# Effects of EGF and PAF on the Hatching and Implantation of Peri-implantation Stage Embryos

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**ABSTRACT** : A fertilized oocyte can get the competence for implantation through cleavage and stage-specific gene expression. These are under the control of autonomous and exogenous regulators including physiological culture condition. Endogenous and exogenous growth factors are considered as critical regulators of cleaving embryos during travel the oviduct and uterus. In this study, an effort was made to evaluate comprehensively the quality of embryos for implantation, grown in media enriched with EGF and PAF. The study evaluated developmental rates on given time, blastulation and hatching rates, and adhesion rates. Developmental rates of blastocyst to the hatching stage were significantly high in PAF treated group compared to the control in a dose-dependent manner but not in EGF group. Implantation rates were significantly high both PAF and EGF in a dose-dependent manner. H7, a PKC inhibitor, blocked the process of hatching of the blastocysts but combined treatment of EGF and PAF enhanced the hatching and implantation of blastocsyts. Based on these results it is suggested that EGF and PAF support acquirement of implantation competence at blastocyst stage through a PKC pathway.

**Key words** : EGF, PAF, PKC, Hatching, Implantation.

## INTRODUCTION

After fertilization, the activated egg starts to duplicate the chromosome and progresses cleavage. During this period the fertilized egg becomes a blastocyst and finally implant on the uterine endometrium. The competence of embryos for implantation is including the embryonic stage paring with the uterine physiological condition, adhesion ability of trophectoderm, and the number of cells in inner cell mass (Bazer et al., 2009; Cheon et al., 2002). The implanting ability of blastocyst is depending on proper differentiation during cleavage, and on the maternal transcripts and embryonic transcripts which are expressed stage-specific manners. Induction of stage- and time-dependent gene expression is primarily autonomous. Secondly, it is accomplished by communication between cells of embryo and female reproductive tracts (Hayashi et al., 2007).

Usually the fertilized rodent eggs progress to the blastocyst stage in the simple or complex media with or without serum. However, retarded cleavage is observed during *in vitro* culture regardless of complexity of media compared with the *in vivo* development and it is hard to get blastocyst stage at 96 hr post hCG injection (Jones et al., 1998; Pribenszky et al., 2010; Van der Auwera & D'Hooghe, 2001). In addition the implantation capacity is dramatically decreased. Therefore to get a high pregnancy rate in embryo transfer usually one-cell stage to the morula stage embryos cultured *in vitro* are transferred into the ampulla of 0.5-day p.c. pseudopregnant recipients, and blastocysts are transferred into the uterine horns of a 2.5 day p.c. pseudopregnant recipient mouse (Hogan et al., 1994; Peters et al., 2006).

It is thought that the physiological microenvironment

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may be the main reason of the delayed development *in vitro*. Growth factors are paracrine factors and may mediate the communication between cleaving embryo and reproductive tracts, oviduct and uterus (Guillomot et al., 1993; O'Neill, 1987). Growth factors and their receptors are also expressed in spatio-temporally in early stag embryos. Although the cleaving embryos can synthesis their own growth factors (Dadi et al., 2004), under the *in vitro* condition the developmental speed of preimplantation embryos is not become similar with those of *in vivo*. However, addition of single growth factors or serum to the medium does not support their extensive development (Pratten et al., 1988). It may be the result of complex interactions between the gene products expressed in embryo and extraembryonic cells.

Embryo expresses its own EGF after 2-cell stage and EGF level steadily increased until the blastocyst stage (Dadi et al., 2004). Post-trascriptional suppressions of EGF and EGFR decrease blastocyst formation, increase the number of apoptotic cells, and reduce the total number of differentiated cells in blastocsyt (Dadi et al., 2009). EGF and EGFR are also expressed in the oviduct and endometrium during early embryo stages (Wollenhaupt et al., 2004). In the case of PAF and PAFR, they are synthesized during preimplantation stages of all mammalian species studied to date (O'Neill, 2005; Purnell et al., 2006). PAF receptor and PAF acetylhydrolase express in the fallopian tube and endometrium (Dearn et al., 2000; Velasquez et al., 2001). PAF acts in an autocrine fashion as a trophic/survival factor for the early embryo (Harper, 1989).

