HB-EGF but not amphiregulin or their receptors HER1 and HER4 is altered in endometrium of women with unexplained infertility

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Abstract

Background: Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and its receptors (HER1 and HER4) play a role in the human implantation process. Amphiregulin is a member of the EGF-family but with unknown function in human fertility. It has been suggested that some women with unexplained infertility have defective endometrial development.

Aim: To determine the presence of amphiregulin and the receptors HER1 and HER4 in normal human endometrium throughout the menstrual cycle. In addition, the present study aimed to compare endometrium from women with unexplained infertility with endometrium from women with male factor infertility and healthy fertile controls.

Methodology: Immunohistochemistry and real-time PCR were used to determine the expression of HB-EGF, HER1, HER4 and amphiregulin.

Result: The stromal staining of HER1 and the epithelial staining of HER4 were most intense in the mid and late secretory phase endometrium. Amphiregulin did not vary during the menstrual cycle. In mid-secretory phase, the protein expression of HB-EGF was lower in endometrium from women with unexplained infertility vs. normal endometrium and endometrium from women with male factor infertility. HB-EGF and HER4 mRNA expression in mid-secretory endometrium of women with unexplained and male factor infertility was increased compared to normal controls.

Conclusion: Impaired endometrial expression of certain members of the EGF family may contribute to infertility in some women with unexplained infertility

Introduction

Infertility is a growing problem, and affects 10-15 % of all couples of fertile age. Traditional fertility tests fail to detect the cause of infertility in as many as 30% of these couples[1] For some infertile women there is reason to believe that development of the endometrium is not optimal. Today there is no suitable diagnostic tool for women with endometrium related infertility, and assisted reproduction is not always successful in these patients[2,3].

The epidermal growth factor (EGF) family consists of EGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin, transforming growth factor alpha (TGF-a), betacellulin (BCL), and epiregulin (EPI). These growth factors are important for proliferation and differentiation in several organs, and have been associated with embryogenesis, development and implantation[4-6]. There are four specific receptors, HER1, HER2, HER3 and HER4, which bind as homodimers or heterodimers to the EGF family growth factors[7,8]. HER1 has the ability to bind both HB-EGF and amphiregulin, while HER4 binds only HB-EGF[7,8]. HER1 and HER4 have previously been shown to be present in the human endometrium and in the human fallopian tube[9,10].

HB-EGF is one of the most intensely studied factors. It plays a significant role in reproduction, including the implantation process[11,12]. HB-EGF exists in two forms, one trans-membrane form and one soluble form. The soluble form results from cleavage of the trans-membrane form, and stimulates proliferation, migration and cell motility[11]. The trans-membrane form of HB-EGF acts as a juxtacrine growth and adhesion factor[11].

Amphiregulin is a less studied member of the EGF family and, in contrast to HB-EGF, it is reported to be expressed in the endometrium in stromal cells and leucocytes and to be decreased at the time of implantation[9].

There is limited knowledge regarding the expression of HB-EGF and amphiregulin and their receptors in the endometrium of infertile women. Therefore, the rationale for this study was to determine the presence and distribution of HB-EGF, amphiregulin and their receptors HER1 and HER4 from three groups of women: (1) women diagnosed with unexplained infertility (2) fertile women and (3) women with male factor infertility. Comparison of the pattern of expression in these three groups may reveal a possible mechanism for unexplained infertility of endometrial origin.

Material and methods

Study material

Endometrial biopsies were obtained from fertile women (healthy volunteers and women undergoing sterilisation by tubal ligation) and from women with the diagnosis 'unexplained infertility' or 'male factor infertility', who attended the Fertility Unit at the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge, Sweden.

The endometrial biopsies from healthy fertile women (n=33) were obtained at various stages of the menstrual cycle: proliferative phase n=5, early secretory phase (LH+0 to LH+5) n=6, mid secretory phase (LH+6 to LH+8) n=14 and late secretory phase (LH+9 to LH+13) n=8. These biopsies were used to detect HER1, HER4 and amphiregulin expression throughout the menstrual cycle. Cyclic changes in endometrial HB-EGF have been reported previously[12,13]. All women had proven fertility and had regular menstrual cycles (range 25-35 days). The mean age of the women was 36.0 ± 6.7 years, BMI 24.1 ± 2.4 and cycle length 28.0 ± 1.5 days. None of them had used steroidal contraceptives or an intrauterine device in the least three months prior to enrolling in the study and none had been pregnant or had had pelvic inflammatory disease during the year preceding the study.

