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Combined Treatment of Activin A and Heparin Binding-EGF (HB-EGF) Enhances *In Vitro* Production of Bovine Embryos

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ABSTRACT

This study was carried out to investigate the effects of tissue inhibitor of matalloproteinase-1 (TIMP-1), Activin A and Heparin binding epidermal growth factor (HB-EGF) on *in vitro* production of bovine embryos. In experiment 1, presumptive zygotes were cultured in the medium supplemented with TIMP-1 (0.5 μg/ml), Activin A (100 ng/ml), or HB-EGF (100 ng/ml) at 39 ℃ in a humidified atmosphere of 5% (v/v) CO_2, 5% (v/v) O_2 and 90% (v/v) N_2. In experiment 2, TIMP-1 + HB-EGF or Activin A + HB-EGF combinations were supplemented in the culture medium. The developmental rate to blastocysts, hatching rate and total cell numbers of the blastocysts were evaluated in both experiments. The embryos cultured in medium without growth factor supplementation was used as control group. In experiment 1, the embryos cultured in medium supplemented with TIMP-1 and Activin A showed significantly higher developmental rate to blastocysts than those cultured with HB-EGF and control (36.9%, 34.1%, 21.2% and 23.1%, respectively) (P<0.0001). However, the hatching rate of blastocyst was significantly higher in embryos with HB-EGF than those with TIMP-1, Actvin A and Control groups (84.4%, 58.8%, 51.4% and 49.3%, respectively) (P<0.001). Total cell number per blastocyst was also significantly higher in embryos with HB-EGF group (174.3±2.5) than those with TIMP-1, Activin A (149.7 and 150.0, respectively) (P<0.05) and Control (119.0) (P<0.001). In experiment 2, embryos cultured with combined treatment of Activin A and HB-EGF resulted in significantly higher rates of blastocysts formation (48.0%), hatching rate (89.7%) and total cell number in blastocyst (182.3±2.1) than those with TIMP-1 and HB-EGF combination group (32.0%, P<0.001; 76.6%, P<0.05; 165.7±4.2, P<0.001, respectively). Our data demonstrate that *in vitro* production of bovine embryos could be improved by combined supplementation of Activin A and HB-EGF in culture medium.

(Key words: HB-EGF, Activin A, TIMP-1, *in vitro* production (IVP), bovine embryo)

INTRODUCTION

To improve embryo quality, various growth factors known to be secreted from the reproductive tract in early embryonic period have been added to *in vitro* culture medium (Park *et al.*, 2010; Vansteenbrugge *et al.*, 1997; Lim *et al.*, 2006), although their expression or secretion patterns showed spatial and temporal manners in reproductive tissues *in vivo* (Rowzee *et al.*, 2008). Some of these factors are also known to be produced by early preimplantation embryo itself in paracrine/autocrine manners (Gandolfi, 1994). Tissue inhibitor of metallo-proteinase (TIMP-1) is secreted from bovine oviduct cells and granulosa cells (Satoh *et al.*, 1994; Gerena and Killian, 1990) and Activin A is secreted from oviductal epithelial cells (Lu *et al.*, 1993), whereas Heparin binding epidermal growth factor (HB-EGF) is known to be secreted solely in the luminal epithelium at the site of blastocyst apposition in mouse (Das *et al.*, 1994). It is thought that HB-EGF may be expressed peri-implantation (blastocyst hatching and attachment to endometrial cells) stage, and Activin A and TIMP-1 are expressed during pre-implantation stage.

TIMP-1, showing embryotrophic activity, can be obtained from bovine oviduct conditioned medium (BOCM) and the mRNA transcript for bovine TIMP-1 is also detectable at the preimplantation embryos such as morula and blastocyst, although it is still unclear how TIMP-1 can improves the development

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of bovine embryos (Vansteenbrugge *et al.*, 1996). Activin A is one of the factors involved in maternal-embryonic interactions, and is known to act on embryogenesis and organogenesis in various species (Jones *et al.,* 2002). Activin subunits and the activin receptor mRNA are expressed in oocytes and in embryos from zygote to morula in mice (Yoshioka *et al.,* 1998). Activins are secreted from the epithelial cells in oviduct and endometrium, and are present predominantly during the estrous cycle and pre-implantation phase (Jones *et al.,* 2002). HB-EGF is identified as an early messenger of implantation. HB-EGF is also associated with the trophectoderm cell surface and its presence is coordinated with the competence of murine blastocyst attachment (Carson *et al.*, 1993).