In the case of some growth factors, their synthetic amounts are lower than that of the embryo of *in vivo* (Stojanov & O'Neill, 2001). On the other hand, some kinds of growth factors have been used to get high quality embryos (Richter, 2008). However it is not completely support the embryonic development and it is needed to more detail research about various kinds of growth factors. EGF and PAF are known as a survival factor in preimplantaiton embryos. However their effects in implantation capacity did not well evaluated. Therefore in this study the possible effects of EGF and PAF were examined.

## MATERIALS AND METHODSES

### 1. Growth Factors and Other Reagents

EGF was purchased from Sigma (St. Louis, MO, USA), PAF was purchased from Biomol (Enzo Life Sciences, Inc. Plymouth Meeting, PA, USA). All other culture grade chemicals and reagents were purchased from Sigma unless otherwise specified.

### 2. Animals

All experiments involving animals were conducted in accordance with NIH standards for the care and use of experimental animals. Female CD-1 mice 6-8 weeks old were superovulated to obtain embryos. Animals were injected with 5 IU (intraperitoneal) of pregnant mare serum gonadotrophin. A second injection of 5 IU of human chorionic gonadotrophin (hCG) was given 48 hr later and the animals were mated with fertile CD-1 male. The day of vaginal plug was designated Day 1 of pregnancy.

### 3. Embryo Collection and Culture

Female CD-1 mice, 6-8 weeks old, were superovulated to obtain 8-cell stage embryos. Animals were injected with 5 IU (interperitoneal) of pregnant mare serum gonadotrophin. A second injection of 5 IU of human chorionic gonadotrophin (hCG) was given 48 hr later and the animals were mated with fertile CD-1 male (day 1 = vaginal plug). The mice were sacrificed at 72 hr post-hCG injection by cervical dislocation and utero-tubal junction area was excised. The utero-tubal junction was flushed with BWW medium. Only healthy 8-cells were used in the examination. The embryos were randomly allocated to the experimental groups. Embryos were cultured in groups of 10 - 12 in 10 $\mu\ell$ media drops over-layered with mineral oil at 37℃ in a humidified incubator with 5% $CO_2$ in air. BWW (containing 0.4% BSA) was used as plane medium. EGF

was prepared as a 10 ㎎/㎖ stock solution and stored in 50 ㎕ aliquots at −20℃ until use. PAF was prepared as a 100 μM stock solution and stored in 20 ㎕ aliquots at −20℃ until use. The embryos were grown in BWW medium alone until 96 hr post hCG injection and further cultured with or without EGF (1 pg, 10 pg, 100 pg, 1 ng, 10 ng, and 100 ng) or PAF (1 pM, 500 pM, 1 nM, 500 nM, and 1 ㎛M). To evaluate the involvement of PKC mediated signaling pathway in the development of blastocsyt, embryos were treated with 1-(5-isoquinolinesulfonyl)-2- methylpiperazine (H7, 50 ㎛M and 100 ㎛M) and growth factors from 96 hr time point post hCG injection to 144 hr time point post hCG injection. Twelve to fifteen replicates were performed for each experiment. The embryos were inspected every 24 hr for progression of growth through blastocyst stage up to the spreading stage, which may represent the ability of early implantation (Hogan et al., 1994). The ability of adhesion *in vitro* was examined and compared between the groups by the appearance of the adhesion.

### 4. Statistical Analysis

All data represent 9 or more experimental replicates. Embryonic development was analyzed using a Chi-square or paired student *t*-test. Rates of hatching and implantation were compared between culture regiments using the Chi-square test. Treatments with a *p*-value of <0.05 were taken to be significantly different. Data was expressed as mean ± SE.

