Improvement of *in vitro*-produced bovine embryo treated with coagulansin-A under heat-stressed condition

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Abstract

Heat stress has large effects on reproduction including conception rate in cattle. In this study, we examined the effects of coagulansin-A (coa-A), a steroidal lactone, on acquired thermo tolerance during *in vitro* production of bovine embryos. Oocytes were incubated in *in vitro* maturation (IVM) media with or without coa-A at two different temperatures, 40.5°C and 42°C, for 20 h. The treatment of coa-A significantly improved blastocyst development only at 40.5°C ($P < 0.05$). Interestingly, immunofluorescence analysis demonstrated that coa-A induced heat shock protein 70 (HSP70) and phosphatidylinositol-3-kinase (PI3K), but significantly attenuated nuclear factor kappa B (NF- κ B) and cyclooxygenase-2 (COX2). To determine the expression patterns of related genes at the transcription level, qRT-PCR was performed. Expression of HSP70 and PI3K was elevated, whereas expression of NF- κ B, COX2 and inducible nitric oxide synthase (iNOS) was significantly ($P < 0.05$) downregulated in the coa-A-treated group compared with the control group. Moreover, pro-apoptotic genes were downregulated, and antiapoptotic genes were upregulated in the coa-A group. We also counted the total cell number and apoptotic nuclei at the blastocyst and found that more cell numbers (143.1 ± 1.5) and less apoptotic damages (6.4 ± 0.5) in the coa-A treatment group comparing to control group (131.4 ± 2.0 and 10.8 ± 0.5), indicating the enhanced embryo quality. In conclusion, our results demonstrate that the coa-A not only improved the blastocyst development *in vitro* but also increased their resistance to heat stress condition through induction of HSP70/PI3K.

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Introduction

Bovine oocytes are used to generate *in vitro*-produced embryos throughout the world. Embryos are derived from immature oocytes and oocytes are obtained from slaughtered cattle, because they are easily available and inexpensive (Jeong *et al.* 2009). However, the efficiency of *in vitro* production (IVP) is poor, because the quality of the embryos produced is lower than that of *in vivo* (Lonergan *et al.* 2006). Exposure of embryos to oxidative stress (OS) has deleterious effects on their development during IVP (Guerin *et al.* 2001). The balance between OS and antioxidants is maintained *in vivo*, whereas the level of reactive oxygen species (ROS) exceeds the cellular antioxidant concentration and causes OS *in vitro* (Combelles *et al.* 2009). Withanolides obtained from *Withania somnifera* are widely used in India and Pakistan (Ihsan-ul-Haq *et al.* 2013). Withanolides are

steroidal lactones that have various pharmacological uses, have positive roles in hemoglobin improvement and weight gain and are antioxidants that scavenge ROS (Ziauddin *et al.* 1996). Withanolides also have a dynamic anticancer role, operating as anticancer agents in cancer cell lines by enhancing apoptosis, and are also used to treat Alzheimer's disease, Parkinson's disease and dementia (Wang *et al.* 2012). Coagulansin-A (coa-A) is a withanolide derivative, and we reported for the first time that it improves bovine embryo development *in vitro* in normal culture conditions (Khan *et al.* 2016). Various environmental factors affect the fertility of dairy animals. Of these, heat stress or heat shock is the most important, because it reduces the developmental competence of oocytes (Yadav *et al.* 2013). During the early stage of folliculogenesis, heat stress damages the ovarian pool of oocytes (Roth *et al.* 2001). Heat stress also greatly affects

the conception rate, and the conception rate is reduced to 20–30% during hot seasons (Cavestany *et al.* 1985, De Rensis *et al.* 2002). Heat stress reduces cytoplasmic and nuclear maturation, the functions of surrounding cumulus cells, and mitochondrial activity and induces apoptosis (Roth 2008, Hansen 2009, Nabenishi *et al.* 2012). Great economic losses are associated with livestock during hot seasons, because the temperature fluctuation causes heat stress that affects the fertility of cattle and other animals (Stott 1961). Special attention is needed to counterbalance the drastic effects of global warming and environmental changes on livestock by reducing losses due to heat stress.

