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Effect of Matrix Metalloproteinases-2 and -9 during IVC-2 on the Development Competence and Gene Expression Profile of Bovine *In Vitro*-Produced Embryos

Kyeong-Lim Lee[1], Jae-Il Bang[1], A-Na Ha[1], Md. Fakruzzaman[1], Chan-Sik Min[3] and Il-Keun Kong[1,2,†]

[1]Department of Animal Science, Division of Applied Life Science (BK21Plus), Gyeongsang National University, Jinju 660-701, Republic of Korea
[2]Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea
[3]Gyeongsangnamdo Agriculture Research & Extension Services, Jinju 660-985, Republic of Korea

ABSTRACT

Matrix Metalloproteinases (MMP)-2 and -9 are participated in embryo development, implantation, remodeling of epithelial cell and ovulation. The objective of this study is to evaluate an impact of MMP2 and MMP9 on embryonic developmental competence as well as gene expression profiles of *in vitro*-produced bovine embryos. After *in vitro* fertilization, embryos of all groups were transferred into IVC-2 medium treated with MMP2 and MMP9 to check the optimum concentration on the basis of embryo development competence and cell numbers. The optimum concentrations for MMP2 and 9 were 1,200 ng/ml and 300 ng/ml. The blastocyst development competence was not different among 1,200 ng/ml of MMP2 vs. 300 ng/ml of MMP9 vs. combined MMP2 + 9 vs. control groups (41.46 ± 10.66 vs. 37.73 ± 8.92 vs. 45.11 ± 11.41% vs. 41.59 ± 11.88, respectively). Furthermore, the developmental competences to hatching and hatched blastocysts were not also different among the same groups (79.84 ± 12.63 vs. 83.3 ± 17.46 vs. 78.55 ± 14.48% vs. 72.02 ± 14.09). In addition, total cell number was significantly (p<0.05) greater in blastocyst treated with MMP9 300 ng/ml among all treatment groups. On the other hand, there was no significant difference of ICM vs. TE ratio in all groups. The expression of five out of six genes (i.e., MMP2, MMP9, IFNt, SSLP1 and HNRNPA2B1) was different among the groups. The expression of IFNt and HNRNPA2B1 genes was significantly greater in MMP9 (p<0.05), but there was no difference of MMP9 expression between MMP2 and MMP9 group (p>0.05). The normalized expression of MMP2 and SSLP1 was greater in MMP2 than other groups (p<0.05). In conclusion, MMPs treatment during IVC-2 medium was remarkably effected on blastocyst developmental competence and gene expression profiles that are related to embryo quality and implantation.

(Key words : matrix metalloproteinase, bovine, embryo developmental competence, gene expression)

INTRODUCTION

Matrix Metalloproteinases (MMPs) are able to be controlled exactly its activation by combined with tissue inhibitors of metalloproteinases. MMPs play very pivotal roles for extracellular matrix (ECM) remodeling during ovarian follicular development, ovulation and atresia (Imai *et al.*, 2003). Moreover, MMPs is participated in embryo development, implantation, remodeling of epithelial cell and formation of bone, etc. MMP9 expressed most strongly in trophoblast cells of embryo being implanted that had been reported in mouse. A well-timed breakdown of ECM composed of structural protein such as collagens, proteoglycans and glucoprotein is essential phenomenon at embryogenesis, morphogenesis, reproduction and absorption and reformation of tissue. Especially, MMPs were denominated matrixins of which perform an important function in this process of remodeling of ECM, implantation and ovulation (Nagase and Woessner, 1999, McCawley and Matrisian, 2000). Most of MMPs expression is become transcriptional control through cell growth factors, hormones, cytokines, transformation of cell and etc. In goats, MMP2 activity is regulated by co-localized membrane-type 1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2), and they control endometrium remodeling during gestation (Uekita *et al.*, 2004). In bovine, MMPs has been played a crucial role during peri-partum, termination of gestation, and post-partum (Walter and Boos, 2001, Takagi *et*

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† Correspondence : E-mail : ikong7900@gmail.com

al., 2007). The detailed expression profiles of gelatinases have not been clarified during implantation; namely, the proteolysis mechanisms of the endometrial ECM are still obscure during implantation in cows (Kizaki *et al.*, 2008). During the implantation process, trophoblast cells eliminate epithelial cells, and the epithelium is reorganized (Yamada *et al.*, 2002a, Yamada *et al.*, 2002b).