## RESULTS

### 1. PAF supported the Hatching

Previously in our study the optimal concentration of PAF for improving the hatching rate of blastocyst was 1 nM (Cheon, 2006). Usually the chemical-signal molecules have dual roles in stimulation role of the concentration. Therefore, to evaluate the optimal dose range, experiment was designed as mentioned in Materials and Methods. As seen in Fig. 1, the results gave more critical information than previous one. The response of blastocyst to PAF was clearly shown a bell-shaped dose-response curve. The hatching rates were significantly high in the doses between 500 pM to 1 nM (Fig. 1).

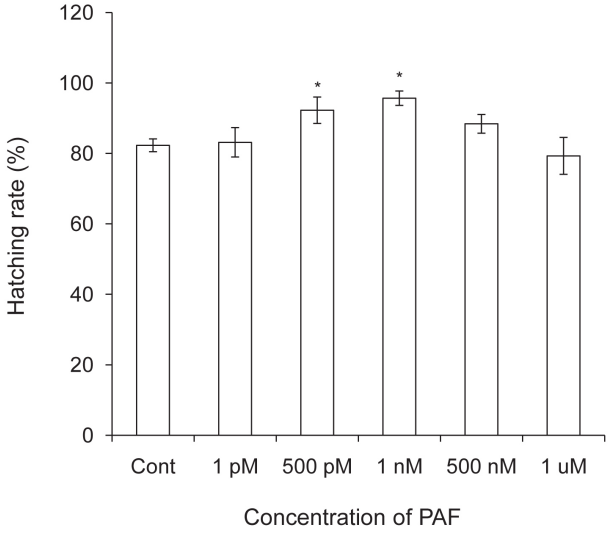


**Fig. 1. PAF enhanced the development of blastocyst to hatching stage embryos.** Embryos were collected at 72 hr post hCG injection from oviduct-uterine junction and cultured in BWW medium. PAF was treated at 96 hr time point post hCG injection and further cultured for 48 hr. **P*<0.05 vs control.

### 2. EGF did not supported the Hatching

Heparin binding-epidermal growth factor, one of the members of EGF family, is known as a regulator of hatching (Seshagiri et al., 2009). However, the role of EGF in hatching is not clear and controversial, so far, in its possible role. In here, the possible role of EGF was examined in blastocyst. EGF did not improve the developmental capacity of blastocyst to the hatching stage. The highest hatching rate in the EGF treated group was 89.58% (100 pg) but there was no statistical significance compared with control (Fig. 2).

### 3. PAF improved the Ability of Implantation

Within a narrow range of doses PAF improved hatching

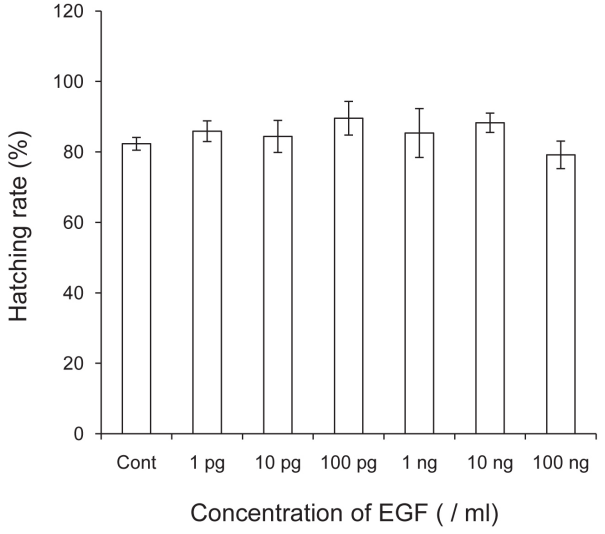


**Fig. 2. EGF did not enhance the development of blastocyst to hatching stage embryos.** Embryos were collected at 72 hr post hCG injection from oviduct-uterine junction and cultured in BWW medium. EFG was treated at 96 hr time point post hCG injection and further cultured for 48 hr.

