy for oocyte growth and development (Nagano and Katagiri 2006; Van Blerkom 2011). In general, lower $\Delta\Psi_m$ and ATP content are associated with poor oocyte fertility, whereas higher $\Delta\Psi_m$ and ATP content are associated with increased oocyte developmental capacity (Fissore *et al.* 2002; Iwata *et al.* 2011). However, higher $\Delta\Psi_m$ and ATP production in the mitochondria also lead to higher levels of ROS production, which perturb physiological processes in oocytes. In this study, we found that the $\Delta\Psi_m$ and ATP content were higher, but ROS levels were lower, in baicalin-treated oocytes compared with control oocytes. These results show that adding 0.1 μg mL^{-1} baicalin to the IVM medium can produce more energy in addition to lowering ROS by protecting the integrity of the mitochondria within the oocytes. This result indicates that baicalin could be used as a potential antioxidant to improve the IVM capacity of pig oocytes.

Expression of genes associated with pluripotency (*Oct4*, *Sox2* and *Nanog*) and apoptosis (*Bax* and *Bcl2*) and the number of cells per blastocyst reflect embryo quality *in vitro* (Yang and Rajamahendran 2002; Sommer *et al.* 2009; Jin *et al.* 2016). In the present study, treatment with 0.1 μg mL^{-1} baicalin during IVM increased the number of cells per blastocyst, upregulated expression of *Sox2* and *Nanog* and downregulated expression of *Bax* after PA. This is similar to the previous finding that 4 μg mL^{-1} baicalin improves the *in vitro* development of mouse embryos by inhibiting apoptosis (Wigglesworth *et al.* 2013). The reason why different concentrations of baicalin elicited similar effects in mice and pigs may be that the sensitivity of oocytes and embryos to baicalin differs between species.

In summary, this study demonstrated that 0.1 μg mL^{-1} baicalin improved pig oocyte maturation and subsequent embryo development *in vitro* by inhibiting ROS production and reducing apoptosis in oocytes. Baicalin increased the

maturation rate, mRNA expression of genes related to nuclear maturation and cumulus cell expansion, $\Delta\Psi_m$ and ATP content in oocytes. Moreover, the blastocyst formation rate, number of cells per blastocyst and mRNA expression of pluripotency-related genes were increased in blastocysts derived from baicalin-treated oocytes. In contrast, baicalin decreased the expression of *Bax* mRNA in oocytes, cumulus cells and blastocysts.

Conflicts of interest

The authors declare no conflicts of interest.

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