However, reciprocal action of growth factors secreted from reproductive tissues during pre- and peri-implantation period is unclear, although each growth factor are identified to support early embryonic development *in vitro*. The present study was performed to investigate the effects of Activin A, HB-EGF and TIMP-1, and their combinations on bovine embryos in a chemically defined culture condition to improve the efficiency of *in vitro* produced (IVP) embryos in cattle.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were purchased from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated in the text.

2. Oocyte Recovery and *In Vitro* Maturation (IVM)

Korean native Hanwoo cattle ovaries were collected at a local slaughterhouse and transported to the laboratory within 2～3 h in saline at 25～35℃. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 8 mm follicles using an 18 gauge hypodermic needle attached to a 10 ml disposable syringe.

After washing three times in HEPES-buffered Tyrode's solution, the COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in Nunc^{TM} 4-well culture dishes(Nunc, Roskilde, Denmark) containing 500 μL of IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 10 μg/ml follicle-stimulating hormone (FSH)-P (Folltrophin-V; Vertrepharm, London, UK) and 10% fetal bovine serum (FBS; Gibco-BRL, NY, USA) under warmed andgas-equilibrated mineral oil for 20～22 hat 38.5℃, 5% CO_2.

3. Sperm Preparation and *In Vitro* Fertilization (IVF)

After IVM, the matured oocytes were washed three times washing in IVF100 medium (Research Institute for the Functional Peptides, Yamagata, Japan), and placed into 45 μl drops of IVF100 medium under mineral oil. A frozen semen straw from the HanWoo cattle was rapidly thawed in a 38℃ water bath and the semen was diluted with Tyrode's albumin lactate pyruvate (TALP) solution and washed twice in a same medium by centrifugation at 503 × *g* for 5 min. The final sperm pellet was resuspended in IVF100 medium and the number of spermatozoa was counted using a hemocytometer then adjusted to 1.0×10^7/ml by further dilution. A 5 μl aliquot of the sperm suspension were introduced to a 45 μl droplet of IVF100 medium containing matured oocytes. Incubation was carried out at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 6 h.

4. *In Vitro* Culture (IVC)

At the end of the insemination period, groups of 10 oocytes were stripped free from cumulus cells, and transferred into 50 μl drops of modified potassium simplex optimized medium containing 70.2 μM myo-inositol and 1 mM GlcNAc (mKSOM/aa) supplemented with 20% RD (RPMI1640 + DMEM, 1:1 v/v) which was described by Momozawa and Fukuda (2011). This medium was used as Control. The incubation was conducted at 38.5°C under the 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere for 7 to 9 days. Following 24 h of culture, the presumptive zygotes which did not undergo cleavage were removed and at this time, the IVC medium was replaced with fresh medium according to each experimental group. Total cell number of Day 7 blastocyst was assessed by nuclear staining with 2 μg/ml of Hoechst 33342 in IVC medium for 10 min. Then, embryos were placed between a slide and a coverslip and nuclei were counted under UV light using an inverted microscope. Hatching rate of the blastocyst was evaluated on Day 9 of IVC.

5. Experimental Design

In Experiment 1, the effect of TIMP-1 (0.5 μg/ml), Activin A (100 ng/ml) and HB-EGF (100 ng/ml) on IVC from cleavage to blastocyst formation and hatching was evaluated. The con-

centration of these factors in the medium was determined in the preliminary experiment of the present study. Two-cell stage embryos were submitted to each culture condition (control, Activin A, TIMP-1 and HB-EGF supplementation), and then cultured for 7 days for the evaluation of blastocyst formation and total cell number, or cultured for 9 days for counting hatching of Day 7 blastocysts. In Experiment 2, the effect of combined treatments (Activin A + HB-EGF or TIMP-1 + HB-EGF) on IVC was evaluated.