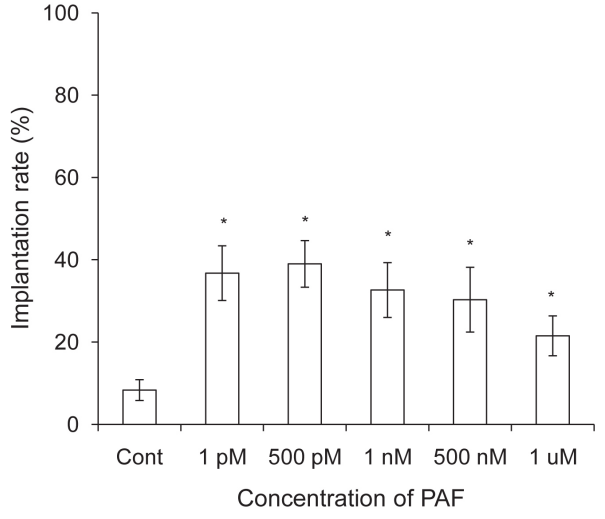


**Fig. 3. PAF enhanced the implantation competence of blastocyst.** Embryos were collected at 72 hr post hCG injection from oviduct-uterine junction and incubated in BWW medium. PAF was treated at 96 hr time point post hCG injection and further cultured for 48 hr. **P*<0.05 vs control.

ability of blastocyst. To evaluate the possible effects in implantation, attachment of blastocsyt was analyzed at 48 hr after PAF treatment (144 hr time point after hCG injection). In control group, the attachment rate was 8.31% but it was dramatically increased by PAF (1 pM; 36.8%, 500 pM; 39.0%, 1 nM; 32.7%, 500 nM; 30.3%, and 1 μM; 21.5%). There was statistical significance in all groups (Fig. 3).

### 4. EGF improved the Ability of Implantation

In contrast to PAF, EGF did not give significant effect on hatching of blastocyst, but the hatching and implantation are different events. So, to examine the effect of EGF on attachment, the embryos were cultured in the media containing EGF. Compared with the control (8.32%) the attachment rate was dramatically high in EGF group. There was statistical significance in 1 pg, 10 pg, 100 pg, 1 ng, and 10 ng groups but in 100 ng group (Fig. 4). It means that EGF can improve the implantation ability of blastocyst to the uterine epithelium.

### 5. PAF and EGF may improve the Implantation through PKC Pathway

To investigate how PAF and EGF show the same effect on implantation, activity of a key molecule of the common cellular pathway of PAF and EGF was inhibited with its specific antagonist. H7 (50 μM) totally block the hatching and there was no attachment (Fig. 5). Cotreatment of 50 μM H7 and 10 ng/㎖ EGF supported to the hatching stage but it was very limited (9.65% hatching). The hatching rate was similar between 50 μM H7+EGF and 100 μM H7+EGF. Implantation rates were 3.5% and 2.9% in 50 μM H7+EGF and 100 μM H7+EGF, respectively. In the case of PAF, it improved dramatically the development of blastocyst into the next steps (hatching and implantation) under the PKC inhibition by H7 treatment. Hatching rate was significantly increased (0% vs. 46%) in 50 μM H7+50 μM PAF. But its recovery effect was dependent on H7 concentration (46% in 50 μM H7 and 0% in 100 μM H7). In the case of implantation, PAF increased the implantation chance up to 18% in 50 μM H7 but was could not at 100 μM H7 (Fig. 5).

