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Glycyrrhizic acid improves fertilization and embryonic development of porcine oocytes fertilized *in vitro*: focus on HMGB proteins in embryo development

Malavi Arachchi Gamage Nayodya Dananjanee Malavi Arachchi Gamage[1], Wijesooriya Mudhiyanselage Nadeema Dissanayake[1,2], Sang-Myeong Lee[3], Young-Joo Yi[1*]

[1]*Department of Agricultural Education, College of Education, Sunchon National University, Suncheon 57922, Korea*
[2]*Department of Animal Science and Biotechnology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 34134, Korea*
[3]*Laboratory of Veterinary Virology, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea*

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***Corresponding author**
Young-Joo Yi
Department of Agricultural Education, College of Education, Sunchon National University, Suncheon 57922, Korea
Tel: +82-61-750-3352
E-mail: yiyj@scnu.ac.kr

ORCID
Malavi Arachchi Gamage Nayodya Dananjanee Malavi Arachchi Gamage
https://orcid.org/0009-0009-3194-6548
Wijesooriya Mudhiyanselage Nadeema Dissanayake
https://orcid.org/0000-0001-7130-7737
Sang-Myeong Lee
http://orcid.org/0000-0002-3624-3392
Young-Joo Yi
http://orcid.org/0000-0002-7167-5123

Abstract

High-mobility group box-1 (HMGB1) is a conserved nuclear protein that stabilizes the nucleosome and regulates gene transcription. Recent studies have reported that glycyrrhizic acid (GA), a HMGB1 inhibitor, blocks extracellular HMGB1 cytokine activity and has a protective effect in various diseases. This study was performed to investigate the effect of GA on *in vitro* fertilization (IVF) and embryo culture of pig oocytes. HMGB1 was detected by immunofluorescence in the boar sperm post-acrosomal sheath and sperm tail mid-piece, as well as in the nucleus of immature, germinal vesicle stage porcine oocytes, the cytoplasm of mature metaphase II oocytes, and in the embryonic nuclei. A related protein, HMGB2, was also observed in spermatozoa and oocytes, co-localizing with HMGB1. Both HMGB1 and HMGB2 were detected in the protein extracts of spermatozoa and oocytes by Western blotting. Total fertilization rates (mono and polyspermic) increased, and more spermatozoa were bound to the zona pellucida of the oocytes when the IVF medium was supplemented with 20 μM GA compared to the control ($p<0.05$). In the presence of 20 μM GA, there was a significant increase in the percentage of cleaved embryos, blastocyst formation, and the mean cell numbers per blastocyst ($p<0.05$). GA treatment increased porcine fertilization rates and improved embryo development *in vitro*, possibly by blocking the cell-survival-limiting activities of HMGB proteins. Thus, GA could be a suitable therapeutic candidate in assisted reproductive technologies.

Keywords: HMGB1 protein; spermatozoa; embryonic structures; fertilization *in vitro*; pig

INTRODUCTION

High-mobility group box-1 and 2 (HMGB1 and HMGB2) are non-histone nuclear proteins belonging to the HMG superfamily that help regulate various genomic operations, including DNA

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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Ethics Approval
Not applicable.

repair, transcription, and nucleosome sliding [1–3]. These two proteins are highly expressed in all mammalian tissues during embryogenesis. In specific tissues, they have been found both inside and outside the nucleus, with the testis and ovary displaying higher levels in the nucleus and cytoplasm [4–6]. HMGB1 initiates inflammatory responses when it interacts with cell surface receptors, such as the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4), to influence the levels of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α [7, 8]. Extracellular HMGB2 promotes the proliferation and migration of endothelial cells and influences these functions by engaging RAGE [9]. The porcine species serves as a distinctive model for conducting research in the field of human developmental biology and assisted reproductive technology, such as *in vitro* fertilization (IVF), and has been extensively employed in investigations pertaining to porcine reproduction and development of early-stage embryos [10]. Developing effective *in vitro* methods for generating porcine embryos has been challenging due to the frequent occurrence of polyspermic fertilization, which is more common in pigs than in other species [11]. Glycyrrhizin (or glycyrrhizic acid [GA]) is a triterpene glycoside, the primary constituent derived from the licorice root (*Glycyrrhiza uralensis*). It has been reported that GA inhibits the Ca^{2+}- and phospholipid-dependent phosphotransferase activity of protein kinase C (PKC), the phorbol ester tumor promoter receptor. GA has also been found to exhibit various pharmacological effects, including anti-inflammatory, anti-ulcer, anti-allergic, anti-carcinogenic, and immunomodulatory actions [12]. In addition, GA has been demonstrated to be a natural inhibitor of extracellular HMGB1 [13, 14]. Previous studies have reported that the addition of an aqueous extract of licorice to the artificial insemination (AI) culture medium increases the IVF rates in mouse oocytes [15]. Therefore, the purpose of this study was to investigate how GA affects the fertilization and early development of porcine oocytes fertilized *in vitro*, specifically focusing on the potential involvement of the HMGB1 protein in the pre-implantation stage of embryo development.