The aim of this study was to examine the positive effect of coa-A on the development of bovine embryos in heat stress conditions *in vitro*.

Materials and methods

Reagents and approval of experiments

All chemicals and reagents were obtained from Sigma-Aldrich. The care and use of laboratory animals as well as the experiments were performed following Gyeongsang National University guidelines and rules (approval no. GAR-110502-X0017).

Isolation and characterization of coagulansin-A

This method has been elaborated in detail in the Supplementary extended section. Briefly, the plant material was collected, sorted out for any foreign material, diseased or deteriorated parts. Then, it was shade dried with continuous agitation every 6 hourly and then crushed in a grinding mill. A total of 10-kg shade dried and crushed aerial parts without fruits (leaves and stems only) were taken and macerated in

30 L of solvent by occasional shaking for 3 days. Mixture of chloroform and methanol (1:1) was used as extraction solvent. Filtrate of extraction was dried by vacuum distillation. It was then subjected to solvent extraction and normal phase preparative chromatography to isolate 50 mg of coagulansin-A. The compound was characterized by performing 2D NMR and LC–MS experiments, and purity was 98.6% (Ihsan-UI-Haq *et al.* 2013).

Experimental design

The goal of this study was to determine the protective effect of coa-A against heat stress in IVM media for blastocyst development. For this purpose, the optimal concentration of coa-A (5 µM) was selected based on our previous data (Khan *et al.* 2016). Oocytes treated with 5 µM coa-A were compared with the untreated control group. Both the coa-A-treated and control groups were exposed to heat stress at 42°C for 2, 5, 10 and 20 h. However, at this temperature, there was no significant difference in blastocyst development between the control and coa-A-treated group. Then, we exposed both groups to a heat stress at 40.5°C for 2, 5, 10, 20 and 24 h and then cultured at a normal temperature (38.5°C). At 40.5°C, we found the significant developmental competence to blastocyst between the control and coa-A-treated group after 2 h (26.6 and 36.1), 5 h (24.7 and 32.6), 10 h (21.8 and 29.2) and 20 h (18.6 and 24.6) except 24 h (14.2 and 15.2). We focused on blastocyst development at 40.5°C after 20-h heat stress, because it was the maximum time up to which the coa-A shows its activity for blastocyst development. The study was completed in six replications.

Recovery of cumulus-oocyte complexes (COCs)

Ovaries of Korean native cows (Hanwoo) were obtained from a local abattoir, placed in physiological saline (0.9% NaCl) at approximately 35°C and transported to the laboratory within

Table 1 Primer list for RT-PCR.

Gene	Primer sequence	Accession no.	Product size (bp)
<i>HSP70.1</i>	F: GAGTCGTACGCCTTCAACAT R: ACTTGTCAGCACCTTCTTC	U09861	94
<i>PI3K</i>	F: TCAACCATGACTGTGTGCCA R: CCATCAGCATCAAATTGGGCA	NM_174574.1	234
<i>NF-κB</i>	F: TGGCGGAATTACCTTCCATAC R: CATCACTCTTGCCACAACCTTC	DQ464067	110
<i>COX2</i>	F: CTGACCCATACAAGCACGATAG R: TCGTAAAGAAGGAAGAGCAATTAGA	DQ347627.1	99
<i>iNOS</i>	F: CGAGCTTCTACCTCAAGCTATC R: CTGGCCAGATGTTCTCTATTT	DQ676956.1	84
<i>P53</i>	F: CTATGAGATGTTCCGAGAGC R: CTCTCTCTTGACATTGGTT	NM_174201.2	153
<i>P21</i>	F: GCAAATATGGGTCTGGGAGA R: AAATAGTCCAGGCCAGGATG	NM_001098958.2	112
<i>BAX</i>	F: CACCAAGAAGCTGAGCGAGTGT R: TCGGAAAAAGACCTCTCGGGGA	NM_173894	118
<i>Caspase 3</i>	F: CCCAAGTGTGACCACTGAAC R: CCATTAGGCCCACTCACTG	NM_001077840	169
<i>BCL-2</i>	F: TGGATGACCGAGTACCTGAA R: GAGACAGCCAGGAGAAATCAAA	NM_001166486.1	123
<i>GAPDH</i>	F: CCCAGAATATCATCCCTGCT R: CTGCTTCACCACTTCTTGA	NM_001034034	185

Table 2 Cleavage and developmental rates of bovine embryos generated from oocytes treated with 5 μ M coagulansin-A at heat stress condition of 40.5°C after various time intervals.