6. Statistical Analysis

All the experiments of treated group were repeated 3～4 times. Results subjected to statistical analyses were expressed as mean ± SD. Data were subjected to one-way ANOVA (PRISM software version 4.0; GraphPad, San Diego, USA). Difference at $P<0.05$ was considered significant.

RESULTS

In Experiment 1, first cleavage was not affected by growth factor supplementation (control, 79.5%; Activin A, 76.9%; TIMP-1, 84.0%; HB-EGF, 80.1%, Fig. 1A). However, the percentage of embryos developed to the blastocyst stage in Activin A (34.1%) and TIMP-1 (36.9%) groups were significantly higher than in Control (23.1%) and HB-EGF (21.2%) groups ($P<0.0001$, Fig. 1B). the rate of blastocyst hatching was significantly higher ($P<0.001$) in embryos cultured with HB-EGF (83.4%) group than in all other experimental groups (Control, 49.3%; Activin A, 51.4%; TIMP-1, 58.8%, Fig. 1C). The mean cell number of blastocysts cultured with HB-EGF on Day 7 (174.3 ± 2.5) were significantly higher than that of other groups (Control, 119.0 ± 3.1; Activin A, 150.0 ± 2.6; TIMP-1, 149.7 ± 1.9; $P<0.001$, Fig. 1D). In addition, total cell numbers in embryos cultured with Activin A or TIMP-1 groups were also significantly higher than those in control groups ($P<0.05$).

In Experiment 2, combination of Activin A and HB-EGF showed significantly increased blastocyst development (48.0%, $P<0.001$) and hatching (89.7%, $P<0.05$) than TIMP-1 + HB-EGF counterpart (32.0% and 76.6%, respectively, Fig. 2A, B). The mean cell numbers in Day 7 blastocysts was significantly higher in Activin A + HB-EGF (182.3 ± 2.1) than the number in TIMP-1 + HB-EGF (165.7 ± 4.2, $P<0.001$, Fig. 2C).