MATERIALS AND METHODS

Preparation of boar spermatozoa

Liquid boar semen was purchased from a local AI center and stored at 17°C for 5 days prior to use.

Collection and *in vitro* maturation of pig oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (3–6 mm in diameter), washed three times in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered Tyrode lactate (TL-HEPES) medium supplemented with 0.01% (w/v) polyvinyl alcohol (PVA; TL-HEPES-PVA), followed by three washes with the oocyte maturation medium [16]. A total of 50 COCs were transferred to 500 μL of the maturation medium and layered with mineral oil in a 4-well multi-dish equilibrated at 38.5°C and 5% CO_2 in air.

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Tissue culture medium (TCM)199 was used as the oocyte maturation medium. It was supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/mL luteinizing hormone (Sigma-Aldrich, Seoul, Korea), 0.5 μg/mL follicle-stimulating hormone (Sigma-Aldrich), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 75 μg/mL penicillin G, and 50 μg/mL streptomycin. The oocytes were cultured in TCM199 for 44 hr at 38.5°C and 5% CO_2 in air.

In vitro fertilization and culture of pig oocytes

After *in vitro* maturation (IVM), the cumulus cells were removed by treatment with 0.1% hyaluronidase in the TL-HEPES-PVA medium, and metaphase II oocytes (MII) were selected by observation under a stereomicroscope. Thereafter, oocytes were placed into four 100 μL drops of modified Tris-buffered medium (mTBM) in a 35-mm polystyrene culture dish and covered with mineral oil. One milliliter of liquid semen stored in Beltsville Thawing Solution was washed twice with phosphate-buffered saline (PBS) containing 0.1% PVA (PBS-PVA), at 800 × g for 5 min. The washed spermatozoa were resuspended and diluted in mTBM. For IVF, 1 μL of the sperm suspension was added to the medium containing the oocytes to give a final sperm concentration of 1×10^5 spermatozoa/mL. Oocytes were co-incubated with spermatozoa for 5 hrs at 38.5°C and 5% CO_2 in air. After IVF, oocytes were transferred to 500 μL porcine zygote medium (PZM-3) [17] supplemented with 0.4% bovine serum albumin (BSA, A0281, Sigma-Aldrich), and cultured for an additional 20, 48, or 144 hr. To observe the effects of GA on IVF and *in vitro* culture (IVC), various concentrations of GA were added into the mTBM (0–200 μM GA) or PZM (0–100 μM GA), respectively. GA (Glycyrrhizic acid ammonium salt G2137, Sigma-Aldrich) was dissolved in DMSO for use, and the addition amount was limited to no more than 0.5% (v/v) in both IVF and IVC media. The IVM, IVF, and IVC studies were repeated four times for each treatment regimen.

Evaluation of fertilization and embryonic development

Oocytes/embryos were fixed with 2% formaldehyde for 40 min at room temperature (RT), washed twice with PBS, permeabilized with PBS-Triton X-100 for 30 min, and stained with 2.5 mg/mL 4′,6-diamidino-2-phenylindole (DAPI; DNA staining; Molecular Probes, Eugene, OR, USA) for 40 min. The fertilization status of the zygotes (unfertilized, fertilized-monospermic, or fertilized-polyspermic), cleaved embryo number, blastocyst formation, and the cell number per blastocyst were assessed under a fluorescence microscope (Nikon Eclipse Ci microscope; Nikon Instruments, Tokyo, Japan).