Mid-secretory endometrial biopsies were obtained from women with unexplained infertility (n=10). The mean age of the infertile women was 33.8 ± 4.4 years, BMI 20.8 ± 1.2 and cycle length 29.7 ± 2.2 days. Unexplained infertility was diagnosed by a standard set of tests that included hormonal analyses and at least two semen analyses. All women had normal ovarian function. Their serum concentration of follicle stimulating hormone (FSH) was not higher than 11 IU/L during the early follicular phase (day 2-5). All women had a serum prolactin concentration below 20μg/L, and normal thyroid stimulating hormone (TSH) and thyroid hormone serum concentrations. The women had normal tubal passage as demonstrated by hysterosonosalpingography and no evidence of endometriosis (i.e. absence of symptoms and negative clinical exam and ultrasonography. Their partners had normal semen analysis results.

Additional biopsies were obtained from patients with male factor infertility (n=14) in mid-secretory phase. The mean age of these women was 34.6 ± 3.2 years, BMI 21.8 ± 2.1 and cycle length 27.9 ± 1.5 days. They had normal ovarian function, FSH levels under 11 IU/L during the proliferative phase, prolactin concentration below 20μg/L, and normal TSH and thyroid hormone levels. The partners of eight women had either low total sperm count (<40 $x10^6$), low sperm concentration (<20$x10^6$/mL) and/or low number of motile sperm (<30%). The partners of four women had performed testicular sperm aspiration (TESA), one had

performed percutaneous epididymal sperm aspiration (PESA) and one had > 97% abnormal sperm morphology. All fertilizations were performed using ICSI.

The Ethics Committees of the Karolinska Institutet approved this study and informed consent was obtained from all participating subjects.

Menstrual cycle monitoring

All women underwent transvaginal ultrasonographic evaluation, and serum progesterone measurement to confirm that cycles were ovulatory, a prerequisite for normal priming of the endometrium.

The day of the luteinising hormone (LH) surge was estimated by using a self-test detecting urinary LH (Clearplan, Searle Unipath, Bedford, UK) twice daily from cycle day 10 to the LH peak. The results of this test have been proven to correlate well with the LH surge determined from peripheral blood[14]. The days of biopsy were selected in relation to the day of the LH surge, LH +0. For fertile women, a single biopsy was taken at one point during the menstrual cycle. For women with male factor infertility or unexplained infertility, a biopsy was obtained once during the period LH+6 to LH+8. All biopsies were taken from the anterior wall of the uterine cavity, without dilatation of the cervix, using a Randall curette (Stille Werner AB, Stockholm, Sweden). The biopsies were used for immunohistochemistry and real-time PCR.

Histological evaluation of all biopsies included in the study showed normal maturation in relation to the cycle day, according to the criteria of Noyes et al[15].

Immunohistochemistry

All biopsies for immunohistochemistry were fixed in 4% formaldehyde for a maximum of 24 hours and then stored in 70% ethanol until embedding. The biopsies were embedded in paraffin, sectioned to 4µm and mounted on glass slides. The paraffin was then removed using Bioclear (CiAB, Stockholm, Sweden) and washed in decreasing concentrations of ethanol.

The samples were rinsed in water and thereafter phosphate buffered saline (PBS), incubated in darkness for 15 min in H_2O_2 (3 % in Methanol) to block endogenous peroxidase activity, and washed with PBS. Thereafter the slides were blocked with 1.5% horse serum in PBS for 30 minutes. The sections were then incubated with the primary antibody over night at 4°C. The primary antibodies for HER1 and HER4 detection were monoclonal mouse anti-human antibodies (18-7329 and 28-8005, Zymed Laboratories Inc., San Francisco, CA, USA), and for HB-EGF and amphiregulin were polyclonal goat anti-human antibodies (AF-259-NA and BAF989, R&D Systems, Oxford, UK). In negative control slides, the primary antibody was

replaced with the equivalent concentration of non-immune IgG from the same species. The slides were washed in PBS with 0.01% Tween and then incubated with the secondary horse anti-mouse and horse anti-goat antibodies diluted 1:100 (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at room temperature. The slides were then washed with PBS-Tween, prior to incubation with ABC complex (Vectastain Elite ABC immunoperoxidase detection kit, Vector Laboratories,) according to the manufacturer's instructions. After washing with PBS-Tween, freshly prepared diaminobenzidine-hydrogen peroxide solution (DAB kit from Vector Laboratories) was added to the slides, which were thereafter rinsed in distilled water. The slides were counterstained with 10% Mayer's Haematoxylin (VWR, Stockholm, Sweden), washed in cold water and then mounted with Pertex (Histolab, Gothenburg, Sweden). Two samples from each patient were stained.

