Coagulansin-A has beneficial effects on the development of bovine embryos in vitro via HSP70 induction

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Synopsis

Coagulansin-A (withanolide) is the steroidal lactone obtained from Withania coagulans which belong to Solanaceae family. The present study investigated the effects of coagulansin-A on bovine oocyte maturation and embryo development in vitro. All these oocytes were aspirated from the ovaries obtained from Korean Hanwoo cows at a local abattoir. To determine whether coagulansin-A has beneficial effects on bovine oocyte maturation in vitro, 355 oocytes per group (control and treated) in seven replicates were subjected with different concentrations (1, 2.5, 5, 7.5 and 10 μM) of coagulansin-A. The coagulansin-A was added in the in vitro maturation (IVM) media followed by in vitro fertilization (IVF) and then in vitro culture (IVC). Only treatment with 5 μM coagulansin-A remarkably (P < 0.05) improved embryos development (Day 8 blastocyst) having 27.30 and 40.01% for control and coagulansin-A treated groups respectively. Treatment with 5 μM coagulansin-A significantly induced activation of heat shock protein 70 (HSP70) (P < 0.05). Immunofluorescence analysis revealed that 5 μM coagulansin-A treatment also significantly inhibited oxidative stress and inflammation during bovine embryo development in vitro by decreasing 8-oxoguanosine (8-OxoG) (P < 0.05) and nuclear factor-κB (NF-κB) (P < 0.05). The expressions of HSP70 and NF-κB were also confirmed through real-time PCR (RT-PCR). Additionally, the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay confirmed that coagulansin-A treatment significantly improved the embryo quality and reduced bovine embryo DNA damage (P < 0.05). The present study provides new information regarding the mechanisms by which coagulansin-A promotes bovine embryo development in vitro.

Key words: bovine embryo, coagulansin-A, DNA damage, heat shock protein 70 (HSP70), nuclear factor-κB (NF-κB).

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INTRODUCTION

In assisted reproductive technology (ART), in vitro maturation (IVM) is an extremely important step, followed by fertilization and embryo culture. For mammals, culture is normally performed in 5 % CO2 and 95 % air (20 % O2) [1]. The main difference between in vivo and in vitro environments is the O2 concentration, which is greater in the latter than in the former [2]. The increased O2 concentration leads to the production of large amounts of reactive oxygen species (ROS), which have detrimental effects on developing embryos [3]. These effects include DNA and protein damage and activation of various signalling pathways (P53 and P38), leading to apoptosis [4].

Withanolide are steroidal lactones obtained from Withania plants, which belong to the Solanaceae family. There are two

Abbreviations: ART, assisted reproductive technology; CDC, cumulus–oocyte complex; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP70, heat shock protein 70; IOD, integral optical density; IVC, in vitro culture; IVF, in vitro fertilization; IVM, in vitro maturation; NF-κB, nuclear factor-κB; 8-OxoG, 8-oxoguanosine; PVP polyvinylpyrrolidone; ROS, reactive oxygen species; RT-PCR, real-time PCR; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

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important Withania species, namely, Withania coagulans and Withania somnifera, which are native to Pakistan and India [5]. Withanolides containing a 14, 20-epoxide bridge are specific to W. coagulans. Hepatoprotective, antihyperglycaemic, anti-inflammatory, hypolipidaemic, free radical scavenging, cardio-vascular, antimicrobial, central nervous system depressant, cytotoxic, antitumour and immunomodulating activities have been studied in W. coagulans [6]. The variety of activities reported for the extracts, fractions and withanolides isolated from W. coagulans provide promising evidence for future research. Withanolides are also reported to have vital role against cancer and enhance the apoptosis in cancer cells as well as prevent tumorigenesis by inhibiting tumour necrosis factor-α (TNF-α) activated nuclear factor-κB (NF-κB) [5,7]. Clinical and animal research confirmed that withanolide has positive effects in the treatment of Alzheimer’s disease, Parkinson’s disease and dementia. It is also used as an anti-ulcer agent, a tonic for liver and an antioxidant due to its scavenging of ROS [8–10]. Derivatives of withanolide have antioxidant properties and reduce lipid peroxidation [11]. Withanolide also inhibits the activities of cyclooxygenase (COX)-1 and -2, which are involved in inflammation [12].

