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RESEARCH ARTICLE

Expression of Epidermal Growth Factor (EGF) and Heparin-Binding EGF (HB-EGF) mRNA in Mare Endometrium [1]

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Summary

EGF and HB-EGF play crucial roles in embryonic development and peri-implantation. In this study, aim was to characterize expression profiles of EGF and HB-EGF in mare endometrium during estrous cycle and early pregnancy. Endometrium biopsies were obtained from mares on day of ovulation (d0), late diestrus (LD) and after luteolysis (AL) in the estrus phase. In pregnant groups, biopsies were taken on days 14 (P14), 18 (P18), 22 (P22) and 60 (P60). Relative expression levels of genes were quantified using real-time RT-PCR. A mixed model was fitted on the normalized data and Least Significant Difference (LSD) test was employed to determine significantly different group(s). EGF mRNA expression was up-regulated at LD compared to d0 while HB-EGF expression was not changed throughout the cycle. EGF expression was also increased during early pregnancy with the highest expression level observed on P60. Similarly, HB-EGF mRNA level was increased on P60. Pregnancy induced EGF expression on P14 and P18 compared to LD and AL whereas expression of HB-EGF was only significantly higher on P18 than that of AL. These results indicate that EGF expression is up-regulated during the cycle at late diestrus when P4 is high and is increased by pregnancy. HB-EGF expression is induced later in the pregnancy. In conclusion, EGF and HB-EGF appear to involve in the events that happen in the mare endometrium during peri-implantation period.

Keywords: *EGF, HB-EGF, Mare, Endometrium, Pregnancy*

Kısrak Endometriumunda Epidermal Growth Factor (EGF) ve Heparin-Binding EGF (HB-EGF) mRNA Ekspresyonu

Özet

EGF ve HB-EGF implantasyon sırasında ve embriyonik gelişim esnasında çok önemli roller oynamaktadır. Bu çalışmanın amacı, erken gebelik ve östrus siklusunda at endometriumunda EGF ve HB-EGF ekspresyon profilini karakterize etmektir. Endometrium biyopsileri östrustaki kısraklardan ovulasyon gününde (d0), geç diöstrusta (LD) ve luteolizis sonrası (AL) dönemlerde toplandı. Gebe kısraklardan ise gebeliğin 14. (P14), 18. (P18), 22. (P22) ve 60. (P60) günlerinde biyopsiler toplandı. Nispi mRNA ekspresyon seviyeleri real-time PZR kullanılarak tespit edildi. Normalize edilmiş veriler karışık model kullanılarak analiz edildi ve istatistiki olarak farklı olan gruplar Asgari Önemli Fark (AÖF) testi ile farklı gruplar tespit edildi. EGF mRNA ekspresyonu LD'de d0 göre artmasına rağmen, HB-EGF ekspresyonu siklus boyunca değişmedi. EGF ayrıca erken gebelik boyunca arttı ve en yüksek seviyeye 60. günde ulaştı. Benzer şekilde HB-EGF mRNA seviyesi 60. günde arttı. Gebelik, EGF ekspresyonunu 14. ve 18. günlerde LD ve AL göre arttırırken, HB-EGF sadece gebeliğin 18. gününde AL'e göre önemli oranda arttı. Bu sonuçlar, EGF'nin P4 seviyesinin yüksek olduğu geç diöstrusta ve gebeliğe bağlı olarak arttığını göstermektedir. HB-EGF ise gebeliğin daha ileri dönemlerinde uyarılmaktadır. Sonuç olarak, kısrak endometriumunda EGF ve HB-EGF'nin peri-implantasyon dönemindeki görülen olaylara katıldığı gözlenmiştir.