Immunofluorescence

Spermatozoa, oocytes, embryos, or blastocysts were fixed with 2% formaldehyde for 40 min at RT, washed with PBS, followed by permeabilization in PBS and 0.1% Triton-X 100 (PBS-TX) for 40 min at RT. The membrane blocking was done by PBS-TX containing 5% normal goat serum for 20 min. Subsequently, the fixed cells were incubated with rabbit monoclonal anti-HMGB1 antibody (ab79823, Abcam, Cambridge, UK) or rabbit monoclonal an-

ti-HMGB2 antibody (1:1,000 dilutions, ab124670, Abcam) for 40 min at RT. After washing with PBS-TX, the cells were incubated with goat anti-rabbit IgG Alexa Fluor™ 488 (A-11008, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 40 min at RT. Spermatozoa were incubated with 5 μM MitoTracker™ Red CMXRos (Thermo Fisher Scientific) for mitochondria labeling, and the DNA was stained with DAPI. All immunofluorescence signals were visualized in a fluorescence microscope, and images were captured using a fluorescence microscope (Nikon Instruments).

Western blot

Total protein was extracted from the testis samples using lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 5.0 Mm sodium pyrophosphate, 1 g/mL leupeptin, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) on ice, and protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were subsequently separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in tris-buffered saline containing Tween-20 for 1 hr at RT, and then incubated with rabbit monoclonal anti-HMGB1 antibody, rabbit monoclonal anti-HMGB2 antibody (1:1,000 dilutions, Abcam) at 4°C overnight. Blots were incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) secondary antibody (#31460, Thermo Fisher Scientific) for 1 hr at RT. The immunoreactive bands were detected by enhanced chemiluminescence detection reagents (#34096, SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific) using the Davinch-Chemi Fluoro imaging system (Davinch-K, Seoul, Korea).

Statistical analysis

Values are presented as the mean ± S.E.M. Data analyses were conducted using one-way analysis of variance (ANOVA) with the SAS package 9.4 (SAS Institute, Cary, NC, USA). A completely randomized design was used, and Duncan's multiple range test was performed to compare the values of individual treatments when the *F*-value was significant ($p<0.05$).

RESULTS

Localization of high-mobility group box-1 and high-mobility group box-2 in germ cells by immunofluorescence

Visualization with immunofluorescence microscopy provided a detailed view of the localization of HMGB1 (Fig. 1). HMGB1 was observed in boar spermatozoa, including the head post-acrosomal sheath and the mid-piece of the sperm tail (white arrows in Fig. 1A). Accumulation of HMGB1 could be observed in the nuclear region of immature oocytes (germinal vesicle [GV] in Fig. 1B), and it migrated into the cytoplasm of the oocyte during the mature metaphase II (MII) stage (Fig. 1C). HMGB1 was seen in the zygotic pronuclei (PN in Fig.

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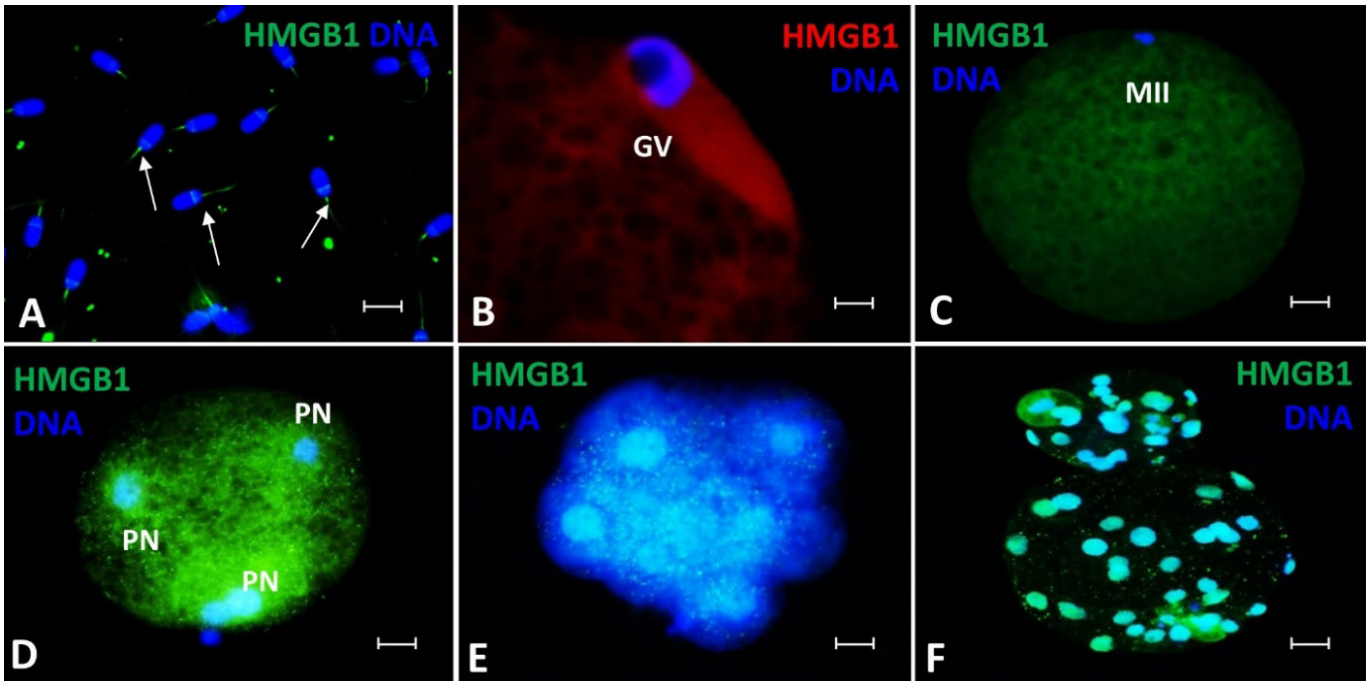