In the present study, we used coagulans-A isolated from the naturally growing W. coagulans, in ART for the first time to test its beneficial effects on bovine embryo development in vitro. For this purpose, different concentrations of coagulansin-A (1, 2.5, 5, 7.5 and 10 μM) were tested. Treatment with 5 μM coagulansin-A has more positive effects on embryo development than other concentrations and so we focused on this concentration in comparison with the control group.

MATERIALS AND METHODS

Reagents
All chemicals and reagents were obtained from Sigma–Aldrich, unless otherwise noted. Experiments were conducted in accordance with the Gyeongsang National University guidelines for the care and use of laboratory animals (approval no. GAR-110502-X0017).

Isolation and characterization of coagulansin-A
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. Total 10 kg of shade dried and crushed aerial parts without fruits (leaves and stems only) were taken and macerated in 30 litres of solvent for 3 days by occasional shaking. 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 (Figure 1) [5].

Experimental design
The ROS-scavenging compound coagulansin-A was added to IVM media in order to overcome the effects of ROS. Embryos were divided into various groups that have 1, 2.5, 5, 7.5 and 10 μM coagulans-A concentration in treatment 1, treatment 2, treatment 3, treatment 4, treatment 5 and control group (without treating compound) respectively. From these different concentrations 5 μM coagulansin-A was selected as an optimal concentration for further study. Real-time PCR (RT-PCR), immunofluorescence technique and TUNNEL were used for developmental analysis in control and coagulansin-A treated groups. The effects of coagulansin-A were determined at a normal culture temperature (38.5°C).

Cumulus–oocyte complex recovery
Ovaries of Korean native cows (Hanwoo) were collected at a local abattoir, placed in physiological saline (0.9% sodium chloride) at approximately 35°C and transported to the laboratory within 2 h after slaughter. Ovaries were washed in fresh Dulbecco’s PBS (D-PBS) and cumulus–oocyte complexes (COCs) were recovered as described by Deb et al. [13]. In brief, COCs were recovered from follicles with a diameter of 2–8 mm using an 18-gauge needle attached to a vacuum pump. Aspirated fluid was expelled into 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, 2.0 mM calcium chloride, 10 mM HEPES, 1 μM/μl 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 homogeneous cytoplasm were selected. COCs were washed three times in TL-HEPES medium.

In vitro maturation
Oocytes were cultured in maturation medium as described by Deb et al. [13]. In brief, COCs were washed three times in maturation medium (TCM-199) supplemented with 10% (v/v) FBS, 1 mg/ml oestradiol-17β, 10 mg/ml follicle-stimulating hormone, 0.6 mM cysteine and 0.2 mM sodium pyruvate, and then transferred to a 4-well plate containing 700 ml of IVM media for 22–24 h at 38.5°C in a humidified atmosphere of 5% CO2 in air.

In vitro fertilization and in vitro culture
In vitro matured COCs were fertilized with frozen-thawed bovine sperm as described by Deb et al. [13]. Semen was thawed at 39°C for 1 min and sperm were washed and pelleted in D-PBS by centrifugation at 750 × g for 5 min at room temperature. The pellet was diluted with 500 μl of heparin (20 μg/ml) prepared in in vitro fertilization (IVF) medium (Tyrodes lactate solution supplemented with 6 mg/ml BSA, 22 mg/ml sodium pyruvate, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) and incubated at 38.5°C in a humidified atmosphere of 5% CO2 in air for 15 min (to facilitate capacitation). Thereafter, sperm were diluted in IVF
medium (final density of $1 \times 10^6$ sperm/ml). 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 zygotes were placed in 700 μl of CR1-aa medium [14] supplemented with 44 μg/ml sodium pyruvate, 14.6 μg/ml glutamine, 10 μg/ml penicillin–streptomycin, 3 mg/ml BSA and 310 μg/ml glutathione for 3 days. Presumed zygotes were then cultured until Day 8 of embryonic development (Day 0 = day of IVF) in medium of the same composition, except that BSA was replaced with 10 % (v/v) FBS. 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 (Figure 3).