Several difficulties are arising during implantation process of *in vitro*-produced embryos. Gelatinases may play a significant role in this process. *In situ* zymography for gelatinolytic activity established a pattern of activity that corresponded with the localization of MMP-2 and MMP-9 mRNA around developing follicles (Curry *et al.*, 2001). Several researches have been conducted about MMP2, 9 regarding their functions related to ovulation, implantation, maturation, and tissue remodeling, etc. To date, no studies have elucidated the exact role of MMP2 and MMP9 added into medium for development of bovine *in vitro*-produced embryo. Therefore, this study is the first to find out the influence of MMP2 and MMP9 addition during IVC-2 on embryo developmental competence and further impact on embryo quality as well as gene expression analysis.

MATERIALS AND METHODS

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

1. Oocyte Retrieval

Ovaries were obtained from Korean native cows (Hanwoo) at a local abattoir and transported to the laboratory within 2 h in physiological saline (0.9% NaCl) maintained at 35 to 37℃. Ovaries were washed in fresh Dulbecco's PBS, and cumulus-oocyte complexes (COCs) were retrieved as described by (Deb *et al.*, 2011). In brief, COCs were recovered from 2 to 8 mm diameter follicles using an 18-G needle attached to a vacuum pump. Only COCs having more than three layers of compact cumulus cells with homogenous cytoplasm were selected in 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 μl/ml phenol red, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) under a stereomicroscope.

2. *In Vitro* Maturation (IVM)

Oocytes were cultured *in vitro* maturation medium according to (Deb *et al.*, 2011). In brief, collected COCs were washed three times in maturation medium (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 μg/ml estradiol-17 β, 10 μg/ml FSH, 0.6 mM cysteine and 0.2 mM sodium lactate. The COCs were then incubated in 700 μl IVM medium at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 23 to 24 h.

3. *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC)

In vitro matured COCs were fertilized with thawed sperm as previously described (Deb *et al.*, 2011). Thawing was performed at 36℃ for 1 min, after which sperm were washed and pelleted in Dulbecco's PBS (D-PBS) by centrifugation at 750 × g for 5 min at room temperature. The pellet was diluted with 500 μl heparin (20 μg/ml) in fertilization (IVF) medium (Tyrode lactate solution supplemented with 6 mg/ml bovine serum albumin (BSA), 22 μg/ml sodium lactate, 100 IU/ml of penicillin, and 0.1 mg/ml of streptomycin and incubated at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 15 min. Capacitated spermatozoa were diluted in IVF medium to 1 to 2 × 10^6 spermatozoa /ml. Matured oocytes were transfer redin to 700 μl IVF medium containing sperms for 18 to 20 h.

After IVF, cumulus cells of COCs were removed by pipetting and denuded presumed zygotes were placed in 700 μl CR1-aa medium (Hurskainen *et al.*, 1996) supplemented with 44 μg/ml sodium lactate, 14.6 μg/ml glutamine, 10 μl/ml penicillin-streptomycin, 3 mg/ml BSA and 310 μg/ml glutathione for 3 days (IVC-I). Cleaved embryos were then cultured until Day 8 of embryonic development (Day 0 = Day of *in vitro* fertilization) in a medium of the same composition (IVC-I), except that BSA was replaced with 10% (v/v) FBS (IVC-II). Day 8 blastocysts were washed three times in TL-HEPES, transferred into fixative (4% [v/v] paraformaldehyde in 1 M PBS), and stored at 4℃ until cell number determination. For gene expression analysis, Day 8 blastocysts were transferred into a 1.5 ml Eppendorf tube, immediately snap frozen in liquid nitrogen, and stored at −80℃ until use.