Fig. 1. High-mobility group box-1 (HMGB1) was localized in the boar sperm head post-acrosomal sheath and sperm tail mid-piece (A; white arrows), as well as in the nucleus/germinal vesicle (GV) of immature porcine oocytes (B), the cytoplasm of metaphase II (MII) oocytes (C), in the zygotic pronuclei (PN; D), and in the embryonic (E) and hatched blastocyst nuclei (F). Immunofluorescence for HMGB1 and DAPI staining for DNA. Scale bar = 50 μm.

1D), embryonic stage (Fig. 1E), and hatched blastocyst nuclei, respectively (Fig. 1F). Particularly, in pronuclear formation stage, strong expression of HMGB1 was observed in both the pronuclei and cytoplasmic regions (Fig. 1D). Subsequently, cytoplasmic expression decreased (Fig. 1E), but its localization was again clearly identified in both the nuclei and cytoplasm of pre-implantation blastocysts (Fig. 1F).

As shown in Fig. 2A, HMGB2 was detected in the post-acrosomal sheath and tail of spermatozoa. In the germinal vesicle breakdown (GVBD) stage, abundant HMGB2 was visualized in the nucleus (Fig. 2B). Similarly, it was strongly expressed in the cytoplasm after the maturation of the oocytes (Fig. 2C). After IVF, HMGB2 localization was detected in the pronucleus (Fig. 2D), the zygote nucleus (Fig. 2E), and the nuclei of pre-implantation embryonic cells (Fig. 2F). Similar to HMGB1 localization, HMGB2 showed strong expression in the pronuclei and cytoplasmic regions during the post-fertilization pronuclear formation stage (Fig. 2D). Cytoplasmic expression decreased somewhat (Fig. 2E), but HMGB2 localization was again clearly observed in the nuclei and cytoplasm of pre-implantation blastocysts (Fig. 2F).

Identification of high-mobility group box-1 and high-mobility group box-2 in spermatozoa and oocyte extracts by Western blot analysis

Western blot analysis was performed using antibodies against HMGB1 and HMGB2 on protein extracts from the spermatozoa and oocytes. HMGB1 and HMGB2 were detected at approximately 25 kDa in both spermatozoa and oocytes. At the upper part of the band, protein degradation was observed in the complex binding form of HMGB1 and HMGB2 (Fig. 3).

