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Effects of Interferon-Tau (IFN-τ) and Progesterone on Transcription Level of Matrix Metalloproteinases-2 (MMP-2) and Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) in Cultured Bovine Endometrial Cells

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Abstract: Interferon-tau (IFN-τ) secreted by blastular trophectoderm was thought to be the primary signal for pregnancy recognition during bovine early pregnancy period after pregnancy recognition, progesterone dominated pregnancy maintaining. Moreover, Matrix Metalloproteinases-2 (MMP-2) and Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) was important for embryo implantation. The objectives of studies were evaluated whether IFN-τ and progesterone regulated MMP-2 and TIMP-2 expression in cultured bovine Endometrial Cells (bECs). In the studies, bECs were cultured in DMEM-Ham's F12 medium with free-serum. These cultured cells were divided into 4 groups and cultured in four types culture medium: culture medium without IFN-τ and progesterone (Co group); culture medium including 100 ng mL^{-1} IFN-τ (I_N group); culture medium including 200 nmol L^{-1} progesterone (Pr group); culture medium including 100 ng mL^{-1} IFN-τ and 200 nmol L^{-1} progesterone (I+P group). Cultured bECs were harvested and extracted total mRNA after being cultured for 1, 3, 6 and 12 h and then expression level of MMP-2 and TIMP-2 were assessed with fluorescence quantitative PCR. The results showed that MMP-2 expression level in Pr, I_N and I+P group were extremely significantly higher than in Co group ($p<0.01$). MMP-2 expression level in I_N and I+P group was significantly higher than in Pr group ($p<0.05$). MMP-2 expression level was no statistical difference between I_N group and I+P group ($p>0.05$); TIMP-2 expression level in Pr, I_N and I+P group was extremely significantly lower than in Co group ($p<0.01$) and TIMP-2 expression level was no statistical difference among Pr, I_N and I+P group ($p>0.05$). The conclusion indicated progesterone and IFN-τ induced MMP-2 expression *in vitro* cultured bECs whereas, progesterone and IFN-τ suppressed TIMP-2 expression *in vitro* cultured bECs.

Key words: MMP-2, TIMP-2, progesterone, IFN-τ, bovine endometrium, China

INTRODUCTION

Matrix Metalloproteinases-2 (MMP-2) belonges to the family of the Matrix Metalloproteinases (MMPs) and it is involved in degradation of Extracellular Matrix (ECM) (Johnsen *et al*., 1998; Westermarck and Kahari, 1999; Visse and Nagase, 2003). Tissue Inhibitor of Metalloproteinases (TIMPs) is inhibitors of MMPs, it suppress MMPs bioactivity by inhibiting pro-MMPs activation and forming stable complex with pro-MMPs. In the other hand, TIMPs inhibited MMPs activity by linkage to highly conserved zinc binding site of MMPs to form stable complex (Robinson *et al*., 1999; Bourboulia and Stetler-Stevenson, 2010). MMP-2 and TIMP-2 regulated the dynamic changes of degrading and reconstruction of ECM and ECM appears to have a key role in many physical process such as: cellular adhesion, cellular migration and proliferation, wound healing, angiogenesis, ovulation and embryo implantation (Meredith *et al*., 1993; Davis and Senger, 2005; Hynes, 2009). Embryo implantation was a complex process during the whole implantation period. The uterus undergoes dynamic changes including zygote migration, inner cell mass proliferation and trophectoderm-endometrial epithelial cellular adhesion (Carson *et al*., 2000). Therefore, MMPs acted as an important role in embryo implantation. In maternal-fetal interface during peri-implantation period, decidual endometrium and embryonic trophoblast secreted MMPs and TIMPs (Menino *et al*., 1997; Salamonsen, 1999) and MMPs induced embryonic tropohblast invade into maternal endometrium in contrast, TIMPs inhibited the invasion

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process. Hence, the equilibrium of MMPs and TIMPs appears to play a vital role in embryo implantation (Red-Horse *et al*., 2004; Kizaki *et al*., 2008; Ren *et al*., 2010). It has been reported that MMP-2 was important for a variety of biological responses in process of embryo implantation of human and mouse including endometrial decidualization, implantation window formation and vascularization (Rundhaug, 2005; Takagi *et al*., 2007; Klemmt *et al*., 2009).