4. Differential Staining

Differential staining was performed according to Thouas *et*

al. (2001) with minor modification. In brief, fixed embryos were washed with 1 mg/ml ployvinylpyrrolidone (PVP) in 0.1 M phosphate buffer saline (PBS) before permeabilization in 0.5% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate. The stock of propidium iodide (PI) (Sigma, P4170) was prepared by dissolving 2.5 mg/ml in PBS and stored at 4℃. For working solution, dilute the stock of PI solution 1:25 in 0.5% Triton X-100 to a concentration of 100 μg/ml. The Hoechst33258 stock was prepared by dissolving 25 mg Hoechst 33258 (Sigma, B2883) in 2.5 ml of distilled water (10 mg/ml) and stored at 4℃. On the day of use, the stock solution was diluted of the ratio of 1:1,000 in 4% paraformaldehyde to give a working concentration of 1 μg/ml.

Five hundred μl of PI solution was placed in one well of a SPL (SPL Life Science Co., Cat#30004) 4-well plate and remaining 3 wells were filled with 500 μl of PBS/PVP and placed the four-well plate on a slide warmer at 39℃ for 5～10 min. After warming, embryos were removed from culture solution as little medium as possible and placed into the PI solution well for 30 sec. After incubation in PI solution, embryos were washed thrice in warm PBS/PVA and moved into a 50 μl droplet of the Hoechst 33258 solution and incubated at room temperature for 15 min. Then the embryos were washed thrice in PBS/PVA. After washing in PVP-PBS, embryos were mounted onto glass slides and covered with a cover slip and their nuclear configuration was analyzed.

5. RNA Extraction and cDNA Preparation

1) RNA Extraction and Isolation

For RNA extraction, Arcturus picopure RNA isolation kit (ARCTURS; Cat# 12204-01) were used. In brief, 100 μl of extraction buffer was added to sample tube and incubated at 42℃ for 30 min. After incubation, the sample tube was centrifuging with 3,000 × g for 2 min, supernatant moved into a new 1.5 ml RNA-free tube. Two hundred μl of conditioning buffer was added into a column tube and incubated at room temperature for 5 min, and then centrifuged at 16,000 × g for 1 min. One hundred μl of 70% ethanol added to extract cells from RNA extraction, and pipetting thoroughly. Then the mixture added into a column tube, centrifuged at 100 × g for 2 min, again with 16,000 × g for 30 sec to remove flow through. Putted 100 μl wash buffer 1 into the column and centrifuged for 1 min at 8,000 × g. DNase I solution (5 μl DNase added to 35 μl buffer RDD) were prepared and mixed gently. Then 40 μl DNase I mixture were putted directly into the purification column membrane and incubated at room temperature for 15 min. Again 40 μl wash buffer 1 were placed into the column tube and centrifuged at 8,000 × g for 15 sec. Then 100 μl wash buffer 2 were putted into the column and centrifuged for 2 min at 16,000 × g followed by 1 min centrifuge at 16,000 × g for complete removed of washing buffer. Finally the purification column was transferred into a new 1.5 ml RNasse-free tube and placed 20 μl of elution buffer into center of column and incubated at room temperature for 1 min and centrifuge at 1,000 × g for 1 min followed by centrifuge again at 16,000 × g for 1minute. The RNA samples were used immediately or stored at －80℃ until use.

2) cDNA Synthesis

RNA concentration and purity were checked by NANO drop machine (Thermo Fisher Scientific, NANO DROP 2000c). The mRNA samples were reverse transcribed into first-stand cDNA using Bio-Rad Company. The 15 μl mRNA samples were transferred into a 200-μl eppendorf tube containing 4 μl 5× iScript Reaction Mixture and 1 μl iScript Reverse Transcriptase. The reactions was terminated by heating at 25℃ for 5 min, 42℃ for 30 min, 85℃ for 5 min and finally hold at 4℃.