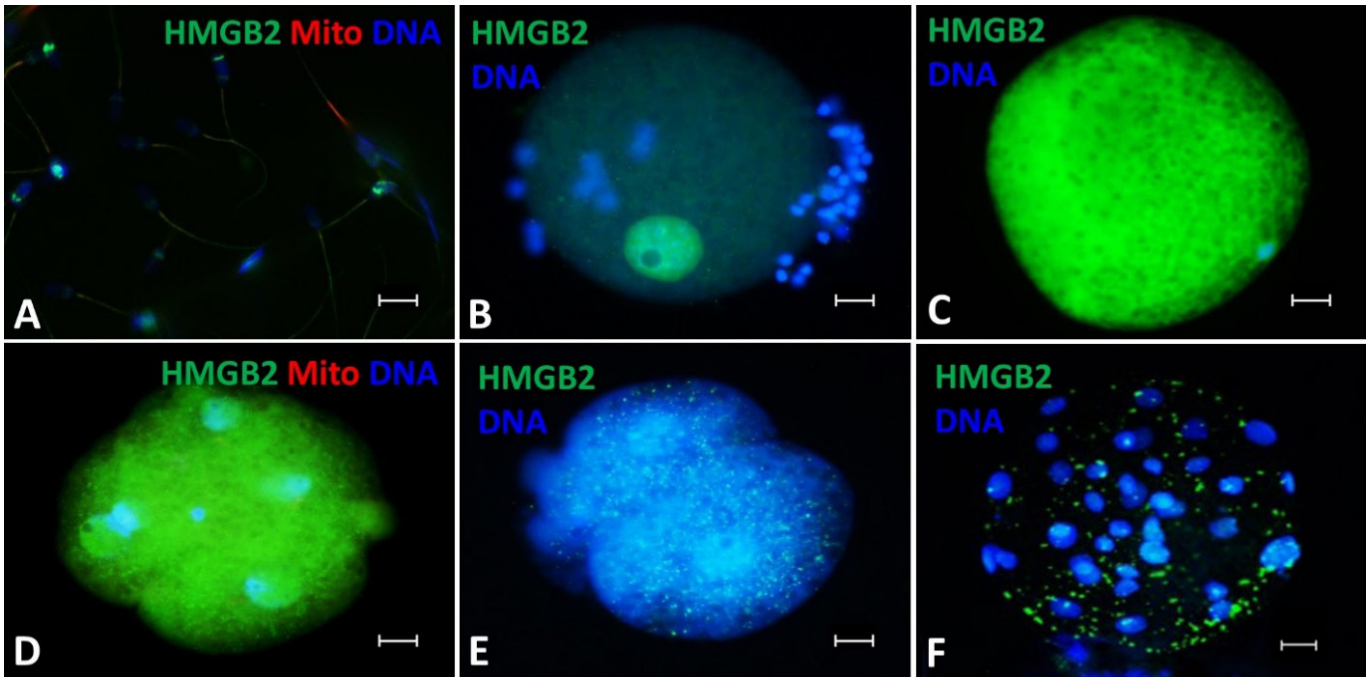

Fig. 2. High-mobility group box-2 (HMGB2), a related protein, was also detected in the boar sperm head post-acrosomal sheath and sperm tail mid-piece (A), as well as in the nucleus of germinal vesicle breakdown (GVBD) stage of porcine oocytes (B), the cytoplasm of metaphase II (MII) oocytes (C), in pronuclei (PN; D), and in embryonic (E) and blastocyst (F) nuclei. Immunofluorescence for HMGB2, MitoTracker dye for mitochondria, and DAPI staining for DNA. Scale bar = 50 μm.

Effect of glycyrrhizic acid on porcine oocyte fertilization *in vitro*

To determine the fertilization rates, the oocytes were subjected to insemination with spermatozoa in the presence of GA for 5 hr, followed by a 20 hr culture period (Fig. 4A). Additionally, one hr after IVF, other batches of oocytes were fixed and stained using DAPI to count the number of spermatozoa bound to the zona pellucida (ZP; Fig. 4B). In Fig. 5A, the total fertilization rate can be observed, which encompasses both monospermic and polyspermic oocytes. There was no significant difference in the rate of normal fertilization (monospermic oocytes; 18.6%–40.7%), while the polyspermy rate increased significantly in oocytes fertilized in the presence of 20 μM GA (70.3%, $p<0.05$) compared to oocytes fertilized with (50–200 μM GA) or without GA (24.8%–63.2%, Fig. 4A). Consequently, a higher rate of total fertilization was observed in oocytes fertilized with 20 μM GA (91.1%, $p<0.05$, Fig. 4A). After co-culturing the sperm and oocytes in medium with (20–200 μM GA) or without GA for 1 hr, the number of sperm attached to the ZP of the oocytes was counted. The results showed that a higher number of sperm bound to the oocytes in the medium containing 20 μM GA (326.5 vs. 203.3–253.8, $p<0.05$, Fig. 4B).