At the end of the luteal phase, release of prostaglandin F2-α ($PGF_{2\alpha}$) by the endometrial epithelial cells in response to Oxytocin (OT) of pituitary and ovarian origin causes luteolysis (Krishnaswamy *et al*., 2009). The IFN-τ is a cytokine belonging to the type I IFN family (Guzeloglu *et al*., 2004). IFN-τ is a major product of ruminant animals conceptuses during the period before the trophoblast makes firm attachment to the uterine wall and begins to form a placenta and it's the pregnancy recognition signal that prevents development of the endometrial luteolytic mechanism (Roberts, 2007).

It has been demonstrated that IFN-τ inhibits $PGF_{2\alpha}$ production in epithelial cells by preventing the up-regulation of Estrogen Receptor-α (ERα) and OT Receptor (OTR) (Spencer and Bazer, 1996). Therefore, IFN-τ prevented development of the endometrial luteolytic mechanism.

Corpus Luteum (CL) maintenance was the basis of pregnancy establishment because its primary function is to produce the steroid hormone, progesterone (Green *et al*., 2005; Roberts, 2007). Hence, the change of oriented hormone from IFN-τ to progesterone leaded to the transformation from pregnancy recognition to pregnancy establishment.

The principle aim of the study was to determine whether IFN-τ and progesteroe could influence the expression of MMP-2 and TIMP-2 *in vitro* cultured bovine Endometrial Cells (bECs) and further reveal the molecular mechanism of pregnancy recognition turn to embryo implantation.

MATERIALS AND METHODS

Bovine uterine tissues were collected in the slaughterhouse from 8 Holstein cows during luteal phase (6-8 days after ovulation) and kept in 20°C PBS solution including 1000 IU mL^{-1} penicillin and 1000 μg mL^{-1} streptomycin and these tissue were transferred into lab in 1 h. Estrus cycle (luteal phase) of Holstein cows were checked in advance by a recto-ovarian palpation techniques. The procedure of all animal slaughter obeyed Chinese acts of animal welfare and animal slaughtering. All chemical reagents were from Sigma Company and Culture medium and Fetal Bovine Serum (FBS) was from Gibco BRL Company.

Cell culture: The explant culture of bovine endometrial tissue was adopted In primary passage and were cultured in DMEM-Ham's F_{12} medium (Sigma, 034K83101) including 100 IU mL^{-1} penicillin, 100 ug mL^{-1} streptomycin and 10% Fetal Bovine Serum (FBS). The detail operations refer to Parent *et al*. (2003). In primary culture, bECs were collected by trypsinization and centrifugation when cultured bECs grew to 80% confluence. After and adjusting cell concentration to 5×10^5 mL^{-1}, the collected primary cells were sub-cultured in DMEM-Ham's F_{12} medium with free serum but including 100 IU mL^{-1} penicillin, 100 ug mL^{-1} streptomycin and 0.2 nM 17 β-hydroxy-estradiol (E_2, Sigma Co., USA).

Experimental design: Four groups were designed by being treated and untreated with IFN-τ and progesterone. Culture medium in every group was as fellow: Control group (Co group) contains basic medium without IFN-τ and progesterone; IFN-τ group (I_N group), basic medium including 100 ng mL^{-1} IFN-τ; progesterone group (Pr group), basic medium including 200 nmol L^{-1} progesterone (Pr group); combination group of IFN-τ and progesterone (I+P group) contains basic culture medium including 100 ng mL^{-1} IFN-τ and 200 nmol L^{-1} progesterone. The basic medium was DMEM-Ham's F_{12} medium including 100 IU mL^{-1} penicillin, 100 ug mL^{-1} streptomycin and 0.2 nM 17 β-hydroxy-estradiol (E_2). At first, the 1st passage of bECs were divided into 4 groups and respectively cultured in basic medium. Then basic culture medium was changed into 4 kinds different experiment culture medium when bECs adhered to the bottom of culture flask (BD Falcon) and grew to 80% confluence.