3) Real Time PCR

The primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and were presented in Table 1. The qPCR was performed in duplicate in a CFX98 instrument (Bio-Rad Laboratories, Hercules, CA) using a 10 μl reaction mixture containing 0.2 mM of each bovine-specific primer, 1× iQ SYBR Green Supermix (iQ SYBR Green Supermix kit, Bio-Rad Laboratories), and 1.0 μl of cDNA. The cycling conditions were as follows: 95℃ for 3 min followed by 44 cycles of 15 sec at 95℃, 20 sec at 57℃, and 30 sec at 72℃, and a final extension of 5 min at 72℃. Amplification was followed by a melting curve analysis using progressive denaturation, during which the temperature was raised from 65 to 95℃ at a transition rate of 0.2℃ per second. Continuous fluorescence measurements were made during the progressive heating. A negative control was performed for each gene. The PCR products that exhibited only a single fusion temperature, which confirms a unique PCR product, were retained for further quantitative analysis. The target genes were quantified by the ΔΔ C(t) method using CFX ma-

Table 1. Information on primers used for quantitative real-time PCR

Gene name	Accession No.	Primer sequence	Gene size (bp)
MMP9	NM_174744.2	F: GAGATGCCCACTTCGACGAT R: GAGCGACCCTCAAAGGTGAA	121
MMP2	NM_174745.2	F: ATCGTCTTCGACGGCATCTC R: GTGGGTCTTCGTACACAGCA	166
GAPDH	NM_173979	F: ATTTTATGGACAGCCATC R: TGTACAGGAAAGCCCTGACT	120
IFNτ	NM_001015511.2	F: CTGGGAAATCATCAGAGTGGAG R: TTAAGGACTCATGCCCCTACAG	279
SSLP1	NM_001105478.1	F: CCTTTAGAATGGACTGGTTGGATC R: AGAAAGTATCCTTGGACCTGGC	236
PLAC8	NM_001025325.1	F: TCGCCATGAGGACAATGTATCGGA R: GCTTGAGTTGACAAAGGGCACAGA	108
HNRNPA2B1	XM_580324.4	F: TATGGAGAAGGACGAGGAGGTT R: AGCCCCTGCCAAATAACAAG	298

nager V1.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

6. Experimental Design

1) Experiment 1 : Find out Optimal Concentration of MMP2, 9 and Embryo Developmental Competence

The first experiment was focused on finding out the optimal concentration of MMP2 and MMP9 during IVC-2 for improvement of embryo development competence. To find out the best concentration we added three different concentration of MMP2 (300, 750, 1,200 ng/ml) and MMP9 (100, 300, 600 ng/ml) including control. On the basis of TE cell numbers per blastocyst, total cell numbers as well as blastocyst development, we selected optimum concentration for 1,200 ng/ml in MMP2 and 300 ng/ml in MMP9 and used in next step experiment including of combined MMP2+MMP9 and control.

2) Experiment 2 : Effect of MMP2 and 9 Additions during IVC-2 on the Gene Expression Profiles of IVP Blastocysts

In this experiment, three treatment groups were used 1,200 ng/ml of MMP2, 300 ng/ml of MMP9, combined MMP2+9 and control groups. The MMP2 and MMP9 were added in IVC2 medium and checked blastocyst developmental competence at D 8. Day 8 blastocysts were washed several times with PBS-PVP and kept in 0.1% pronase for 2～5 min to dissolve zona pellucida. The blastocysts were putted into a 1.5-ml RNase free tube with minimum volume of solution, immediately snap frozen in liquid nitrogen, and stored at −80℃ until use.

7. Statistical Analysis

All experiment was performed at least three replications. The embryo development rate (cleavage and blastocyst) and quality (total cell, ICM and TE cell numbers) were expressed as Mean ± SD. Significant differences between groups were detected using Turkey's and Duncan's multiple range test (SPSS Inc., Chicago, IL, USA). Differences with $p<0.05$ were considered significant.