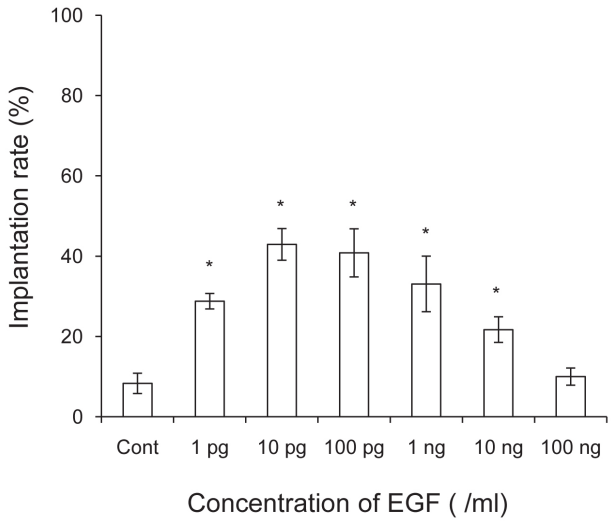


**Fig. 4. EGF enhanced the implantation competence of blastocyst.** Embryos were collected at 72 hr post hCG injection from oviduct-uterine junction and incubated in BWW medium. EGF was treated at 96 hr time point post hCG injection and further cultured for 48 hr. **P*<0.05 vs control.

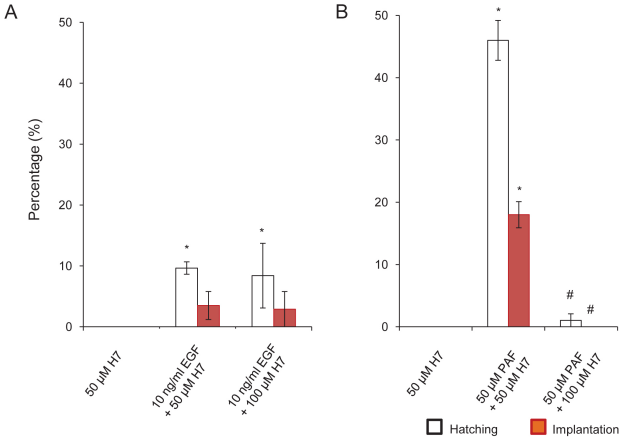


**Fig. 5. PKC inhibitor, H7 blocked the differentiation of blastocyst but EGF and PAF counter act the effect of H7.** H7 inhibit the effects of EGF on the development of blastocyst to hatching stage in a dose independent manner. On the other hand, H7 inhibit the effects of PAF on the development of blasytocyst to hatching stage in a dose dependent manner. The implantation competence of blastocyst also showed same pattern both in PAF and EGF with the development of blastocyst to hatching stage. **P*<0.05 vs control; #, P, 0.05 vs 50 $\mu$M PAF+100 $\mu$M H7.

## DISCUSSION

Implantation competence of blastocyst was accomplished during cleavage. It is known that many factors are involved in the process of that. In pluriparious animal, a cooperative interaction occurs among preimplantation embryos in culture, and it is mediated by certain growth factors in an autocrine manner (Kang et al., 1997; Paria & Dey, 1990). During development of early stage embryo, EGF plays mitogenic growth and works as a survival factor. siRNA of EGF increases the number of apoptotic cells and reduced the total number of differentiated cells in blastocyst (Dadi et al., 2009). EGF significantly increases blastocyst formation when embryos are cultured in EGF containing media from very early stage (Dadi et al., 2009; Kurzawa et al., 2001). Our early study tested whether exogenous EGF has beneficial effects on the expression of EGF receptor. EGF caused change in the expression level of its receptor in preimplantation embryo (Cho et al., 1998). It means that, exposing of exogenous EGF can stimulate the embryonic development autonomously. Although EGF is a supporter for viability of early stage embryos, the effect of EGF in hatching is controversy (Kurzawa et al., 2001). Some groups suggested that EGF is a helper to sustain the viability of preimplantation embryo but it is not improving the hatching of blastocyst (Kurzawa et al., 2001). On the other hand, it was suggested that EGF helped the development of preimplantation embryos and hatcing (Kim & Fortier, 1995; Yeo et al., 2008). Interestingly, when EGF was treated at blastocyst stage, it did not improve the development of blastocyst to hatching stage. On the other hand, however, it could improve significantly the implantation ability of blastocyst. Based on these results it is suggested that EGF regulate the differentiation of trophectoderm for the ability of implantation.