RNA extraction: In every group (Co, I_N, Pr and I+P group), monolayer cultured bECs were grown in 75 mL culture flake (Falcon®, Becton Dickinson) loaded 5 mL corresponding culture medium. bECs were harvested and after being cultured for 1, 3, 6 and 12 h. After bECs adhered on the bottom of culture flask were washed 3 times with PBS, the bECs were collected by trypsinization and centrifugation and were washed 3 times with PBS again. Finally, total RNA were extracted from collected bECs with Trizol kit (Invitrogen, 1321067) and a standard chloroform-isopropanol method.

cDNA synthesis: After total RNA concentration were measured and treated with Dnase I, total RNA were

Table 1: Primers information and common PCR reaction condition

Genes	Primer sequence (5'-3')	Reaction condition for common PCR	Product size (bp)	Accession no.
MMP-2	F: CGCCATCCCTGATAACCT	-	-	-
	R: TCCGAACTTCACGCTCTTC	94°C/5 min-(94/30-54°C/30 sec and 72°C/1 min)*30 cycles-7°C/8 min	121	NM_174745
TIMP-2	F: GACTCTGGCAACGACATCTAC	-	-	-
	R: AGGTCCCTTGAACATCTTTATC	94°C/5 min-(94/30-54°C/30 sec and 72°C/1 min)*35 cycles→7°C/8 min	81	NM_174472
GAPDH	F: CGTAACTTCTGTGCTGTGC	-	-	-
	R: GGTGGAATCATACTGGAACA	94°C/5 min-(94/30-54°C/30 sec and 72°C/1 min)*30 cycles-72°C/8 min	190	NM_001040552

astemplate and RT-PCR were executed in 20 uL reaction system (5×RT buffer: 4 uL; 2.5 mM dNTP mixture: 2 μL; 40 IU uL^{-1} RNase inhibitor: 1 uL; 10 pmol uL^{-1} Oligo $(dT)_{20}$: 1 uL; 200 IU uL^{-1} ReverTra Ace: 1 uL; RNA: 2 ug and then added water to 20 μL) under 42°C for 15 min and stopped the reaction under 95°C for 2 min.

Fluorescence Quantitative Real-Time PCR (FQ-PCR): BLAST analyses revealed coeden sequence of MMP-2, TIMP-2 and GAPDH. Real time PCR primers were designed with Oligo 6.0 software (Table 1). Common PCR were performed with cDNA template and then FQ-PCR were executed using different concentration PCR product as template and cDNA as template The detail steps were as following. Firstly, a common PCR was performed for MMP-2, TIMP-2 and GAPDH in 50 uL reaction system (cDNA: 1.5 μL; 2×PCR MasterMix: 25 μL; 10 μmol L^{-1} upstream and downstream primer: 1.5 μL and then added water to 50 μL) and reaction condition as shown in Table 1. After this, PCR products were recollected by Gel-Recovery kit (TakaRa Co., Japan) after 1% agarose-gel electrophoresis. Recollected PCR products were diluted to $1/10^4$ to $1/10^8$ and then real-time PCR were performed with diluted PCR products as template for gaining the standard curve. Finally, real time PCR were performed using cDNA of MMP-2, TIMP-2 and GAPDH as template in order to assess their expression level.

Real time PCR was performed in 20 uL reaction system (2×SYBR® Premix Ex Taq™ (Takara, BK3602): 10 uL; upstream and downstream primer: 0.8 uL, respectively; cDNA: 2 uL and then add water to 20 uL) and reactive condition was 95°C for 10 sec for pre-denaturation then a cycle including 95°C for 10 sec for denaturation, 60°C 30 sec for annealing, 45 cycles. After amplification, melt curve were gained by a melt-curve program.