Anahtar sözcükler: *EGF, HB-EGF, Kısrak, Endometrium, Gebelik*

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INTRODUCTION

The several signaling pathways inside uterus regulated by both embryonic and maternal factors are crucial for establishing and maintaining of pregnancy. As material side, endometrium has to proliferate, reorganize and secrete to support successful pregnancy [1]. All these processes are thought to be controlled by ovarian steroid hormones and local growth factors [2]. Effects of autocrine, paracrine and endocrine factors are coordinated during this dynamic process. When compared to the other farm animals, mare implantation process starts later and molecule(s) which plays roles in maternal recognition of pregnancy are unknown [3,4]. It is only known that equine embryo has to move between uterine horns from day 10 to 16 of pregnancy in order to block luteolytic activity [5]. During pre-implantation period, nutritional requirements of embryo have to be supplied by the uterus. Therefore, the secretory products of endometrial glands appear to be a primary source of nutrition for embryonic growth during the first 40 days of early pregnancy in mares [4,6].

The epidermal growth factor (EGF) families, which are secreted signaling molecules, have capacity for inducing proliferation and differentiation in all cell types. They are synthesized as transmembrane protein and need a proteolytic process to reach their mature forms [7]. These factors bind to their specific cell surface receptors that have a tyrosine kinase domain. When ligand binds, intracellular signaling cascade are activated by phosphorylation of some mitogen-activated protein kinase (MAPK) and Sma- and Mad-related family (SMAD) proteins [8,9]. Both EGF and heparin-binding EGF (HB-EGF) belong to EGF family which plays important roles in proliferation and differentiation of several organs [10]. EGF is accepted as a powerful mitogen and has several important regulatory roles in endometrium-trophoplast interaction, endometrial and placental growth in sheep, mare, human and rat [11-15]. EGF interacts with ErbB gene family-I including ErbB1 (EGF-R), ErbB2, ErbB3 and ErbB4 to transmit its message [8]. HB-EGF requires heparine sulfate proteoglycan as a cofactor for binding to its receptor. Similar to EGF, HB-EGF involves in endometrial functions [10]. Especially, implantation process is regulated by HB-EGF in rat and human [16,17] but there is no data to demonstrate expression profiles of HB-EGF in equine endometrium during peri-implantation period.

Therefore, the present study aimed to evaluate expression profiles of EGF and HB-EGF genes at mRNA level during early pregnancy and estrous cycle in order to make a contribution to understanding establishment and maintaining of early pregnancy in mares.

MATERIAL and METHODS

All experimental procedures were approved by the Ethics Committee of Faculty of Veterinary Medicine at Selcuk University (#2007/40 on 23.08.2007). Ten reproductively sound mares and a stallion were used as animal materials. Fertility examination, housing and feeding of animals, insemination procedure, and detection of pregnancy, definition of estrous cycle in mares and collection of biopsy samples were described by Atli et al.[18]. Biopsy samples were collected from mares on day of ovulation (d0, n=4) and on days of 14 (P14, n=4), 18 (P18, n=4), 22 (P22, n=4), 60 (P60, n=2) of pregnancy. Endometrial biopsies were also obtained from mares on late diestrus (LD, high progesterone [P_4], on day of 13.5-14, no edema, n=4), and after luteolysis in the estrus phase (AL, low P_4, on day of 17.5-18 obvious edema, n=4) of the cycle that were comparable days to pregnancy sampling days (P14 and P18). Isolation and quality control procedures of RNA and preparation of cDNA samples were described by Kurar et al.[19].

Primers for EGF and HB-EGF genes were derived from equine sequences by using Primer3 from NCBI *(http://www.ncbi.nlm.nih.gov/)* database. The primer pair sequences and product sizes are shown on *Table 1*.

Real-time PCR was used to evaluate the expression profiles of HB-EGF and EGF. The reaction was set up as follows: 10 µl SYBR Green Master Mix (2´), 5 pMol of each primer, 1µl cDNA and ddH_2O up to 20 µl of final volume. Thermal cyclic conditions were initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation, annealing and amplification (95°C 30 sec, 60°C 1 min, 72°C 30 sec) on a Mx3005P ™ 3005 real-time PCR system. From the RNA extraction to the real-time PCR, whole procedure was performed twice as technical replicate. As for house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected according to Kayis et al.[20].