Terminal deoxynucleotidyl transferase dUTP nick-end labelling
Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) was performed according to the manufacturer’s protocol using an In Situ Cell Death Detection Kit (Roche Diagnostics Corp.). Briefly, fixed embryos (n = 68) were washed twice with 0.3 % (w/v) polyvinylpyrrolidone (PVP) prepared in 1 M PBS (PVP–PBS) before being permeabilized [0.5 % (v/v) Triton X-100 and 0.1 % (w/v) sodium citrate] for 30 min at room temperature [13]. After permeabilization, embryos were washed twice with PVP–PBS and incubated in the dark with fluorescently conjugated terminal deoxynucleotidyl transferase dUTP for 1 h at 37°C. TUNEL-stained embryos were then washed with PVP–PBS and incubated in 10 μg/ml Hoechst 33342 for 10 min. After washing with PVP–PBS, blastocysts were mounted on to glass slides and their nuclear configuration was analysed. The number of cells per blastocyst was determined by counting Hoechst-stained cells under an epifluorescence microscope (Olympus IX71) equipped with a mercury lamp. TUNEL-positive cells were bright red, indicating the occurrence of apoptosis.

Immunofluorescence analysis
Bovine embryos were fixed in 4 % paraformaldehyde solution. Fixed embryos were washed twice with 0.01 M PBS for 10 min. Embryos were incubated in proteinase K solution at 37°C for 5 min. After washing with PBS, embryos were incubated in blocking solution (D-PBS containing 5 % non-immune goat serum and 0.3 % Triton X-100) for 1 h. Embryos were incubated overnight with primary antibodies including mouse-derived anti-heat shock protein 70 (HSP70), anti-NF-κB (Santa Cruz Biotechnology) and anti-8-oxoguanosine (8-OxoG; Millipore, Santa Cruz Biotechnology), 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 antifade reagent (Molecular Probes). All stained embryos were examined using a confocal laser-scanning microscope (Flouview FV 1000, Olympus).

mRNA extraction and cDNA reverse transcription
The quantitative RT-PCR was performed according to Deb et al. [13]. In brief, mRNA was extracted from Day 8 blastocysts using a Dynabeads mRNA direct kit (Dynal AS). Embryos were re-suspended in 100 μl lysis buffer and vortexed at room temperature for 2 min. Pre-washed Dynabeads oligo (dT; 20 μl) were mixed with lysate and annealed by rotating for 3 min at...
RESULTS

Cleavage and developmental rates of embryos generated from oocytes treated with various concentrations of coagulansin-A

The percentage of cleaved embryos was determined on Day 3 of culture. The cleavage rate was greater in the group treated with 5 μM coagulansin-A (90.31%) than in the control group (86.75%), but this was not statistically significant. The percentage of Day 8 blastocysts was greater (\( P < 0.05 \)) in the 5 μM coagulansin-A treated group (40.01%) than in the 1, 2.5, 7.5 or 10 μM coagulansin-A groups and the control group (32.56, 35.12, 20.7, 15.59 and 27.30% respectively) (Figure 2).

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

The total number of cells per Day 8 blastocyst was greater (\( P < 0.05 \)) in the 5 μM coagulansin-A group (150.1 ± 3.624) than in the control group (127.7 ± 4.161) (Table 2 and Figure 3). The number of apoptotic cells per Day 8 blastocyst was lower (\( P < 0.05 \)) in the 5 μM coagulansin-A group (5.667 ± 0.2873) than in the control group (7.400 ± 0.3754) (Table 2 and Figure 3).

Coagulansin-A-induced activation of HSP70 in bovine embryo development

Among the three concentrations of coagulansin-A tested, only 5 μM had beneficial effects on bovine embryos (Table 3). We performed immunofluorescence to analyse the possible involvement of HSP70 induction in the mechanism by which coagulansin-A affects embryo development. This indicated that integral optical density (IOD) of HSP70 was significantly greater (\( P < 0.05 \)) in the 5 μM coagulansin-A group than in control group (Figure 4).

Statistical analysis

Results are expressed as percentages (%). Transformed data and total cell numbers per blastocyst were analysed using Prism 5. Significant differences between groups were detected using the simple \( t \) test and one-way ANOVA. \( P < 0.05 \) was considered significant.
Table 1: Forward and reverse primer pairs designed for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Accession no.</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70</td>
<td>F: GAGTCGTACGCCCTCAAAT</td>
<td>U09861</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>R: ACTTGTCCAGACCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>F: TGGCGGAATTACCTTAC</td>
<td>DQ464067</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>R: CATCACCTGTGCAACACCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AATTTGAAGGCCACCTC</td>
<td>NM173979</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>R: TGTACAGGAAAGCCTGACT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Characteristics of Day 8 blastocysts in the two groups*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of blastocysts examined</th>
<th>Total no. of cells per blastocyst</th>
<th>No. of apoptotic cells per blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>127.7 ± 4.161^a</td>
<td>7.400 ± 0.3754^a</td>
</tr>
<tr>
<td>5 μM coagulansin-A</td>
<td>15</td>
<td>150.1 ± 3.624^b</td>
<td>5.667 ± 0.2873^b</td>
</tr>
</tbody>
</table>