Data treatment and statistical analysis: When the difference of PCR efficiency of MMP-2, TIMP-2 and GAPDH was <5%, relative expression level = $2^{-\Delta Ct}$. In the formula:

$$-\Delta Ct = (Ct_{Sample\ 1}-Ct_{GAPDH})-(Ct_{Sample\ 2}-Ct_{GAPDH}),\ Ct_{Sample\ 1}$$

Threshold cycle number of MMP-2 or TIMP-2 in I_N, Pr and I+P group; $Ct_{Sample\ 2}$: threshold cycle number of MMP-2 or TIMP-2 in Co group; Ct_{GAPDH}: threshold cycle number of GAPDH. When the difference of PCR efficiency of MMP-2, TIMP-2 and GAPDH was >5%:

$$\text{Relative expression level} = \frac{(1+Ef)^{Ct_{Sample\ 1}-Ct_{GAPDH}}}{(1+Ef)^{Ct_{Sample\ 2}-Ct_{GAPDH}}}$$

Ef: amplification effeciency of real-time PCR. MMP-2 and TIMP-2 relative expression level in different group were compared by one way ANOVA.

RESULTS AND DISCUSSION

Cell culture: In explant culture of bovine endometrial tissue, primary bECs grew out of the tissue after being cultured for 5 days (Fig. 1) and grew to 80% confluence for approximate 11-12 days. bECs could grow in cultured medium with free serum.

FQ-PCR: Regression equations of FQ-PCR standard curve were shown in Table 2. PCR efficiency of GAPDH, MMP-2 and TIMP-2 were 98.2, 100.2 and 98.7%, respectively. The difference of PCR efficiency of MMP-2, TIMP-2 and GAPDH was <5% so, relative expression level were calculated with formula $2^{-\Delta Ct}$ formula. Standard curve, melt curve and PCR amplification curve of G APDH, MMP-2 and TIMP-2 as shown in Fig. 2. That melt curve of GAPDH, MMP-2 and TIMP-2 only had single peak suggested FQ-PCR products were particular.

Relative expression of MMP-2 and TIMP-2: Expression level of MMP-2 and TIMP-2 as shown in Fig. 3. The results showed: MMP-2 expression level in Pr, I_N and I+P group were extremely significantly higher than in Co group ($p<0.01$). MMP-2 expression level in I_N and I+P group was significantly higher than in Pr group ($p<0.05$). MMP-2 expression level has not statistical difference between I_N group and I+P group ($p>0.05$). The results showed progesterone and IFN-τ maybe induced MMP-2 expression *in vitro* cultured bECs. TIMP-2 expression level in Pr, I_N and I+P group was extremely significantly lower than in Co group ($p<0.01$) and TIMP-2 expression level was no statistical difference among Pr, I_N and I+P group ($p>0.05$). The results indicated progesterone and IFN-τ maybe can suppress TIMP-2 expression *in vitro* cultured bECs.

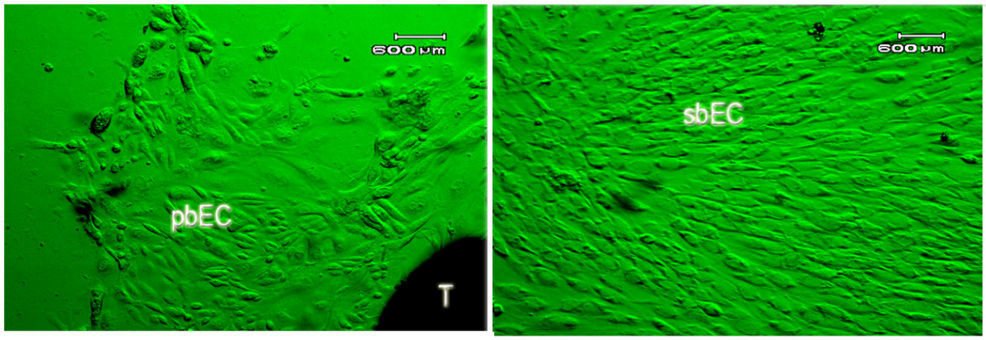

Fig. 1: Culture bovine endometrial cells (Ph1, 100×). pbEC: primary bovine Endometrial Cells; sbEC: subculture bovine Endometrial Cells in cultured medium free serum; T: Tissue explant