Two persons evaluated the immunohistochemical staining independently, blinded to the identity of the samples. When the evaluation of the slides turned out differently, the average value was used. The staining was graded on a scale of 0 = no staining of cells, 1 = faint staining, 2 = moderate staining and 3 = strong staining.

Nonparametric statistical evaluation, ANOVA on ranks was performed analyze any differences in staining intensity during the menstrual cycle. Mann-Whitney Rank Sum Test was performed to compare staining intensity between fertile and infertile women. $P<0.05$ was considered statistically significant.

RNA preparation

Samples for PCR were snap-frozen and stored in liquid nitrogen until RNA preparation. Total tissue-RNA was isolated using RNeasy Mini-kit (Quiagen, Venlo, The Netherlands) according to the manufacturer's protocol. cDNA was prepared from 1µg of the total RNA by reverse transcription using deoxynucleotide triphosphates (10 mM each), random hexamer (250 ng/ml), ribonuclease inhibitor (40 U/µl), and Superscript reverse transcriptase (200 U/µl; using the Superscript^{TM} II RNase H^{-} Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA).

Real Time -PCR

Real-time PCR reactions were performed on RNA from seventeen samples (normal proliferative phase n=2, normal early secretory phase n=3, normal mid-secretory phase n=3, normal late secretory phase n=2, male factor infertility n=4, unexplained infertility n=4) using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). SYBR

green was used to detect the amplification. The reaction mixture consisted of 1x SYBR green PCR mastermix, 4 µl of the RT reaction mixture, (corresponding to 4 ng cDNA), and primers at a concentration of 250 nM. The final reaction volume was 15 µl. The mixture was heated to 50 °C for 2 minutes and the cDNA denaturated at 95 °C for 10 minutes. This was followed by 45 cycles of denaturation at 95 °C for 15 seconds, and combined primer annealing/extension at 60 °C for one minute. Fluorescence data was acquired by measurements taken after each extension step and presented as a plot of fluorescence intensity versus cycle number.

Standard curves were constructed using duplicates of seven serial dilutions of cDNA prepared from RNA of known concentration. Standard curves were created and run in parallel with the unknown samples in each experiment. Fluorescence was measured and the threshold cycle (C_t) values at each point in the standard curve were plotted against the log (ng) of the initial concentration[16]. The standard curves displayed a linear correlation for all genes. The relative concentration of unknown samples was calculated from the standard curves. Each sample was run in duplicate and the target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as endogenous control.

The forward and reverse primers were: for HB-EGF 5'-GTC TCT CCC CGT GTC CTC TCC-3' and 5'-GCT CCA ATG TTC CCT GTT CCT-3', for HER1 5'-GTG ACC GTT TGG GAG TTG ATG A-3' and 5'-GGC TGA GGG AGG CGT TCT C-3', for HER4 5'- TGC CCT ACA GAG CCC CAA CTA-3' and 5'-GCT TGC GTA GGG TGC CAT TAC -3', for AR 5'- TGG AAG CAG TAA CAT GCA AAT GTC-3' and 5'- GGC TGC TAA TGC AAT TTT TGA TAA-3', and GAPDH 5'-CAG AGT TAA AAG CAG CCC TGG T-3' and 5'-GAA GGT GAA GGT CGG AGT CAA C-3' respectively. The amplified product size was for HB-EGF 252bp, for HER1 104bp, for HER4 105bp, for AR 116bp and for GAPDH 71bp respectively.

Relative expression was calculated as change in target/change in GAPHD.

Differences in expression levels between different study groups were analyzed using Kruskal Wallis one-way Analysis of Variance on ranks and post hoc test according to Dunns's method. Significance was accepted at a p-value ≤ 0.05.