Exposed time for heat shock (h)	Treatments	No. of oocytes	No. of presumed zygote	No. (%) of embryos developed to	
				Cleavage	Blastocyst
2	Control	195	172	138 (80.2) ^a	46 (26.6) ^a
	Coa-A	195	170	140 (82.3) ^a	60 (36.1) ^b
5	Control	195	168	131 (78.5) ^a	41 (24.7) ^a
	Coa-A	195	165	130 (79.3) ^a	53(32.6) ^b
10	Control	195	167	129 (77.3) ^a	35 (21.8) ^a
	Coa-A	195	165	129 (78.1) ^a	47 (29.2) ^b
20	Control	265	227	172 (75.7) ^a	42 (18.6) ^a
	Coa-A	265	231	176(76.2) ^a	57 (24.6) ^b
24	Control	195	161	84 (52.1) ^a	23 (14.2) ^a
	Coa-A	195	164	88 (53.7) ^a	25(15.2) ^a

This study was completed in six replications.

^{a,b}Values with different superscripts are significantly different ($P < 0.05$).

2 h after slaughter. Ovaries were washed with Dulbecco's phosphate-buffered saline (D-PBS), and COCs were recovered as described by Deb and coworkers (Deb *et al.* 2011). In brief, an 18-gauge needle attached to a vacuum pump was used to aspirate COCs from follicles with a diameter of 2–8 mm. The fluid obtained from follicles was expelled into ϕ 100 mm petri dishes containing TL-HEPES medium (114 mM

sodium chloride, 3.2 mM potassium chloride, 2 mM sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2 mM calcium chloride, 10 mM HEPES, 1 μ L/mL phenol red, 100 IU/mL penicillin and 0.1 mg/mL streptomycin) and imaged with a stereomicroscope. Good-quality oocytes with more than three layers of compact cumulus cells and homogenous cytoplasm were selected for maturation and then washed three times in TL-HEPES medium.

Figure 1 Representative images of bovine embryos stained with Hoechst 33342. Apoptotic cells were identified by TUNEL. The corresponding images were merged. (A, B, C) Control group. (A', B', C') Group treated with 5 μ M coagulansin-A. Scale bar, 200 μ m. Apoptotic cells are indicated by yellow arrows. The graph shows the number of apoptotic cells per embryo. The symbol a shows the significant difference ($P < 0.05$) between the control and treated group.

In vitro maturation (IVM) of oocytes

After selection, COCs were cultured in IVM medium as described by Deb and coworkers (Deb *et al.* 2011). In brief, the COCs were washed three times in IVM medium (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mg/mL estradiol-17 β , 10 mg/mL follicle-stimulating hormone, 0.6 μ M cysteine and 0.2 μ M sodium pyruvate, and then transferred to a 4-well dish containing 600 μ L of IVM media in a humidified atmosphere of 5% CO₂ in air at 38.5°C for 22–24 h.

In vitro fertilization (IVF) followed by in vitro culture

Matured COCs were fertilized as described by Deb and coworkers (Deb *et al.* 2011). Semen was thawed in water at 37°C for 1 min, and sperm were washed and pelleted in D-PBS by centrifugation at 750 g, room temperature for 5 min. The pellet was resuspended in 500 μ L of heparin (20 μ g/mL) prepared in IVF medium (Tyrode's lactate solution supplemented with 6 mg/mL bovine serum albumin (BSA), 22 mg/mL sodium pyruvate, 100 IU/mL penicillin and 0.1 mg/mL streptomycin) and incubated in a humidified atmosphere of 5% CO₂ in air at 38.5°C for 15 min (to facilitate capacitation). The sperm pellet was diluted in IVF medium (final density of 1–2 $\times$ 10⁶ sperms/mL).