RESULTS

1. Developmental Competence of Blastocysts, Hatching and Cell Number according to Different Concentration of MMP2 and MMP9 during IVC2 Period

The blastocyst development competence of MMP2 and 9 treatment during IVC-2 was not different among control vs. 300, 750, 1,200 ng/ml of MMP2 vs. 100, 300, 600 ng/ml of MMP9 groups (43.4 ± 11.9 vs. 51.7 ± 12.1, 44.4 ± 5.9, 47.1 ± 10.2 vs. 33.0 ± 5.5, 36.6 ± 5.4, 40.7 ± 6.1%, $p>0.05$). Also hatching blastocysts competence was not different among control vs. same MMP2 vs. same MMP9 groups (59.8 ± 19.2 vs. 58.0 ± 15.3, 55.1 ± 17.8, 64.6 ± 12.9 vs. 73.0 ± 24.1, 70.0 ± 12.9, 67.8 ± 17.8%, $p>0.05$), respectively (Table 2). In addition, total cell

number was significantly ($p<0.05$) greater in 300 ng/ml of MMP9 treated blastocysts among the all treatment groups, whereas significantly ($p<0.05$) lower in 100 ng/ml of MMP9 blastocysts. On the other hand, there was no significant difference of ICM vs. TE ratio in all groups (Table 3).

We selected optimal concentration of MMP2 and MMP9 from above experiment (Table 1 & 2) and then evaluated blastocyst developmental competence after addition of 1,200 ng/ml of MMP2, 300 ng/ml of MMP9 and 1,200 + 300 of MMP2+9 and control during IVC2. The blastocysts development competence was not significantly different among control vs. 1,200 ng/ml of MMP2 vs. 300 ng/ml of MMP9 vs. MMP2+9 groups (41.59 ± 11.88 vs. 41.46 ± 10.66 vs. 37.73 ± 8.92 vs. 45.11 ± 11.41%. respectively, $p>0.05$). Furthermore, the development competence of hatching and hatched blastocysts was not also significantly different among control vs. 1,200 ng/ml of MMP2 vs. 300 ng/ml of MMP9 vs. MMP2+9 groups (72.02 ± 14.09 vs. 79.84 ± 12.63 vs. 83.30 ± 17.46 vs. 78.55 ± 14.48%, $p>0.05$) (Table 4).

2. Gene Expression Profiles of *In Vitro*-Produced Blastocysts Derived from Different Groups

Table 4. Effect of selected concentration of MMP-2 and -9 on the development of blastocysts and hatching competence

Treatment groups (ng/ml)	IVC2	No. and (%) of embryos developed to (Mean ± SD)	
		Blastocyst	Hatching rate/blastocysts
Control	349	149(41.59 ± 11.88)	104(72.02 ± 14.09)
1,200 MMP2	313	130(41.46 ± 10.66)	103(79.84 ± 12.63)
300 MMP9	349	129(37.73 ± 8.92)	108(83.30 ± 17.46)
1,200+ 300 MMP2+9	345	155(45.11 ± 11.41)	118(78.55 ± 14.48)

Table 2. Effect of MMP-2 and -9 during IVC-2 on the developmental competence of blastocyst and hatching embryos

Treatment groups (ng/ml)		IVC2	No. and (%) of embryos developed to (Mean ± SD)	
			Blastocysts/cleavages	Hatching rate/blastocysts
Control	0	539	234(43.4 ± 11.9)	140(59.8 ± 19.1)
MMP2	300	267	138(51.7 ± 12.1)	80(58.0 ± 15.3)
	750	266	118(44.4 ± 5.9)	65(55.1 ± 17.8)
	1200	276	130(47.1 ± 10.2)	84(64.6 ± 12.9)
MMP9	100	270	89(33.0 ± 5.5)	65(73.0 ± 24.1)
	300	273	100(36.6 ± 5.4)	70(70.0 ± 12.9)
	600	275	112(40.7 ± 6.1)	76(67.8 ± 17.8)