Results

HB-EGF expression in endometrium from fertile women

In our previous study we demonstrated the most intense staining intensity of HB-EGF in the mid-secretory phase[13]. The highest relative mRNA level was seen during the late secretory phase p=0.08 (Table I).

HER1 expression in endometrium from fertile women

There was no immunostaining of HER 1 in the glandular or luminal epithelium in any of the samples analyzed. The staining of HER 1 was solely seen in the stromal cells (Figure 1A). It was more intense during the secretory phase than during the proliferative phase, p=0.003, (Figure 2A) There was no statistical variation in relative mRNA expression throughout the secretory phase of the cycle (Table I). Due to the small sample size in PCR analysis in proliferative phase (n=2), statistical evaluation was not performed on that samples.

HER4 expression in endometrium from fertile women

Immunostaining of HER 4 was more intense in the epithelial cells than in the stromal cells (Figure 1B). Its immunoexpression in the luminal epithelial cells increased in the mid secretory phase (p=0.008) and remained high throughout the rest of the cycle (Figure 2B). In the glandular epithelial cells, the increase in the mid luteal phase (p=0.031) followed the same pattern as seen in the luminal epithelial cells (Figure 2C). Stromal staining did not vary throughout the cycle. The highest relative HER4 mRNA expression was seen during the late secretory phase, corresponding to highest HB-EGF mRNA level (Table I).

Amphiregulin expression in endometrium from fertile women

Immunostaining was observed in luminal epithelium, glandular epithelium and stroma (Figure 1C). The immunostaining for amphiregulin was both nuclear and cytoplasmic, and was generally more intense in the cytoplasm (p=0.024). There was no variation in staining intensity during the menstrual cycle (Table II). Real-time PCR did not reveal any variation in mRNA expression during the cycle (Table I).

HB-EGF expression in endometrium from fertile women vs. endometrium from infertile women

The immunostaining of HB-EGF was significantly more intense in luminal epithelium from fertile women than in women with unexplained infertility, p>0.001, (Figure 3A). A similar pattern was seen in the glandular epithelium, p=0.002, (Figure 3B). However, on the gene level, endometrium from women with unexplained infertility had higher relative HB-EGF mRNA levels than fertile control women, p=0.034 and 0.049 respectively (Figure 4A).

HER1 expression in endometrium from fertile women vs. endometrium from infertile women

Staining intensity of HER1 in endometrial stroma from fertile women or from women with male factor infertility did not differ significantly from endometrium from women with unexplained infertility (Table III). The relative mRNA expression did not differ between the two groups of infertile women (Figure 4B).

HER4 expression in endometrium from fertile women compared to endometrium from infertile women

There was no difference in HER4 immunostaining in either luminal or glandular epithelium or in stroma between endometrium from women with unexplained infertility and endometrium from women with male factor infertility or fertile control women (Table III). When comparing HER4 mRNA expression in fertile and infertile women, the relative mRNA expression of HER4 was higher in endometrium from women with unexplained infertility and with male factor infertility than in fertile control women, (p=0.049 and 0.034 respectively) which correlated with HB-EGF mRNA levels in those groups of women (Figure 4C).

Amphiregulin expression in endometrium from fertile women compared to endometrium from infertile women

There was no difference in staining intensity or mRNA expression of amphiregulin in endometrium from women with unexplained infertility compared to male factor infertility or healthy fertile controls (Figure 4D and Table IV).

Discussion

Communication between the embryo and the endometrium is needed to facilitate a normal implantation process. Based on earlier reports, HB-EGF is likely to be involved in embryo

development, endometrial maturation and implantation[12,13,17-20]. HB-EGF plays a role in the embryo implantation process in the mouse where it is expressed in the luminal epithelium surrounding the embryo[21]. It has also been shown that HB-EGF coated beads induce a local stromal including BMP-2, FGF-2 and WNT-4[22], suggesting a role for HB-EGF during implantation.

It is known that HB-EGF is present in endometrial glandular and luminal epithelium[12,13,23] and in the fallopian tube[10]. The highest level of HB-EGF in the endometrium is seen during the receptive phase[13]. This is not reflected in the mRNA levels in the present study, the HB-EGF concentration mRNA being highest during the late secretory phase.