Table 3 Characteristics of Day 8 blastocysts in the two groups.*

Treatment	No. of blastocysts examined	Total no. of cells per blastocyst	No. of apoptotic cells per blastocyst
Control	17	131.4 $\pm$ 2.05 ^a	10.80 $\pm$ 0.52 ^b
5 μ M coa-A	17	143.1 $\pm$ 1.51 ^b	6.400 $\pm$ 0.47 ^a

This study was completed in four replications.

*Total number of cells and number of apoptotic cells per blastocyst are shown (mean $\pm$ S.E.); ^{a,b}values with different superscripts in the same column are significantly different ($P < 0.05$).

Matured oocytes were transferred to IVF medium (600 µL) containing sperm for 18–20 h. After IVF, cumulus cells were removed by pipetting, and denuded presumed zygotes were placed in 600 µL of CR1-aa medium (Rosenkrans *et al.* 1993) supplemented with 44 µg/mL sodium pyruvate, 14.6 µg/mL glutamine, 10 µL/mL penicillin–streptomycin, 3 mg/mL BSA and 310 µg/mL glutathione for 3 days. After checking cleavage, 8-cell stage embryos were cultured until Day 8 of embryonic development (Day 0 = day of IVF) in medium of the same composition, except that FBS replaced BSA. Day 8 blastocysts

were washed three times in TL-HEPES, transferred to fixative (4% (v/v) paraformaldehyde prepared in 1 M PBS) and stored at 4°C until cells were counted.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

TUNEL was performed using an In Situ Cell Death Detection Kit according to the manufacturer's protocol (Roche Diagnostics Corp.). Fixed embryos were permeabilized (0.5% (v/v) Triton

Figure 2 Confocal microscopy, showing HSP70 expression at a magnification of 10×. (A, B) Control group, (C, D) coa-A treated group. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated group. The scale bar for A and C is 10 µm, whereas for B and D is 20 µm.

Figure 3 Confocal microscopy of bovine embryos *in vitro* showing PI3K expression in two groups at a magnification of 10×. (A, B) Control group, (C, D) coa-A treated group. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated group. The scale bar for A and C is 10 µm, whereas for B and D is 20 µm.

X-100 and 0.1% (w/v) sodium citrate) at room temperature for 30 min and then washed twice with 0.3% (w/v) polyvinyl alcohol prepared in 1 M PBS (PVA-PBS) (Deb et al. 2011). After permeabilization, embryos were washed twice with PVA-PBS and incubated in the dark with fluorescently conjugated TUNEL at 37°C for 1 h. TUNEL-stained embryos were then washed with PVA-PBS and incubated in PVA-PBS containing 10 µg/mL Hoechst 33342 for 10 min. After washing, blastocysts were mounted on a glass slide, and their nuclear configuration was analyzed. An epifluorescence microscope (Olympus IX71) equipped with a mercury lamp was used to determine

the number of cells per blastocyst by counting Hoechst-stained cells. Red cells (TUNEL positive) were apoptotic.

Confocal microscopy (immunofluorescence analysis)

Fixed embryos were washed twice with 1 M PBS for 10 min. Embryos were incubated in proteinase K solution at 37°C

Figure 4 Confocal microscopy of NF-κB in two groups at a magnification of 10×. (A, B) Control group, (C, D) coa-A treated group. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated group. A and C have a scale bar of 10 μm, whereas B and D have a scale bar of 20 μm.