Effect of glycyrrhizic acid on the embryonic development *in vitro*

After IVF, the fertilized oocytes were subjected to culture in the PZM-3 medium in the absence or presence of various concentrations of GA (10–100 μM) for 48 and 144 hr (Fig. 5). The percentage of cleaved embryos was significantly higher in fertilized oocytes cultured in the presence of 20 μM GA (78.8%, $p<0.05$) than those in fertilized oocytes cultured with (10,

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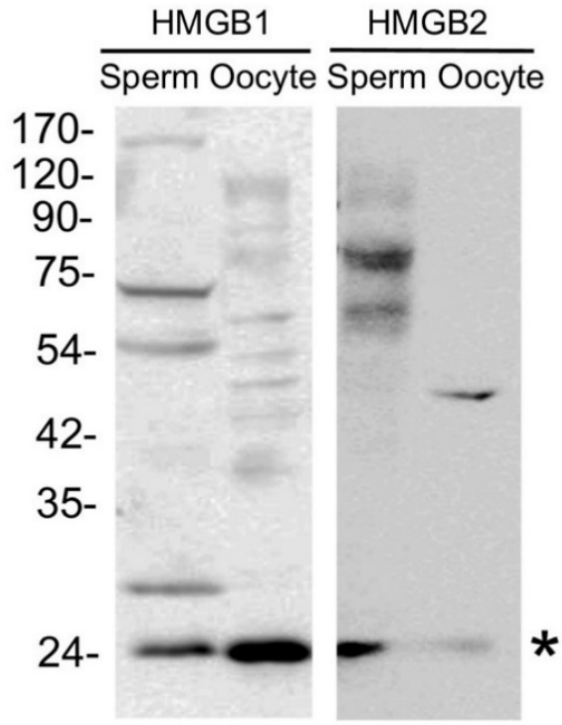

Fig. 3. High-mobility group box-1 and 2 (HMGB1 and HMGB2) were detected in protein extracts of spermatozoa and oocytes by Western blot analysis (*predicted band size 25 kDa).

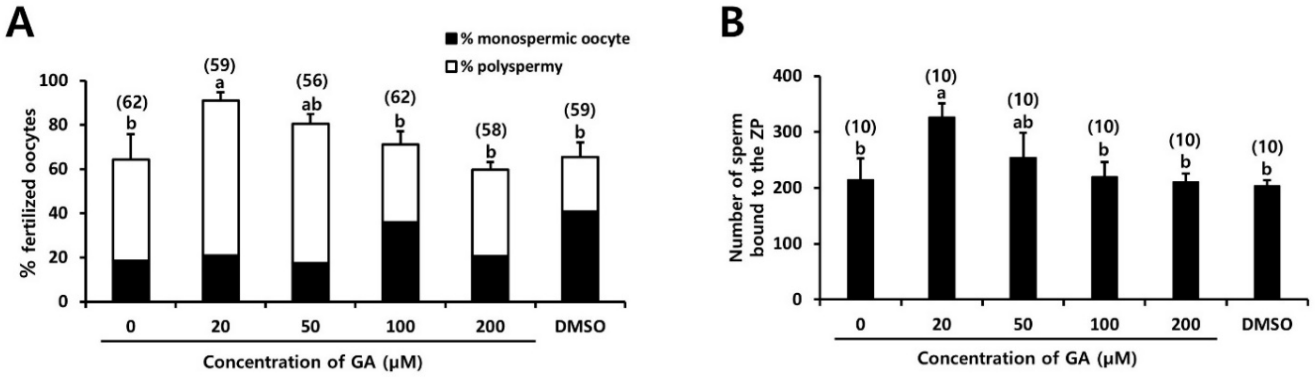

Fig. 4. Effect of glycyrrhizic acid (GA) on porcine oocyte fertilization *in vitro*. Porcine oocytes were fertilized in the presence of GA for 5 hr and then cultured for 20 hr (A). One hour after IVF, other batches of oocytes were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) to count the number of spermatozoa bound to the zona pellucida (ZP; B). Each experiment was repeated 5 times. Data is expressed as the mean ± S.E.M. The numbers on each column indicate the number of oocytes. [a,b] Superscripts denote significant differences (p<0.05). IVF, *in vitro* fertilization.