For statistical analysis, data normalization process was performed via $2^{-\triangle C'T}$ method, where $\triangle C'_T = C_{T,\text{ target}} - C_{T,\text{ reference}}$ (where $C_{T,\text{ target}}$ and $C_{T,\text{ reference}}$ are threshold cycles for target and reference genes amplifications, respectively). A mixed model was fitted on the normalized data and least significant difference (LSD) test was employed to determine significantly different groups [18]. All analyses were carried out by using Genstat [21] (Release 7).

RESULTS

Expression of EGF and HB-EGF were detected in equine endometrium *(Fig. 1)*. The steady-state concentrations of mRNA for EGF are shown in *Fig. 2*. The concentration of mRNA for EGF was increased significantly at LD during the estrous cycle. Expression of EGF mRNA was also up-regulated both on days P14 and P60. The greatest elevation of EGF expression was observed at P60. When compared to LD, EGF mRNA level was increased significantly on P14.

The steady-state concentrations of mRNA for HB-EGF

are shown on *Fig. 3*. Compared to d0, HB-EGF mRNA did not show significant changes in both LD and AL. There was only a dramatic increase in mRNA concentration for HB-EGF on P60 during the pregnancy. When AL and P18 were compared, expression level of HB-EGF mRNA increased on P18.

DISCUSSION

In the present study, expression of HB-EGF and EGF at mRNA levels were determined in equine endometrium and also their expression profiles during the early pregnancy and estrous cycle were shown. Biopsy day ranges (d0, P14, P18, P22 and P60) allow us to examine expression of EGF and HB-EGF throughout early pregnancy including pre-implantation and implantation periods. To understand effect of embryo, expression of EGF and HB-EGF on day of P14 and P18 were also compared to LD and AL, respectively. Although expression of HB-EGF mRNA did not show any significant changes during the estrous cycle, EGF mRNA increased at LD. Furthermore, while EGF was up-regulated at both P14 and P60, expression of HB-EGF was only increased at P60. When compared to cyclic days (LD and AL), it can be suggested that EGF expression is regulated by embryonic factors in pre-implantation period.

Table 1. *Primers used for real-time PCR analyses*
Tablo 1. *Real-time PZR analizlerinde kullanılan primerler*

Locus	Primer Sequence	Product Size (bp)
EGF	5′-TGTGGTTCTCTGATTGGACT-3′	233
	5′-GGCTTTCATAAAACCTTCAC-3′	
HB-EGF	5′-AGGGTTAGGGAAGAAGAGAG-3′	240
	5′-ACGATGACAAGCAGACAGAC-3′	
GAPDH	5′-ATCACCATCTTCCAGGAGCGAGA-3′	341
	5′-GTCTTCTGGGTGGCAGTGATGG-3′	