* Total number of cells and number of apoptotic cells per blastocyst are shown (mean ± S.E.M.). ^a,b^ Values with different superscripts in the same column are significantly different (P < 0.05).

Table 3: Cleavage and developmental rates of embryos generated from oocytes treated with various concentrations of coagulansin-A

<table>
<thead>
<tr>
<th>Coagulansin-A concentration (μM)</th>
<th>Total no. of oocytes</th>
<th>No. of presumed zygotes</th>
<th>No. of cleaved embryos (%)</th>
<th>No. of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>355</td>
<td>317</td>
<td>275 (86.75)</td>
<td>90 (27.30)^a</td>
</tr>
<tr>
<td>1</td>
<td>355</td>
<td>310</td>
<td>279 (90.00)</td>
<td>102 (32.56)^bd</td>
</tr>
<tr>
<td>2.5</td>
<td>355</td>
<td>316</td>
<td>267 (84.49)</td>
<td>111 (35.12)^ab</td>
</tr>
<tr>
<td>5</td>
<td>355</td>
<td>320</td>
<td>289 (90.31)</td>
<td>120 (40.01)^a</td>
</tr>
<tr>
<td>7.5</td>
<td>355</td>
<td>318</td>
<td>239 (75.15)</td>
<td>66 (20.7)^c</td>
</tr>
<tr>
<td>10</td>
<td>355</td>
<td>314</td>
<td>228 (72.61)</td>
<td>50 (15.59)^c</td>
</tr>
</tbody>
</table>

^a,b,c,d^ Values with different superscripts are significantly different (P < 0.05).

Figure 3: Representative images of bovine embryos stained with Hoechst 33342

Apoptotic cells were identified by TUNEL. The corresponding images were merged. (A–C) Control group. (A’–C’) Group treated with 5 μM coagulansin-A. Scale bar, 100 μm. Apoptotic cells are indicated by yellow arrows. The graph shows the number of apoptotic cells per embryo. Columns with different superscripts are significantly different (P < 0.05).
Coagulansin-A reduces NF-κB protein expression during bovine embryo development

The expression level of NF-κB protein was investigated by performing immunofluorescence analysis. Expression (IOD) of NF-κB protein was greater in control embryos than the coagulansin-A embryos, because treatment with 5 μM coagulansin-A induced HSP70 activation which inhibited NF-κB protein expression during embryo development (Figure 5).

Coagulansin-A reduces oxidative stress by inhibiting 8-OxoG expression during bovine embryo development

The 8-OxoG is a well-known marker of oxidative stress. To investigate whether coagulansin-A treatment reduces the level of ROS in bovine embryo development, we performed immunostaining of 8-OxoG. Surprisingly, expression (IOD) of 8-OxoG was significantly lower in the 5 μM coagulansin-A group than in control group, which suggests that coagulansin-A inhibits oxidative stress by reducing the expression level of 8-OxoG (Figure 6).

Gene expression profile in control and coagulansin-A treated groups

The expression of two candidate genes HSP70 and NF-κB were also confirmed through RT-PCR. Expression amounts were normalized against a housekeeping gene, GAPDH. The expression of HSP70 is greater in coagulansin-A treated group than control group. Similarly the expression of NF-κB was reduced by coagulansin-A than control group (Figure 7).

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**Figure 4** Confocal microscopy, images of bovine embryos in vitro

(A and B) Control group. (C and D) Coagulansin-A treated group. Columns with different superscripts are significantly different ($P < 0.05$).

**Figure 5** Confocal microscopy

(A and B) Control groups. (C and D) Coagulansin-A treated groups. Columns with different superscripts are significantly different ($P < 0.05$).
Coagulansin-A improves bovine embryo development

Figure 6  Confocal microscopy of 8-OxoG
(A and B) Control embryos. (C and D) Coagulansin-A treated embryos. Columns with different superscripts are significantly different ($P < 0.05$).