50 and 100 μM GA) or without GA (49.8%–69.5%, Fig. 5A). The blastocyst formation rate was also significantly higher in the fertilized oocytes cultured in PZM-3 medium containing 20 μM GA (26.5%, p<0.05) than in the other groups (7.0%–20.5%, Fig. 5A). A higher mean cell number was observed in the blastocyst in the presence of 20 μM GA (43.3 vs. 28–35.2, p<0.05, Fig. 5B). These results indicate that the addition of 20 μM of GA in the culture medium may have a significant influence on cleavage, blastocyst formation, and the average cell

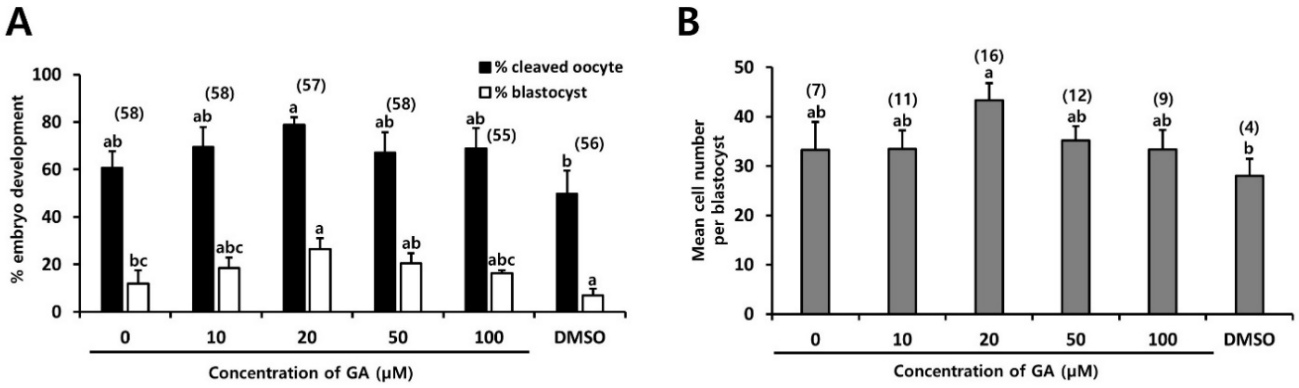

Fig. 5. Effect of glycyrrhizic acid (GA) on porcine embryo development *in vitro*. After *in vitro* fertilization (IVF), zygotes were cultured for 144 hr in porcine zygote medium (PZM)-3 medium in the presence of GA (A). The average cell number per blastocyst was counted after 4′,6-diamidino-2-phenylindole (DAPI) staining (B). Each experiment was repeated 5 times. Data were expressed as the mean ± S.E.M. The numbers on each column indicate the number of oocytes. [a,b] Superscripts denote significant differences (p<0.05).

count of blastocysts. This suggests that GA has the potential to enhance the development of porcine embryos during IVC.

DISCUSSION

In this study, following IVF and IVC with the addition of GA, improvements were observed in certain parameters associated with oocyte and embryo development. The results showed the ability of GA to suppress HMGB1. It is known that HMGB1 is an evolutionarily conserved protein, widely distributed in the nuclei and cytoplasm of nearly all cell types [8]. HMGB1 consists of two contiguous DNA-binding domains, specifically the HMG A box (comprising 9–79 amino acids) and the B box (comprising 89–162 amino acids), along with a C-terminal tail (comprising 186–215 amino acids) that possesses a significantly negative charge. It has been shown that HMGB1 has an N-terminal region that plays a key role in cellular processes involving DNA regulation, repair, and gene transcription [8, 18, 19]. As previously mentioned, HMGB1 binds to chromatin and is predominantly found in the nucleus. It is capable of moving from the nucleus to the cytoplasm and to extracellular vesicles in response to high levels of reactive oxygen species (ROS) production [20]. The presence of higher concentrations of free oxygen radicals suggests that increased oxidative stress could reduce the rate of embryonic development [21]. ROS are produced through IVF culture media as a result of high oxygen concentration, metabolic activities of oocytes and embryos, and influence on both oocyte maturation and embryo development [22]. Furthermore, free radicals have the potential to damage various cellular components, including DNA, adenosine triphosphate synthesis, and the mitotic spindle [23]. The higher level of HMGB1 expression in endometrial epithelial cells can diminish the adhesive capacity of these cells, thereby impacting blastocyst implantation. It has been suggested that the amount of HMGB1 protein in the follicular fluid is linked to the growth of the follicle and the success of IVF [24] and that higher levels lead to repeated implantation failure [25]. Therefore, the increase in HMGB1 levels during embryo development

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necessitates the development of a defense mechanism against HMGB1.