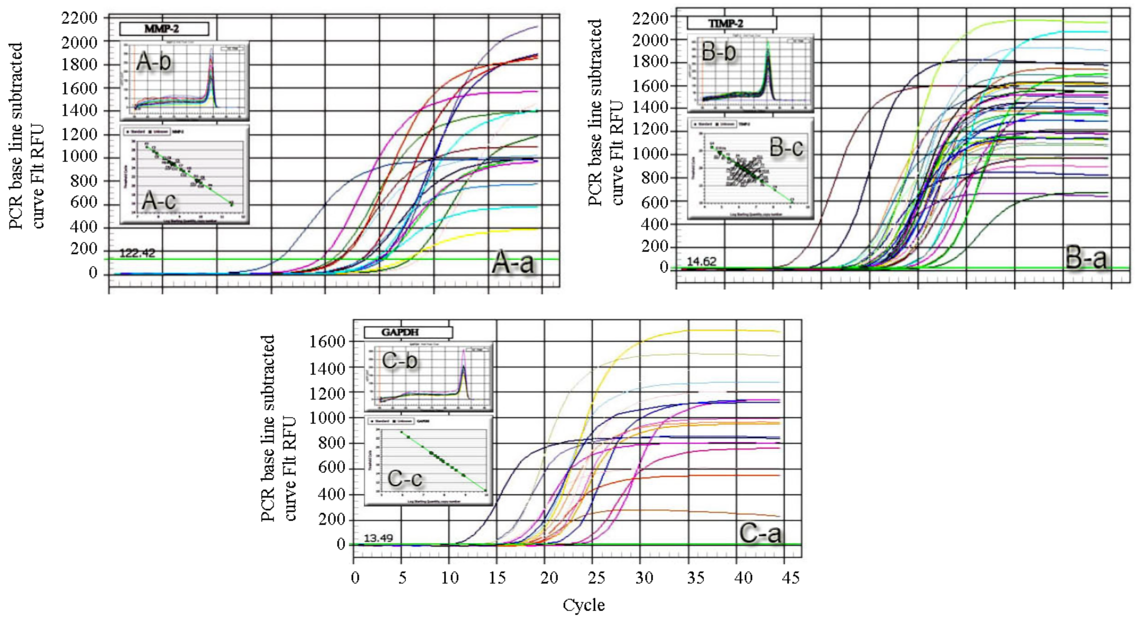

Fig. 2: Standard curve, melt curve and PCR amplification curve of GAPDH, MMP-2 and TIMP-2. A-a: PCR amplification curve of MMP-2 gene; A-b: Melt curve of MMP-2 PCR product; A-c: Standard curve of MMP-2 amplification; B-a: PCR amplification curve of TIMP-2 gene; B-b: Melt curve of TIMP-2 PCR product; B-c: Standard curve of TIMP-2 amplification; C-a: PCR amplification curve of GAPDH Gene; C-b: Melt curve of GAPDH PCR product; C-c: Standard curve of GAPDH amplification

Relative expression level of MMP-2 and TIMP-2 with time-course: Relative expression level of MMP-2 and TIMP-2 with time-course as shown in Fig. 4. In Pr, MMP-2 expression level kept increasing with progesterone treatment time extending, similarly TIMP-2 expression level kept decreasing with progesterone treatment time extending. The result indicated the action that progesterone induced MMP-2 expression and suppressed TIMP-2 expression was durative. Whereas in I_N group, MMP-2 expression level did not kept increasing with progesterone treatment time extending though, the change tendency of MMP-2 expression was increased and TIMP-2 expression level did not keep decreasing with progesterone treatment time extending though the change tendency of TIMP-2 expression was decreased. The results shown the action that IFN-τ induced MMP-2