Table 3. Comparison of cell numbers of Day 8 blastocysts among the groups

Treatment groups (ng/ml)		No. of blastocysts differentially stained	No. of cells counted (Mean ± SD)			ICM : TE ratio
			Total	ICM	TE	
Control		33	140.6 ± 48.3[a,b]	31.8 ± 12.9	108.6 ± 47.9	1 : 4.1 ± 2.7
MMP2	300	20	130.5 ± 33.2[a,b]	40.8 ± 11.9	89.6 ± 24.7	1 : 2.3 ± 0.5
	750	14	120.4 ± 26.5[a,b]	32.3 ± 11.1	88.1 ± 17.7	1 : 3.0 ± 1.0
	1,200	12	124.0 ± 34.5[a,b]	32.2 ± 9.7	91.8 ± 28.5	1 : 3.1 ± 1.3
MMP9	100	13	116.1 ± 24.8[a]	28.1 ± 10.9	88.0 ± 27.3	1 : 3.9 ± 3
	300	11	157.3 ± 39.4[b]	25.4 ± 8.0	132.4 ± 42.6	1 : 5.9 ± 3.1
	600	13	137.6 ± 32.6[a,b]	27.6 ± 10.7	110.0 ± 33.2	1 : 4.7 ± 2.8

[a,b] Values with different superscripts in same column denoted were significantly different($p<0.05$).

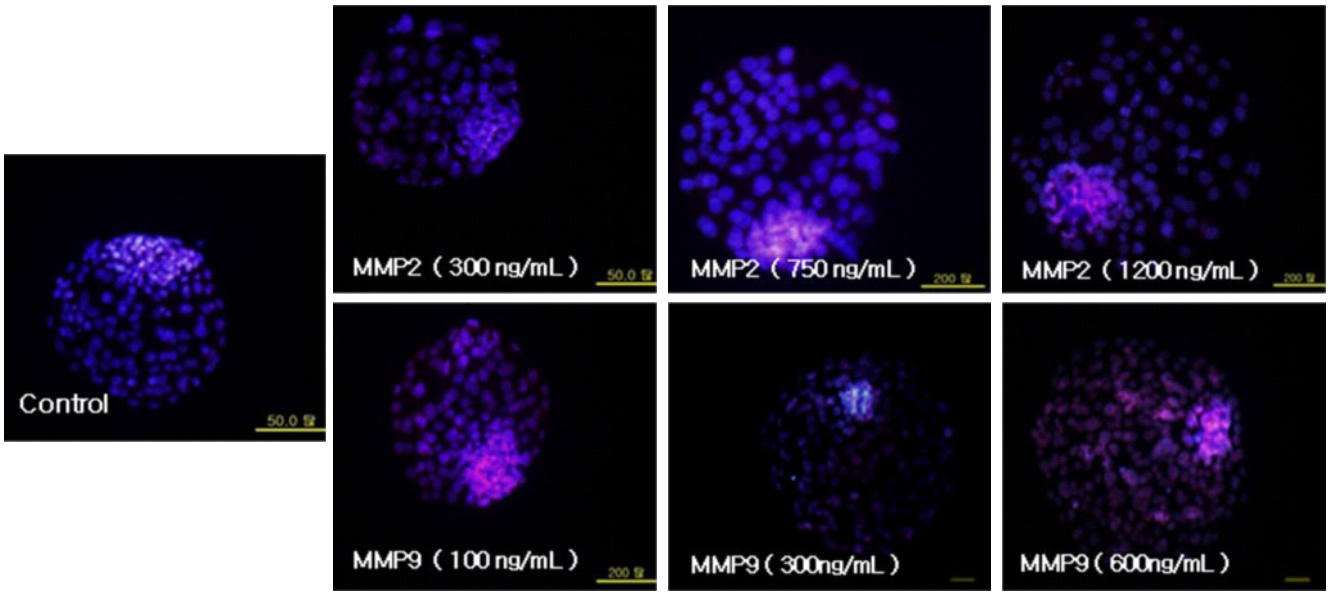

Fig. 1. Representative images of differential stained blastocyst derived from different treatments of MMP2 and MMP9 and control.