Figure 5 Confocal microscopy of COX2 in two groups at a magnification of 10×. (A, B) Control group; (C, D) coa-A treated group. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated group. The scale bar for A and C is 10 μm, whereas for B and D is 20 μm.

for 5 min. Embryos were incubated in blocking solution (PBS containing 5% non-immune goat serum and 0.3% Triton X-100) for 1 h after washing with PBS. Embryos were incubated overnight with primary antibodies including mouse-derived anti-phosphatidylinositol-3-kinase (PI3K), anti-heat shock protein 70 (HSP70), anti-nuclear factor kappa B (NF- κ B) (Santa Cruz Biotechnology) and anti-cyclooxygenase (COX2) (Millipore, Santa Cruz Biotechnology) antibodies, and then with secondary FITC- and TRITC-conjugated antibodies (diluted 1:50 in D-PBS; Santa Cruz Biotechnology) at room temperature for 90 min. DAPI was used as a counterstain for 5 min. Embryos were mounted on glass slides with Prolong anti-fade reagent (Molecular Probes). Stained embryos were examined using a confocal laser-scanning microscope (Fluoview FV 1000, Olympus). Then, the integral optical density (IOD) has been analyzed using ImageJ software (<https://imagej.nih.gov/ij/>). Negative control was obtained by replacing the primary antibody with goat serum, whereas positive control was obtained by checking the expression of various proteins in cumulus cells (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

mRNA extraction and cDNA formation

An Arcturus PicoPure RNA Isolation Kit (Arcturus, Foster, CA USA; Cat# 12204-01) was used to extract RNA. In brief, 100 μ L of extraction buffer was added to the tube and

incubated at 42°C for 30 min. After incubation, the tube was centrifuged at 3000g for 2 min, and the supernatant was transferred to a new 1.5 mL RNA-free tube. Thereafter, 200 μ L of conditioning buffer was added to a column tube, incubated at room temperature for 5 min and then centrifuged at 16,000g for 1 min. Thereafter, 100 μ L of 70% ethanol was added and pipetted thoroughly. Then, the mixture was added to a column tube, centrifuged at 100g for 2 min, and then centrifuged at 16,000g for 30 s to remove the flow-through. Thereafter, 100 μ L of wash buffer 1 was added to the column and centrifuged at 8000g for 1 min. DNase I solution (5 μ L DNase added to 35 μ L of buffer RDD) was prepared and mixed gently. Then, 40 μ L of the DNase I mixture was added directly to the purification column membrane and incubated at room temperature for 15 min. Thereafter, 40 μ L of wash buffer 1 was added to the column and centrifuged at 8000g for 15 s. Then, 100 μ L of wash buffer 2 was added to the column, centrifuged at 16,000g for 2 min, and then centrifuged at 16,000g for 1 min to completely remove the wash buffer. Finally, the purification column was transferred to a new 1.5 mL RNase-free tube. Thereafter, 20 μ L of elution buffer was placed into the center of the column, incubated at room temperature for 1 min, centrifuged at 1000g for 1 min and then centrifuged at 16,000g for 1 min. RNA samples were used immediately or stored at -80°C until use.

The RNA concentration and purity were checked using a NANO DROP 2000c machine (Thermo Fisher Scientific).

Figure 6 Confocal microscopy of HSP70 and ROS in oocytes at a magnification of 40 $\times$. (A) ROS (8-OxoG) expression, (B) HSP70 expression. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated groups. The scale bar is 700 μ m.

The mRNA samples were reverse-transcribed into first-strand cDNA using a kit from Bio-Rad Laboratories. The 15 µL mRNA samples were transferred to a 200-µL Eppendorf tube containing 4 µL of 5× iScript Reaction Mixture and 1 µL of iScript Reverse Transcriptase. The reactions were terminated by heating at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min, and finally samples were held at 4°C.