Table 2: Equation of standard curve of real-time FQ-PCR (Ct: threshold Cycle number; St: Starting quantity; copy number; R^2: determination coefficient)

Genes	PCR efficiency (%)	Equation of standard curve	R^2
GAPDH	98.2	Ct = -3.366LgSt+46.487	1.000
MMP-2	100.2	Ct = -3.197LgSt+44.715	0.993
TIMP-2	98.7	Ct = -3.353LgSt+41.105	0.999

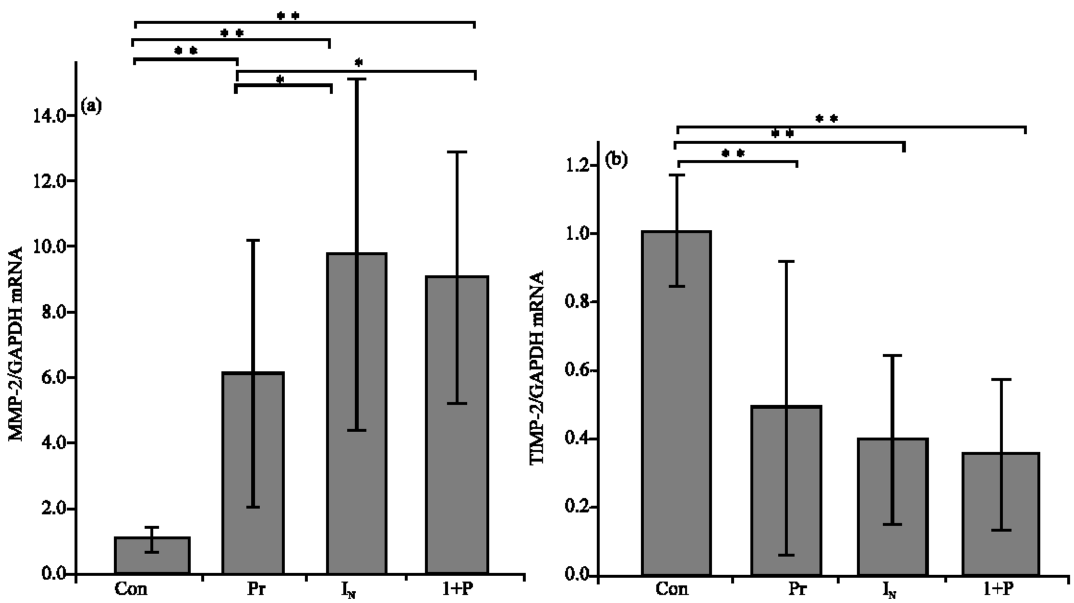

Fig. 3: Expression level of MMP-2 and TIMP-2 in different experiment groups. Con-controlled group, culture medium without progesterone and IFN-τ; Pr-progesterone group, culture medium with 200 nmol L^{-1} progesterone; I_N-IFN-τ group, culture medium with 100 ng mL^{-1} IFN-τ; I+P-Combination of IFN-τ and progesterone group, culture medium with 200 nmol L^{-1} progesterone and 100 ng mL^{-1} IFN-τ. *Means significantly difference ($p<0.05$); **Means extremely significantly ($p<0.01$)