DISCUSSION

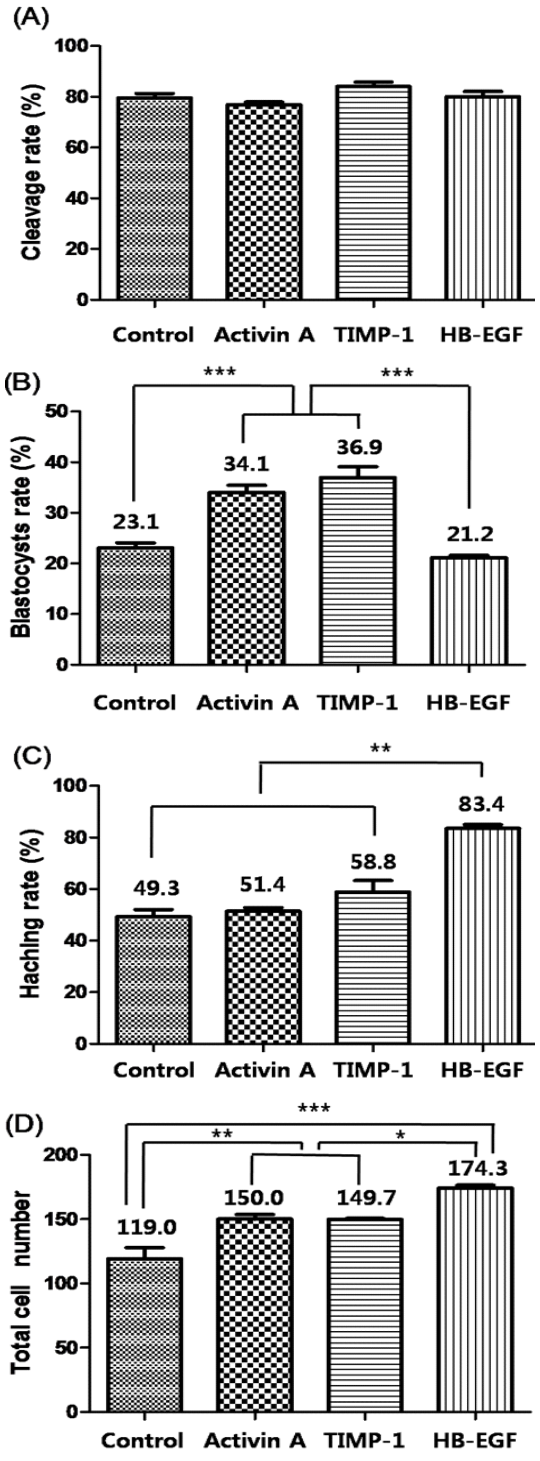

Fig. 1. Effects of Activin A, TIMP-1 and HB-EGF on 2-cell cleavage (A), blastocyst formation from 2-cell embryos on Day 7 (B), hatching per total blastocysts (C) and total cell number in the blastocyst (D). Different letters indicate significant differences ($^{***}P<0.0001$; $^{**}P<0.001$ and $^{*}P<0.05$). Data were expressed as mean ± SD from 3～4 replicates.

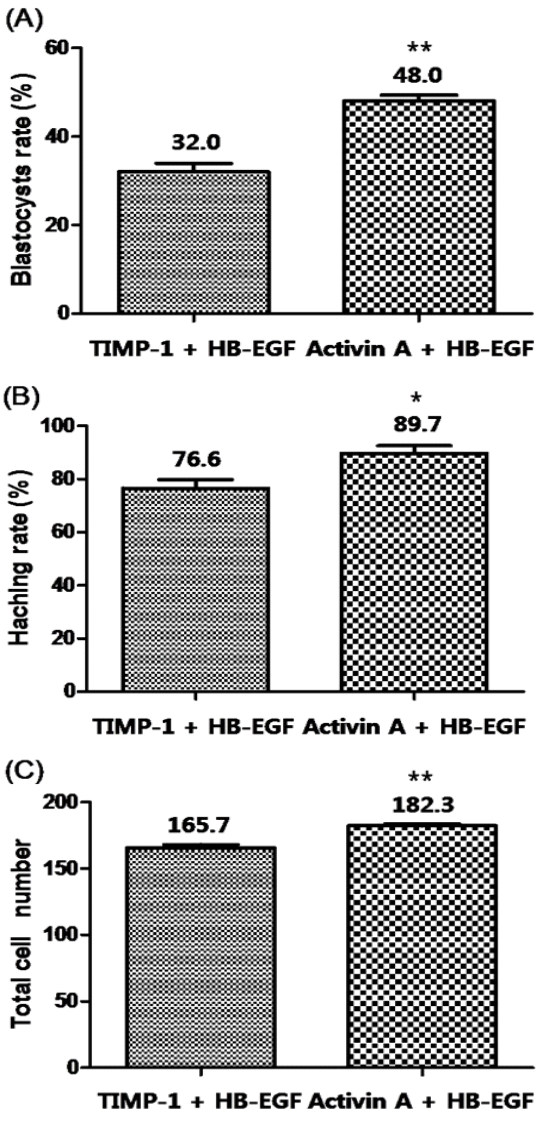

Fig. 2. Effect of combined treatment of 'Activin A and HB-EGF' or 'TIMP-1 and HB-EGF' on blastocyst formation from 2-cell embryos on Day 7 (A), hatching per total blastocysts (B) and total cell number in the blastocyst (C). Different letters indicate significant differences ($^{**}P<0.001$ and $^{*}P<0.05$). Data were expressed as mean ± SD from 3～4 replicates.

The present investigation showed that combination of growth factors, Activin A and HB-EGF, which expressed in the reproductive tissue at different period of embryonic stages (early and late pre-implantation embryonic stages, respectively) can improve IVP of bovine embryos. In Experimental 1, Activin A and TIMP-1 significantly increased blastocyst formation and total cell number, whereas they did not influence blastocyst hatching. In contrast, HB-EGF improved hatching rate and blastocyst cell number when compared with the other groups, although it did not affect blastocyst development. The result implies that each factor has different role (s) for bovine embryo development *in vitro*.