The normalized expression of six embryo biomarker genes namely matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), placenta-specific 8 (PLAC8), interferon-t (IFNt), secreted seminal-vesicle Ly-6 protein 1 (SSLP1) and heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1) were investigated. The relative amounts of the studied genes were calculated following ΔΔ C(t) method that normalized against expression of GAPDH reference gene (Fig. 2). The expression of five out of six genes (i.e., MMP2, MMP9, IFNt, SSLP1 and HNRNPA2B1) was different among the groups. The expression of IFNt and HNRNPA2B1 genes were greater in MMP9 compared to control, MMP2 and MMP9+MMP2 embryos ($p<0.05$), but there was no significant difference of MMP9 expression between MMP2 and MMP9 embryos ($p>0.05$). The normalized expression of MMP2 and SSLP1 were significantly greater in MMP2 than other groups ($p<0.05$).

DISCUSSION

MMPs play very important role of the embryos hatching and implantation. Process of embryo implantation was invasion and adhesion that takes place between the embryo and the endometrium (Kim *et al.*, 2002). In human, MMP2 and MMP 9 made in trophoblast and the cultured embryos secreted MMP2 (Puistola *et al.*, 1989; Unemori *et al.*, 1991). In the first trimester of human trophoblast was producing MMP2 and MMP9, and also the cultured embryos secreted MMP2. Fibronectin and laminin were secreted from the embryos that promoted the formation of MMP2 during implantation (Turpeenniemi *et al.*, 1995). MMP9 was highly expressed in mouse blastocysts, and inhibited of extracellular matrix degradation. Extracellular proteases such as serine proteases and MMPs are thought to play pivotal roles for extensive tissue remodeling during both follicular development and the breakdown of the follicular wall at the time of ovulation (Liu *et al.*, 1998). Another report showed that an extracellular matrix degrading metalloproteinases and their inhibitor are expressed during early mammalian development (Brenner *et al.*, 1989).

MMP2 and MMP9 had a crucial impact on ovulation, implantation, remodeling and hatching (Alexander *et al.*, 1996; Huppertz *et al.*, 1998; Xu *et al.*, 2002; Isaka *et al.*, 2003). In current study, there was no significant difference in blastocyst development and cell numbers among the treatment groups. But several biomarkers gene expression has shown significant differences among the groups. In our research, IFNτ gene was over-expressed in MMP treatment groups and this was consistent of other researches. Interferon-t originally named trophoblastin or trophoblast protein-1, is the best known specific pregnancy perception signal involved in the establishment of early pregnancy in ruminants (Wang *et al.*, 2003). Interferon-t was found to be over-expressed in the hatched blastocyst when compared with the expanded and early stages (Rekik *et al.*, 2011). IFNτ pro-