Real-time PCR analysis of target genes

Selected genes of heat shock protein 70 (*HSP70*), phosphatidylinositol-3-kinase (*PI3K*), nuclear factor kappa B (*NF-κB*), cyclooxygenase-2 (*COX2*), inducible nitric oxide synthase (*iNOS*), bcl-2-associated X protein (*BAX*), B-cell lymphoma 2 (*BCL-2*), cyclin-dependent kinase inhibitor (*P21*), tumor protein (*P53*) and caspase 3 were analyzed by real-time PCR. Quantitative RT-PCR was performed in duplicate using a CFX98 instrument (Bio-Rad Laboratories) with a 10-µL reaction mixture containing 0.2 mM of each bovine-specific primer (Table 1), 1× iQ SYBR Green Supermix (iQ SYBR Green Supermix kit, Bio-Rad Laboratories) and 3 µL of diluted cDNA. All cDNA samples were subjected to real-time PCR using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers to detect any variation in expression of this internal control gene. After confirming that relative *GAPDH* expression did not significantly differ among the samples, all transcripts were quantified using independent real-time PCR analyses. The cycling conditions were as follows: 95°C for 3 min, followed by 44 cycles of 95°C for 15 s, 57°C for 20 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Amplification was followed by melting curve analysis using progressive denaturation, during which the temperature was increased from 65°C to 95°C at a rate of 0.2°C per second. Fluorescence was continuously measured during this incremental heating. Quantitative analysis was performed using the $\Delta\Delta C(t)$ method. The results are reported as the relative expression or *n*-fold difference compared with the calibrator after normalization of the transcript to the average value of the endogenous control *GAPDH*. The coefficients of variation of the intra- and inter-assay variance were calculated according to the formula, $s.d./\text{mean} \times 100$, for all genes profiled by real-time PCR.

Statistical analysis

Results are presented as percentages. Prism 5 was used to analyze the data and total cell numbers per blastocyst. The data are presented as the mean $\pm$ standard error of mean (S.E.M.). A simple *t*-test and one-way ANOVA were used to detect significant differences between groups. $P < 0.05$ was considered significant.

Results

Cleavage and developmental rates of embryos in heat stress conditions

The cleavage rate was determined on the third day of culture. The cleavage rate was not significantly higher in the 5 µM coa-A-treated group than that in the control group

after exposure to heat shock for 2 h (82.3% and 80.2%), 5 h (79.3% and 78.5%), 10 h (77.3% and 78.1%), 20 h (76.2% and 75.7%) and after 24 h (53.7% and 52.1%), respectively, but this increase in cleavage between different groups was not statistically significant. However, the percentage of Day 8 blastocysts was significantly higher ($P < 0.05$) in the 5 µM coa-A-treated group than that in the control group after exposure to heat shock for 2 h (36.1% and 26.6%), 5 h (32.6% and 24.7%), 10 h (29.2% and 21.8%) and 20 h (24.6% and 18.6%) respectively, but for 24-h exposure to heat shock there was no significant difference between the coa-A-treated group and the control group (15.2% and 14.2%) (Table 2).

Determination of the numbers of total cells and apoptotic cells per blastocyst

The total number of cells per Day 8 blastocyst was significantly higher ($P < 0.05$) in the 5 µM coa-A-treated group (143.1 ± 1.5) than that in the control group (131.4 ± 2.0) (Fig. 1 and Table 2). The number of apoptotic cells per Day 8 blastocyst was significantly lower ($P < 0.05$) in the 5 µM coa-A-treated group (6.4 ± 0.5) than that in the control group (10.8 ± 0.5) (Fig. 1 and Table 3).

Induction of HSP70 and PI3K through coa-A in heat stress conditions

The role of HSP70 in the development of blastocyst was analyzed through immunofluorescence analysis. The integral optical density (IOD) of HSP70 was significantly higher ($P < 0.05$) in the 5 µM coa-A-treated group than that in the control group (Fig. 2). In the same way, the expression of PI3K protein was analyzed and the IOD was higher ($P < 0.05$) in the 5 µM coa-A-treated group than that in the control group (Fig. 3).

Coa-A attenuates NF-κB and COX2 expression

Immunofluorescence analysis revealed that the IOD of NF-κB protein was lower ($P < 0.05$) in the 5 µM coa-A-treated group than that in the control group (Fig. 4). Similarly, it was also confirmed through confocal microscopy that the COX2 expression was significantly attenuated ($P < 0.05$) in the 5 µM coa-A-treated group than that in the control group (Fig. 5).

Immunofluorescence analysis of HSP70 and ROS (8-OxoG) in oocytes

The immunofluorescence analysis shows that the IOD of HSP70 in oocytes is significantly higher in the 5 µM coa-A-treated group than that in the control group (Fig. 6A). Similarly, the IOD of ROS (8-OxoG) in oocytes is lower in the 5 µM coa-A-treated group than that in the control group (Fig. 6B).