TIMP-1 is an inhibitor of matrix metalloproteinases (MMPs) in human (Moore and Crocker, 2012). TIMP-1 promotes the prehatched development of bovine embryos (Hwang *et al.*, 2000), and MMP-2 is known as the key regulator of trophoblast invasion during early pregnancy period (Seval *et al.*, 2004). TIMP-1 also acts as a growth factor in various cells (reviewed by Denhardt *et al.*, 1994). It was previously reported that transcripts for MMPs and TIMPs are expressed at various stages during mouse preimplantation embryo development (Brenner *et al.*, 1989). Although major function of TIMP-1 is invasion, Satoh *et al.* (1994) suggested that TIMP-1 is one of the major factors for improving embryo development in cattle. On the other hand, Vansteenbrugge *et al.* (1997) suggested that TIMP-1 do not play an important role in sustaining bovine embryo development, and also do not influence blastocyst quality in terms of total number of cells per embryo when the embryo was cultured in BOCM which contains TIMP-1. In the present study, TIMP-1 supplementation increased blastocyst formation and total blastocyst cell number except for hatching and this may be caused by promoting actions of cell migration, proliferation and cell-to-cell interaction (Wiley *et al.*, 1992).

In recent studies, it has been reported that the expression of Activin A decreased in the ooplasm after maturation, and then increased after fertilization (Silva *et al.*, 2003). Activin A is also known to be expressed in bovine oviducts (Gandolfi *et al.,* 1995). These findings suggest an active role of Activin A in early embryonic development in mammals. Park *et al.* (2010) suggested that Activin A supports blastocyst formation and hatching when Activin A is supplemented at second half culture period in two-step culture system. However, Trigal *et al.* (2011) reported that although Activin A supplementation in second half culture stage support blastocyst formation, it increased apoptotic cell population and also decreased trophoblastic cells. In contrary, supplementation of Activin A in first half culture stage did not enhance blastocyst formation whereas the factor supported blastocyst hatching. In present study, Activin A significantly elevated blastocyst development and total cell number of the blastocyst. However, it did not affect hatching rate. Therefore, role of Activin A in this experiment seems to be similar to the role of TIMP-1 in our experiment.

HB-EGF is expressed on the surface of trophoblasts and is related with acquisition of attachment competence for implantation and blastocyst hatching (reviewed by Jade Lim and Dey, 2009; Carson *et al.*, 1993). The supportive role of EGF-related growth factors including HB-EGF for blastocyst hatching is caused by plasminogen activator in the blastocyst (reviewed by Paria and Dey, 1990). Previous report suggests the possibility of a ligand-receptor signaling of the EGF family at the initiation of implantation process in the mouse (Paria *et al.*, 1993). EGF receptor (EGF-R) is expressed in the trophectoderm of a blastocyst, and this expression is tightly regulated by the status of maternal steroid hormones at the time of implantation (Paria *et al.*, 1993). However, effect of exogenous HB-EGF on bovine embryo itself is unknown yet. Our data show that HB-EGF support hatching, not blastocyst formation. This is in agreement with previous reports mentioned above and also implies that HB-EGF affect to bovine embryos after blastocyst formation.

Since our data demonstrated that HB-EGF supported blastocyst hatching, and Activin A and TIMP-1 improved blastocyst formation and its cell number increase, combined treatment of HB-EGF and Activin A or TIMP-1 was performed as the next step.

In Experiment 2, combination of Activin A and HB-EGF showed significantly higher blastocyst formation, hatching and total cell number in the blastocyst than TIMP-1 and HB-EGF combination group. The result indicates that embryonic quality was improved by synergetic effect of Activin A and HB-EGF. Activin A might affect at the early stage as it is known that it induces fast embryonic cell cycle especially at 4～8 cell stage bovine embryos (Yoshioka *et al.*, 2000). On the other hand, HB-EGF could improve embryo quality in late stage blastocysts because expression of integrin αv and β3 subunits reached peak levels in the outgrowth stage embryo and HB-EGF support this (Lim *et al.*, 2006). However, TIMP-1 and HB-EGF combination showed less synergic effect than Activin A and HB-EGF although it is known that EGF mediates TIMP-1 expression in cultured bovine trophoblasts (Dilly *et al.*, 2010).

In conclusion, the present study suggests combined treatment of Activin A and HB-EGF for IVP of bovine embryos.

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