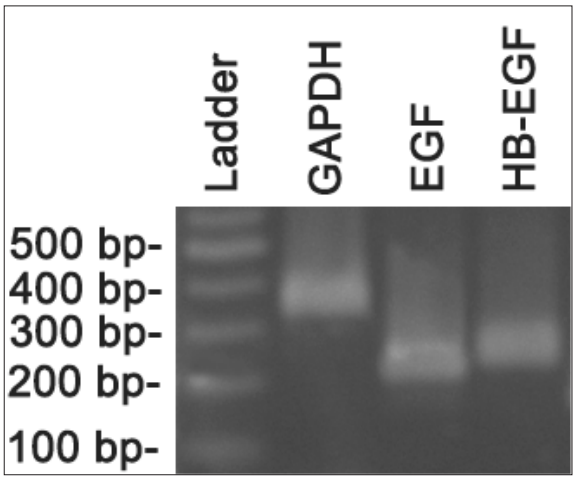


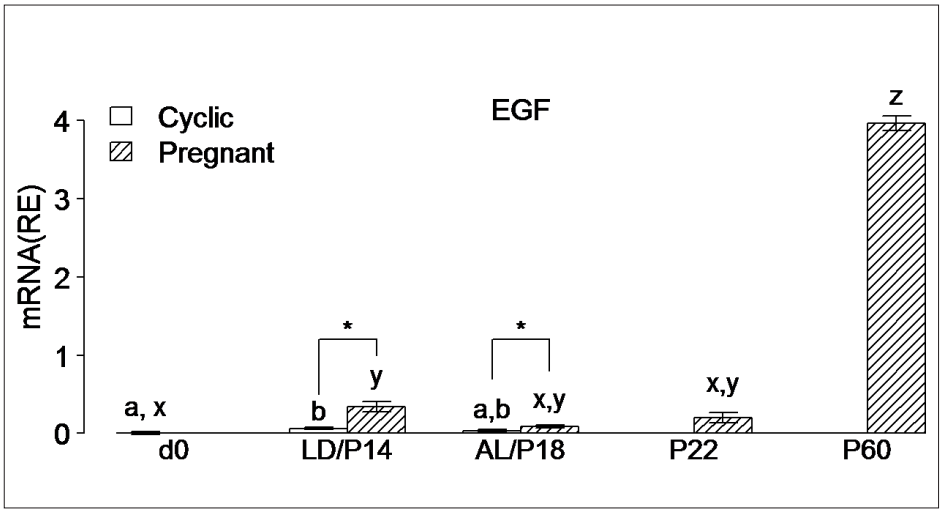
Fig 1. Agarose gel electrophoresis of GAPDH (341 bp), EGF (233 bp) and HB-EGF (240 bp) PCR amplification products

Şekil 1. GAPDH (341 bp), EGF (233 bp) and HB-EGF (240 bp) PZR ürünlerinin agaroz gel elektroforezi

Fig 2. Mean relative expression (RE) (± SEM) of EGF on day 14 (P14), 18 (P18) 22 (P22) and 60 (P60) of pregnancy and on day 0 (d0), late diestrous (LD) and after luteolysis (AL) of estrous cycle in equine endometrium

Şekil 2. EGF'nin at endometriumunda gebeliğin 14. (P14), 18. (P18) 22 (P22) ve 60 (P60) ile östrüs siklüsunun ovulasyon günü (d0), geç diöstrüs (LD) ve luteolizis sonrası (AL) dönemlerinde ortalama (± SEM)

*Different letters on columns (a,b and x,y,z) indicates significant differences at sample collection days within cycle and pregnancy. * indicates significant differences between cycle and pregnancy in a given day (P<0.05)*



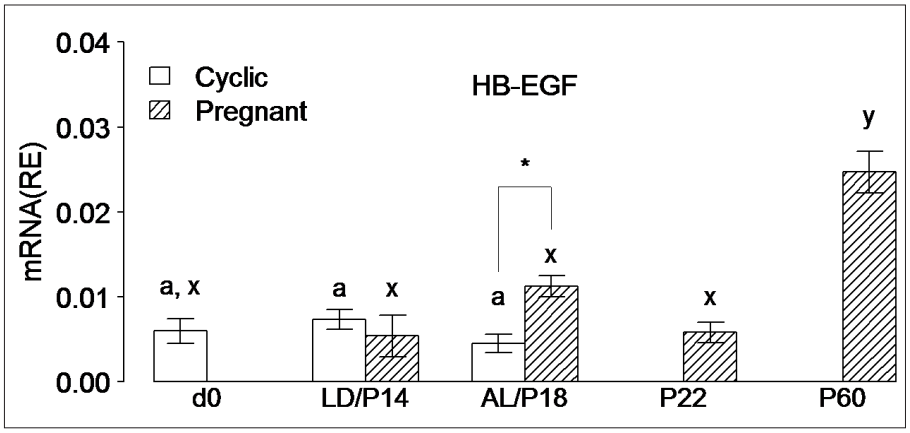


Fig 3. Mean relative expression (RE) (± SEM) of HB-EGF on day 14 (P14), 18 (P18) 22 (P22) and 60 (P60) of pregnancy and on day 0(d0), late diestrous (LD) and after luteolysis (AL) of estrous cycle in equine endometrium