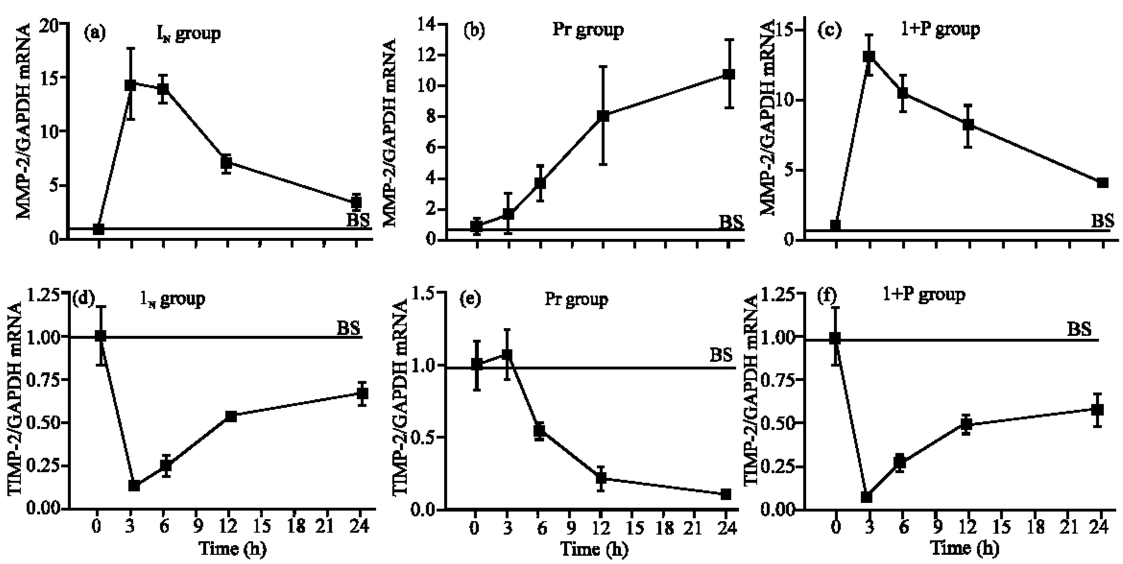

Fig. 4: Relative expression level of MMP-2 and TIMP-2 with time-course. Con-controlled group, culture medium without progesterone and IFN-τ; Pr-Progesterone group, culture medium with 200 nmol L^{-1} progesterone; I_N-IFN-τ group, culture medium with 100 ng mL^{-1} IFN-τ; I+P-Combination of IFN-τ and progesterone group, culture medium with 200 nmol L^{-1} progesterone and 100 ng mL^{-1} IFN-τ. BL-Baseline: relative expression fold (ratio of target gene and inner reference gene) is equal to 1

expression and suppressed TIMP-2 expression was not durative. In I+G group, the change tendency of MMP-2 expression and TIMP-2 expression with time-course was similar with the I_N group. MMP-2 and TIMP-2 regulated the dynamic changes of degrading and reconstruction of ECM and ECM was important for many physical process such as: cellular adhesion, cellular migration and proliferation, wound healing, angiogenesis, ovulation and embryo implantation (Yamamoto *et al.*, 2000) so, MMP-2 and TIMP-2 widely expressed in many kinds of cells and tissue. Many factors such as stress, pathogen infection, homeostasis status and so, on could impact on *in vivo* expression level of MMP-2 and TIMP-2. Therefore *in vitro* cultured bECs was as a model to assess the effects of INF-τ and progesterone on expression level of MMP-2 and TIMP-2. It was an effective method that *in vitro* cultured cell acted as a model to assess MMP-2 and TIMP-2 expression (Classen-Linke *et al.*, 1997, 2000). In the present studies, primary bECs were cultured in medium including FBS and FBS could promote cell adhesion and proliferation but FBS contained much complicated substance such as hormone, cytokines, chemokines and so on. Therefore, cultured medium was not including FBS in subculture and different experiment group. A 24 h serum starvation method often was used in *in vitro* cultured cell model (Zhang *et al.*, 1994; Wang *et al.*, 2003). E_2 was important for female animal to maintain normal physical process, likewise embryo implantation and pregnancy depended on the subtle regulation of E_2 (Hulboy *et al.*, 1997; Milligan and Finn, 1997). Therefore, the objective that basic culture medium was added a low-concentration of E_2 (0.2 nM) was to provide similar *in vivo* hormone environment to bECs. Progesterone is a type of steroid hormone, it was mainly secreted by CL and widely regulated embryonic imbedding, pregnancy establishment, gestation and estrus cycle (Barrera *et al.*, 2007).