Figure 7 Relative mRNA expression profile of various genes (I–X) in control and coa-A treated blastocysts by real-time PCR. The symbol a shows the significant difference ($P < 0.05$) between the control and coa-A treated group.

mRNA expression of related genes

RT-PCR was performed to determine the expression levels of ten genes including heat stress-related gene *HSP70*, oxidative stress-related genes; *NF-κB*, *COX2*, *iNOS*, *PI3K*, pro-apoptotic genes; *P53*, *P21*, *caspase 3*, *BAX* and anti-apoptotic gene *BCL-2* and normalized against the housekeeping gene *GAPDH*. Expression levels of *HSP70*, *PI3K* and *BCL-2* were significantly higher ($P < 0.05$) in the 5 μM coa-A-treated group than that in the control group. However, the expression levels of *NF-κB*, *COX2*, *iNOS*, *P53*, *P21*, *caspase 3* and *BAX* were significantly lower in the 5 μM coa-A-treated group than that in the control group (Fig. 7).

Discussion

During summer, when the temperature increases from June to September, the fertility rate of cattle decreases (Hansen 1997). Heat stress reduces the developmental competence of oocytes, which leads to poor fertilization (Roth et al. 2001). Heat stress perturbs oocyte maturation,

Figure 8 Proposed pathway of coagulansin-A.

which leads to poor blastocyst development (Roth & Hansan 2004a). Here, we are reporting for the first time that 5 μM coa-A treatment in IVM stage improves blastocyst development in heat stress conditions of 40.5°C for 20 h. The blastocyst formation rate at 40.5°C after 20-h heat shock in the control group was 18.6% in the current study, which is in close agreement with a previous study (Roth & Hansan 2004b); however, addition of 5 μM coa-A to the IVM media significantly improved this rate to 24.6%.

The numbers of apoptotic and total cells are key factors for embryo quality; the higher the number of cells in an implanted embryo, the higher the chance of live offspring being delivered (van Soom et al. 2007). In the present study, coa-A treatment in IVM media at a heat stress of 40.5°C for 20 h improved blastocyst quality by increasing the total number of cells and decreasing the apoptotic index compared with the control group, which is in agreement with our previous findings at a normal culture temperature (Khan et al. 2016).

Heat shock proteins counterbalance the detrimental effects of any type of stress (Lindquist & Craig 1988). Among the heat shock proteins, HSP70 is very important, because its production is higher *in vitro* than *in vivo* due to the high level of stress *in vitro* (Sharma et al. 2012). HSP70 also protects cells during heat stress condition (Hendrey & Kola 1991) and works as a molecular chaperone in normal physiology (Ellis & van der Vies 1991). Heat shock induces apoptosis, which is blocked at various points by HSP70 (Mosser et al. 1997). We previously reported that the coa-A induces HSP70 in bovine, which improves blastocyst quality and the blastocyst formation efficiency at normal culture condition (Khan et al. 2016). In the present study, at heat stress condition, we also confirmed that coa-A improves

the blastocyst formation efficiency via induction of HSP70. This improvement of blastocyst through HSP70 induction is consistent with Matwee and coworkers (Matwee *et al.* 2001).

The PI3K lipid kinase pathway has a very important role in cell survival and is activated during mild oxidative stress (Datta *et al.* 1999, Sonoda *et al.* 1999). PI3K is also involved in growth factor-dependent survival of diverse types of cultured cells (Scheid *et al.* 1995, Ulrich *et al.* 1998, Fruman *et al.* 1999). In the current study, coa-A treatment increased the expression of PI3K compared with that in the control group, and this in turn improved the survival of blastocysts through the suppression of apoptosis, which is in agreement with a previous study (Yao & Cooper 1995).

NF- κ B gene was first identified as a transcription factor in 1986 (Sen & Baltimore 1986). NF- κ B has a critical role in all cellular processes (Hayden & Ghosh 2008). It is a multidirectional nuclear transcription factor that has roles in inflammation, oxidative stress and apoptosis (Brasier 2006, Gilmore 2006). NF- κ B is activated in the nucleus, but can be inhibited by I κ B inhibition in the cytoplasm. In the present study, coa-A improved blastocyst development in heat stress conditions by attenuating NF- κ B expression, which is in agreement with previous studies (Heyninck *et al.* 2014, Khan *et al.* 2016).