GA is a natural compound commonly found in licorice roots [12]. It is a triterpene diol conjugate that forms a direct bond with HMGB1 [26] when GA binds two flat curved surfaces of HMG boxes that are created by hydrophobic and electrostatic interactions with key residues, while its sugar moieties are directed outward [27]. The expression level of HMGB1-TLR4-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) mRNA can be inhibited by GA, and molecular docking studies have revealed that GA has an antiepileptic effect to suppress the inflammatory conditions [28]. Furthermore, GA has demonstrated significant potential in the treatment of pregnancy-related complications, such as reducing endothelial cell permeability in preeclampsia and suppressing HMGB1–induced inflammation in hypoxic trophoblastic tissue, establishing itself as a valuable agent in maintaining maternal and fetal health [1]. In this study, HMGB1 and HMGB2 were detected in spermatozoa, oocytes, and embryonic nuclei by immunofluorescence (Figs. 1 and 2). Similarly, in an earlier study, HMGB1 and HMGB2 were detected in the mice testis using immunohistochemistry, with a complementary expression pattern. Thus, HMGB2 expression was high in spermatocytes and elongated spermatids, while HMGB1 expression was low in spermatocytes and absent in spermatids [29]. Also, the HMGB1 expression was high in zygotes and decreased in the 2-cell stage of embryos, and again increased gradually during the development to the morula and blastocyst stage in mice [30].

Polyspermy frequently occurs in IVF of pig oocytes, and these embryos face difficulties in developing to the blastocyst stage [31]. Our study found a significantly higher ($p<0.05$) rate of total fertilization (mono and polyspermic oocyte) in 20 μM of GA compared to the control following IVF. Similarly, in another study, the IVF rate was significantly increased ($p<0.05$) with aqueous licorice extract (*Glycyrrhiza uralensis*), with an optimal concentration of 0.02 mg/mL, using sperm from BALB/c mice [15]. Another study found that sperm from BALB/cA mice preincubated with 0.03 mg/mL licorice extract showed an excellent concentration of IVF rate [32]. Similarly, a significantly higher ($p<0.001$) fertilization rate was observed with 10% *Glycyrrhiza glabra* extract treatment when compared with the control group in mice during IVF. These results are important as they help identify the substances in licorice that influence the efficacy of IVF [33]. We found that there was a significant increase in the percentages of cleaved embryos and blastocyst formation in the presence of 20 μM GA, and the mean cell numbers per blastocyst were also significantly higher in embryos cultured with 20 μM GA compared with the control group ($p<0.05$). In a previous study, an optimal percentage of two-cell embryos was achieved with 0.02 mg/ml of licorice extract containing isoliquiritigenin and formononetin after IVF in mice [15]. Licorice root has approximately 500 components, and the main active ingredients are GA and several flavonoids. Earlier reports suggested that the addition of GA alone to the incubation medium had no significant effect on sperm fertilization ability, and that other components of licorice, either alone or combined with GA, may result in an increase in IVF rates without any damage to fertilized eggs in mice *in vitro*. This suggests that licorice extract maintains sperm motility in the medium but does not induce the acrosome reaction, thereby potentially improving fertilization rates by prolonging sperm pen-

etration time into the oocyte [32]. In our experiment, we observed improved fertilization and blastocyst formation rates following a single treatment of GA in IVF or IVC medium (Fig. 5). This suggests that GA may modulate HMGB1, which is expressed during the early stages of embryo development [30]. In conclusion, the presence of HMGB1 and HMGB2 was confirmed in porcine germ cells, and it was found that adding GA, an HMGB inhibitor, to IVF or IVC media increased fertilization rates and preimplantation blastocyst formation rates. This suggests that GA could be utilized as a potential agent to enhance fertilization and embryonic development in assisted reproduction.

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