Şekil 3. HB-EGF'nin at endometriumunda gebeliğin 14. (P14), 18. (P18) 22 (P22) ve 60 (P60) ile östrüs siklüsunun ovulasyon günü (d0), geç diöstrüs (LD) ve luteolizis sonrası (AL) dönemlerinde ortalama (± SEM)

*Different letters on columns (a, b and x,y,z) indicates significant differences at sample collection days within cycle and pregnancy. * indicates significant differences between cycle and pregnancy in a given day (P<0.05)*

Progesterone and embryonic factors (embryonic estrogen, PGF2α, PGE2) play a major role during initial days of equine pregnancy [4]. Especially, progesterone regulates endometrial secretions [6]. Considering that equine embryo remains un-attached to endometrium until day 40 of pregnancy, critical effects of progesterone regulated embryonic nutrients including uterolipocalin, retinol binding protein and transferrin are more understandable for equine pregnancy [3,22]. During the estrous cycle, EGF mRNA increased at LD where progesterone level was high. In contrast to the present study, Gesternberg et al.[11] failed to show effect of progesterone on EGF expression in late diestrus mares by in situ hybridization but observed an increase for EGF mRNA at day 36 or more after exogenous progesterone administration. In human, rat and rhesus monkey, regulation of EGF is associated with ovarian steroids [23-25]. Similarly, expression of EGF increased in estrus stage compared to proestrus in bitch endometrium [26]. Samuel et al.[27] proposed that EGF may involve in maintaining endometrial gland integrity and the ongoing histotrophe secretion. In the present study, compared to cyclic days (LD and AL) early increase in EGF mRNA in pregnant endometrium at P14 and P18 may support this idea.

Compared to other large domestic animals, equine embryo has been accepted as the slowest in its achievement of implantation. Non-invasive part of equine trophoblast cells and endometrium start to form the microcotyledonary epitheliochorial placenta around day 40 of pregnancy. Coincident with placenta formation, some part of equine trophoblast cells, invasive components, connect with endometrium for the formation of endometrial cups. All those processes are accepted a true implantation [3,4]. Considering that a magnitude up-regulation for expression profiles of both EGF and HB-EGF at P60, it can be easily seen that EGF and HB-EGF play critical roles in implantation of equine embryo. Similarly, important roles of HB-EGF during implantation have been detected in uterus of cow [28,29], human [16], baboon [30], hamster [31] and mice [17]. HB-EGF regulates stromal cells for accumulating lipid and glycogen vacuoles and induces vascular permeability in rats [32]. Moreover, expressions of integrin beta-3 and LIF (leukemia inhibitor factor) mRNA levels are up regulated by HB-EGF [33,34]. Among those, integrin B3 provides embryonic attachment into uterus with osteopontin-1 and LIF induces embryonic developments [35,36]. Furthermore, HB-EGF dependent intracellular signaling Ca^{+2} are also important for lysophoshatidic asid (LPA) pathway in blastosist development [37].

EGF expression profiles observed in this study is consistent with former studies [11,38] which also show a dramatic increase for EGF at day 40 of equine pregnancy. HB-EGF was not demonstrated previously in equine endometrium and is up-regulated in equine endometrium during the implantation process. However, understanding exact functions of HB-EGF in equine implantation needs more detailed studies. Functions of dramatic increase in EGF during the implantation reveal that EGF has a mitogenic role in the formation and growth of choriallantoic placenta in equine.

There is evidence that HB-EGF mRNA expression in the endometrium of d0, late diestrus and estrus mares is not influenced by both estrogen and progesterone. However, the greatest increase during the implantation process indicated that expression of HB-EGF appears to play important roles for equine implantation as described by former studies for the other mammals [16,17,28,30,31]. Similarly, the present study also demonstrated that EGF expression has important roles in equine pregnancy including both earlier (P14) and later (P60) stages. According to these results, it can be suggested that embryonic factors are more effective on regulation of HB-EGF and EGF mRNAs in equine endometrium than steroid hormones.

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