PAF is also known as a trophic ligand in preimplantation embryo. Early preimplantation embryos express PAF and its receptor (Stojano & O'Neill, 1999). Endogenous PAF actions are mediated through its membrane receptor

to enhance embryo survival by 1-0-phosphatidylinositol-3-kinase (Lu et al., 2004). Blockade of PAF signaling may results in increased TRP53 expression and the trophic signaling that reduce normal embryo development to blastocyst. The increased TRP53 expression is responsible for the loss of developmental competence. High level of TRP53 expression is detected in PAF receptor knockout periimplantation embryo. PAF caused increase of phosphorylation of MDM2, to ubiquitinate and to degrade the TRP53 (Jin et al., 2009). Therefore, PAF does not act as a classical growth factor (works through specific cell-cycle checkpoints) but rather act to enhance the survival of cells within the embryo (Stojano & O'Neill, 1999). As expected, the exogenous PAF improved development of blastocyst to hatching stage embryo in a concentration dependent manner. It showed a bell-shaped growth response to the concentration showed other growth factors (Rankin & Rozengurt, 1994; Schwall et al., 1996).

Ryan and colleague (1990) cultured 2-cell embryo in the media containing PAF, and showed that PAF could not increase the development rate to expanded blastocyst stage. Although the treated embryonic stage was different from their experiment (1990), the high dose of PAF (1 $\mu$M) could not support the development of blastosyst to expansion and hatching. The low doses of PAF can support significantly the development of blastocyst. PAF was work as a supporter for the differentiation of blastocyt to hatching and attachment.

The effects on implantation were same both in EGF and PAF. They support significantly the attachment of trophectoderm compared with the control. Tyrosine phosphorylation of the EGFR leads to the recruitment of diverse signaling proteins, including phospholipase-C-Gamma (PLC-$\gamma$), Signal transducer and activator of transcription (STAT), and several other proteins. PLC-$\gamma$ catalyses the hydrolysis of PIP2, generating the second messengers 1,2-diacylglycerol (DAG) and inositol triphosphae (IP3). DAG is the physiological activator of PKC, which in turn leads to phosphorylation of various substrate proteins that are involved in an array of cellular events. PKC also leads to the activation of I-KappaB-Kinases (IKKs), and finally nuclear factor (NF)-KappaB-dependent transcription (Wang et al., 2006). PAF receptor signaling also includes the PKC pathway (Dearn et al., 2000; Kim and Fortier, 1995). Based on them it is expected that EGF and PAF improved the implantation capacity of blastocyst through PKC mediated pathways. This expectations was conformed to PKC inhibitor, H7 (Romero et al., 1992). H7 (50 $\mu$M) totally blocked the hatching of blastocyst, but cotreatment with EGF or PAF supported the hatching of the blastocyst. In implantation capacity, PAF had more strong effect than EGF because the attachment rate was significantly high in PAF-cotreated group.

In summary, this study explored the effect of PAF and EGF in blastocyst. PAF could support the hatching of the blastocyst and implantation. On the other hand EGF could support the implantation but not hatching of the blastocyst. It means that PAF and EGF work through their membrane receptors and share common signaling pathway. One of the possible signaling pathways is PKC mediated signal transduction. Cotreatment of PAF and EGF with H7 could support the implantation. However, other pathway which is not shared between PAF and EGF may involve in the blastocyst development to get implantation competency. The finding of the present study provides a foundation for future experiment directed at uncovering the pathways of hatching and attachment of trophectoderm.

## ACKNOWLEDGEMENTS

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