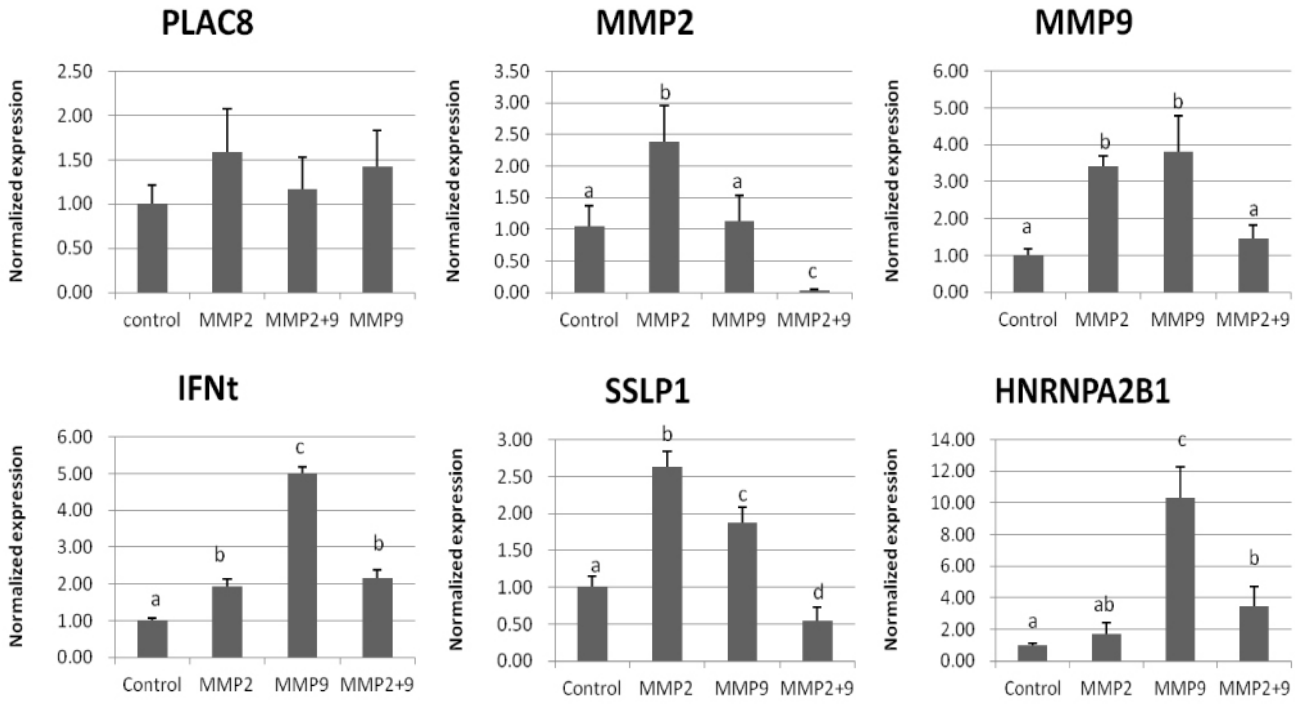

Fig. 2. Normalized expression levels in Control, MMP2, MMP9 and MMP2+9 embryos by RT-PCR.

duction by bovine embryo begins at the blastocyst stage, and then increases as the conceptus starts to extend (Ealy *et al.*, 2001). HNRNPA2/B1 was over-expressed in MMP treatment groups. The main functions of HNRNPA2B1 included transcription, alternative pre-mRNA splicing, cytoplasmic trafficking of mRNA and translation (Hutchison *et al.*, 2002; Rekik *et al.*, 2011). HNRNPA2/B1 was confirmed to be down-regulation in the expanded and the hatched blastocysts by 4.8-fold (Rekik *et al.*, 2011). SSLPI gene was up-regulation in MMP treatment groups in this study. SSLP1 was over-expressed in hatched blastocysts (Rekik *et al.*, 2011) and expressed in fetal tissues (Wright *et al.*, 1990), and also be involved in the remodeling of the extracellular matrix and the organization of the mesenchymal villi of ruminant cotyledons (Ushizawa *et al.*, 2009).

In bovine, PLAC8 gene was reported to be up-regulated in biopsies from blastocysts that led to calf delivery when compared with imbibition. PLAC8 is substantially expressed in trophectoderm in preimplantation embryos, and in the trophoblast giant cells and spongiotrophoblast layer at later stages in development. It was also reported that PLAC8 be up-regulated in the endometrium of pregnant compared to non-pregnant cows (Galaviz-Hernandez *et al.*, 2003). PLAC8, like IFNt, was up-regulated in hatched compared to early blastocysts, which might confirmed the importance of these two marker genes for embryo apposition and pregnancy induction (Rekik *et al.*, 2011). In our result showed that although there was no significant difference among the groups but the fold changes was higher in MMP2 and MMP9 groups than others.

In conclusion, the present study showed that there was no significant difference of embryo development and cell numbers of embryo, but the gene expression profiles related to pregnancy were up-regulated in addition of MMP2 and 9 during IVC-2. So, MMPs addition during IVC-2 has positive effect on gene expression profiles.

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