Inflammation in mammals is mediated and enhanced by various pro-inflammatory mediators, among which COXs are very important (Matheus *et al.* 2007). The COX family comprises COX1, COX2 and COX3. COX2 is the most important COX, induced in the cytoplasm and is involved in inflammatory and neoplastic processes (Thorat *et al.* 2013). In the current study, COX2 expression was attenuated in the 5 μ M coa-A-treated group compared with that in the control group. This decrease in COX2 expression is in agreement with a previous study (Yan *et al.* 2015).

All organisms respond to the environmental stress through heat shock proteins (Becker & Craig 1994). HSP70 is one of the most important heat shock protein, which is found on the acrosome of bovine spermatozoa and plays a role in oocyte and sperm interaction (Kamaruddin 1988). The mRNA microinjection of HSP70 gene to oocyte increased their resistance to heat stress (Hendrey & Kola 1991). In the present study, the coa-A protects the oocytes against the heat stress through the induction of HSP70 proteins, which is in agreement with a previous report (Hendrey & Kola 1991).

Reactive oxygen species (ROS) are very harmful in high concentration and damages the spermatozoa leading to male infertility (Iwasaki & Gagnon 1992). ROS and antioxidant should be balanced for normal embryo development (Patel *et al.* 2007). The 8-OxoG is a common marker for ROS detection (Floyd 1990). In the present study, we found that the 5 μ M of coa-A supplementation in IVM significantly reduces the ROS concentration in oocytes compared to control

group, which is parallel to our previous finding (Khan *et al.* 2016).

In the present study, the expression levels of HSP70, PI3K, NF- κ B, COX2, iNOS and BAX genes were investigated in the control and 5 μ M coa-A-treated groups. Supplementation of coa-A in heat stress conditions upregulated expression of HSP70, which is in agreement with a study by Wang and coworkers (Wang *et al.* 2012). The coa-A increased the expression of PI3K, and this improved the blastocyst quality by suppressing apoptosis, which is in agreement with a previous study (Yao & Cooper 1995). NF- κ B, COX2 and iNOS are very important genes, which favor inflammation and play a main role in cancer and neoplastic anemia (Karin *et al.* 2002, Thorat *et al.* 2013). Our results are in agreement with our previous findings (Khan *et al.* 2016, Zhao *et al.* 2016) because coa-A suppresses the expression of NF- κ B and COX2 as well as iNOS. The tumor suppressor P53 and cyclin-dependent kinase inhibitor P21 have main role in early apoptosis and cell cycle arrest (Ko & Prives 1996). Similarly, the BAX and caspase 3 are also very important and working as pro-apoptotic genes (Pang *et al.* 2016). In contrast, the BCL-2 is an anti-apoptotic gene that suppresses the apoptosis (Guénal *et al.* 1997). In the present study, we found that the 5 μ M coa-A significantly suppresses the expression of P53, P21, BAX and caspase 3 while induces the expression of BCL-2 and hence reduces the apoptosis, which is in agreement with Guénal and coworkers (Guénal *et al.* 1997).

In conclusion, as shown in Fig. 8, our results demonstrated that the heat shock (at 40.5°C) increases the ROS concentration, which results in the inflammation due to the induction of NF- κ B and their subsequent COX2 and iNOS, similarly the P53 and BAX are also induced by the ROS which compromise the blastocyst quality and efficiency. The 5 μ M coa-A when supplemented in the IVM media after a heat shock of 20 h at 40.5°C induced the HSP70 and PI3K while offset the ROS and block their subsequent pathway leading to improve the blastocyst development. Furthermore, although the current findings indicate the beneficial effect of coa-A against heat shock at 40.5°C, but we also observed that this effect of coa-A was completely diminished by the condition like high temperature (42°C). This shows that this beneficial effect of coa-A is limited specifically to heat shock 40.5°C.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0530>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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