The present studies shown that progesterone promoted expression of MMP-2 moreover the tendency of MMP-2 expression increasing possessed time-dependent. The reason maybe was that progesterone is steroid and it has long half-life period and biological effect (Wang *et al.*, 2008; Goldman *et al.*, 2009). The studies reveal that progesterone from trophoblast directly and indirectly inhibited MMP-2 expression and secretion *in vivo* experiment.

The inhibitory effect of MMP-2 expression and secretion would blocked once progesterone antagonist was administrated. Zashizume *et al.* (2003) have investigated effects of progesterone on MMP-2 expression *in vitro* culture cell, the results indicated that low-dosage of progesterone (3 nM) suppressed MMP-2 expression and high-dosage of progesterone (>30 nM) induced MMP-2 expression. Different dosage of progesterone leaded to the different changes of MMP-2 expression which revealed progesterone acted as a subtle mediator in embryo implantation. In mouse embryo implantation, VEGF induced MMP-2, MMP-9 (Li *et al.*, 2002) and MMP-26 (Zhang *et al.*, 2002) and then induced formation of implantation window in endometrium. Hicks *et al.* (2003) studied MMPs expression in sow, mare and bovine endometrium in gestation with immnuohistochemistical localization. The results shown that MMPs expression obviously increased. TIMP-2 inhibited mouse embryonic trophoblast invaded maternal endometrium (Behrendtsen *et al.*, 1992). Actually, the reason that TIMP-2 blocked trophblast invasion was that MMP-2 expression and secretion was suppressed by TIMP-2 and then implantation window couldn't form for lack of MMP-2.

The results indicated the equilibrium of MMP-2 and TIMP-2 was important to embryo properly implant into endometrium. In the present studies, bIFN-τ induced MMP-2 expression andinhibited TIMP-2 expression therefore, bIFN-τ promoted the formation of implantation window in endometrium.

In vitro cultured bECs experiment, 100 ng mL^{-1} IFN-t in cultured medium did not effect MMP-2 expression but 600 ng mL^{-1} IFN-τ in cultured medium obviously inhibited MMP-2 expression *in vitro* cultured bECs. When 3 pM E_2 was added into cultured medium MMP-2 expression obviously increased (Zashizume *et al.*, 2003). Progesterone Antagonist (PA, PRM) inhibited expression of MMPs and TIMPs in human and macaco endometrium (Marbaix *et al.*, 1996; Rudolph-Owen *et al.*, 1998; Hampton *et al.*, 1999) the results implied progesterone maybe could induce expression of MMPs and TIMPs.

CONCLUSION

In the present studies, progesterone inhibited TIMP-2 expression whereas MMP-2 expression was induced. MMP-2 expression increasing and TIMP-2 expression decreasing was better for embryo implantation so, progesterone prompted embryo implantation. But progesterone promoted TIMP-2 expression in primate animal endometrium. TIMP-2 expression difference between cattle and primate animal in per-implantation endometrium maybe was due to the number difference of implantation window: Endometrium of primate animal formed one large implantation window in during pre-implantation period (Xia *et al.*, 2001) whereas, bovine endometrium formed many implantation windows in caruncular area and bovine endometrial surface exhibits deep caruncular crypts which are penetrated by long, profusely branched cotyledonary villi of fetal chorioallantois (Davis and Senger, 2005). Similarly, MMP-2 expression level was associated with the number of implantation window in endometrium, MMP-2 had a relative high expression in bovine endometrial caruncular areas but endometrium of primate animal, MMP-2 only had a relative high expression in implantation window. The process of progesterone regulated expression of TIMP-2 and MMP-2 in endometrium was complicated. In different endometrial area (caruncular or intra caruncular) and different uterine phase (embryonic implantation period, estrus cycle, gestation), MMP-2 and TIMP-2 expression should be different. Therefore, the more studies on localization and expression of MMP-2 and TIMP-2 in bovine endometrium was necessary.

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