Figure 7  Relative mRNA expression levels in control and coagulansin-A treated blastocysts by RT-PCR.

**DISCUSSION**

The selection of competent oocytes is extremely important for assisted reproductive technologies (ARTs). A number of studies reported that embryonic development depends upon the culture conditions and oocyte quality [15,16]. In this regard one study also reported that the blastocyst development can also be improved by decreasing the O$_2$ concentration of the incubator [17]. We reporting for the first time that coagulansin-A supplementation in ART have beneficial effects on embryo development in vitro. In the present study we found that among the different concentrations only 5 μM of coagulansin-A treatment more significantly improve the blastocyst development (40.01 %) than the control group (27.30 %). Consistent with a previous report [18], there was no significant difference in the cleavage rate between the treated group and control group embryos.

Embryo quality is a key factor for successful embryo implantation [19]. Both the cell number and apoptosis play an important role in embryo quality because an increase in apoptosis significantly reduces the total cell number in blastocysts and reduces blastocyst quality [20]. In the present study, the total number of cells per blastocyst was greater in the coagulansin-A treated group than in the control group. Similarly, the number of apoptotic cells per blastocyst was lower in the coagulansin-A treated group than the untreated group (control group). We propose that the improvement in embryo quality is due to coagulansin-A-induced HSP70 activation, which improved the blastocyst quality.

Heat shock proteins act as cellular antioxidants [21]. Most cells produce these proteins, which belong to the HSP70 family [22].
HSP70 protects cells from adverse effects in stress conditions [23,24] and functions as a molecular chaperone in the absence of stress [25,26]. HSP70 also worked as a defensive protein against ROS during the inflammation [27]. The HSP70 level increases as bovine embryos develop in vitro [28]. In the present study we found that the coagulansin-A induced the HSP70 expression which is in agreement with a previous report [7], and this protein after inducing in the culture system improves the embryo quality and efficiency.

The NF-κB pathway plays an important role in many developmental and cellular processes [29]. NF-κB is activated at specific stages of murine spermatogenesis and induces the transcription of various genes in testes [30]. NF-κB activation is also extremely important for the development of mouse embryos beyond the 2-cell stage [31]. In murine and bovine oocytes, changes in NFκBIA and IKBα occur, which are related to aging [32,33]. Similarly, NFκB pathway activation is also involved in various inflammatory diseases [34]. In the present study treatment of coagulansin-A in IVM medium reduces the activation of NFκB pathway in bovine embryo, which is in agreement with a previous report [35].

For embryo development, the balance between ROS and scavengers (antioxidants) is extremely important [36]. ROS causes the dysfunction of DNA, RNA and proteins [37], and affect sperm and oocyte fusion [38]. The ROS also causes the induction of inflammation as it activates and induces NFκB pathway [39]. The 8-OxoG is produced as a result of ROS and is commonly used to determine the ROS concentration [40]. Currently we found that the expression of 8-OxoG was greater in control embryos than coagulansin-A treated embryos, indicative of increased ROS concentration in control than treated group.

In previous literature it is already reported that culture condition effect the expression level of various genes of blastocyst [41,42]. In the present study supplementation of the IVM medium with coagulansin-A increases the expression of HSP70 which is in agreement with [7]. NFκB transcription factor is activated by ROS [39]. In the present study, we also found that the HSP70 induction by coagulansin-A reduces the ROS which ultimately down-regulate the expression of NFκB transcription factor which is in agreement with [39].

We conclude that addition of coagulansin-A, a steroidal lactone, to IVM media induces HSP70 expression in bovine embryos, which reduces the ROS concentration, inhibits NFκB activation and improves embryo development. When NFκB is down-regulated, inflammatory markers such as inducible nitric oxide synthase, COX-2 and Toll-like receptor 4 are also down-regulated which needs further confirmation.

**AUTHOR CONTRIBUTION**

All the authors contributed to this manuscript; Imran Khan and Kyeong-Lim Lee designed and performed the experiments, Md. Fakruzzaman and Seok-Hwan Song analysed the data, Ihsan-ul-Haq and Bushra Mirza isolate and provide compound (coagulansin-A), Chang Yan checked the revised manuscript and Il-Keun Kong wrote the manuscript and manage this work.

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