The regulation of HB-EGF in the endometrium is unclear. It has been shown that although HB-EGF increases at the time of implantation[13], treatment with mifepristone on LH+2 does not alter HB-EGF expression[10]. However, in the mouse, estrogen alone, or a combination of estrogen and progesterone increases HB-EGF in the mouse uterus[24]. HB-EGF is also been shown to be involved in decidualization process[18].

Presence of the HER1 receptor has previously been demonstrated in human endometrium[9,10]. However, the data on the localization of HER1 are conflicting. The present study shows specific immunostaining of HER1 in the stromal cells only, in contrast to previous studies which have found HER1 in both stroma and epithelial cells [10] or solely in the epithelial cells[9]. The discrepancies could to some extent be explained by the use of different fixation techniques or different antibodies. In the previous study[10], HER1 in epithelial cells was only seen after mifepristone treatment, supporting the lack of epithelial staining in the present study, where hormonal treatment was absent.

In the present study, the highest level of HER1 was observed during the luteal phase. This is in line with earlier studies using immunoblotting where HER1 levels increased at the late follicular and luteal stages[25,26]. Real-time PCR data did not reveal any differences in mRNA levels of HER1 throughout the cycle, in contrast to an earlier report where the highest levels of HER1 mRNA were seen in the proliferative phase[9]. This difference could be due to varying stromal and epithelial cell ratios in the biopsies.

The present study demonstrates less HB-EGF immunostaining in endometrium from women with unexplained infertility compared to fertile control women. At the same time, mRNA levels of HB-EGF were higher in the endometrium from the infertile women. The high mRNA levels might not necessarily result in high protein levels, due to several factors, such as RNA instability or altered translation[27,28]. If this is the case, endometrial development might be delayed in women with unexplained infertility, and the lack of synchronization with

a developing embryo may hinder successful implantation. The protein expression of HB-EGF receptors HER1 and HER4 was similar in fertile and infertile women in our study. These data suggest that defective regulation of mainly HB-EGF in the endometrium, possibly on the post-transcriptional level, could contribute to infertility. It has previously been suggested that the interaction of HB-EGF with ErbB4, the mouse equivalent of HER4, is important for embryo implantation in the mouse[29]. Recently, it was shown that HB-EGF could regulate trophinin-mediated adhesion of trophoblast cells[20]

Women with male factor infertility and unexplained infertility demonstrated high HER4 mRNA levels compared to fertile control women, which does not correlate with the immunohistochemistry data. This is probably due to inherent differences in the two techniques: During preparation for real time PCR the whole tissue (epithelial and stromal cells) is homogenized but when using immunohistochemistry variations in specific cell types can be detected.

HER4 staining was more intense in glandular and luminal epithelium in the mid and late secretory phase, which is in accordance with some earlier reports[9,18]. Levels of HB-EGF mRNA correlated to the levels of HER4 (specific HB-EGF receptor), but not HER1, in endometrium from fertile and infertile women.

We observed a weak immunostaining and low mRNA levels of amphiregulin, without cyclic variations. The presence of amphiregulin in the endometrium is controversial: one group found some positive cells (stromal cells and leucocytes) expressing protein and mRNA which decreased at the time of implantation[9], while a second group did not find any amphiregulin immunoexpression in the endometrium[30]. Hamster and mouse decidual cells have been reported to express amphiregulin[31]. It has further been shown that amphiregulin is present at the time of implantation in the mouse uterus[32]. Progesterone seems to regulate amphiregulin in the mouse uterus[32], but this has so far not been confirmed in the human endometrium. Amphiregulin has also been found in human syncytiotrophoblasts of up to 18 weeks of gestation[33].

Leukemia inhibitory factor (LIF) is a prerequisite for implantation in the mouse (Stewart et al., 1992), and probably in humans as well[13,34]. It has been shown that LIF injection increases the expression of amphiregulin in the mouse uterus[35]. In addition, HB-EGF was shown to up-regulate LIF expression in human endometrial epithelial cells in vitro[36].

There was no difference in amphiregulin expression between endometrium from fertile women and endometrium from women with unexplained infertility. This suggests that amphiregulin does not play a major role in the process of human embryo implantation and

that it may not be responsible for the altered endometrial receptivity in women with unexplained infertility.

Women with unexplained infertility are not a homogenous group, and there may be several possible reasons for their fertility problems[37]. However, in some of these women defective endometrial maturation may be cause of their fertility problems. Previous attempts to find an endometrial factor that could explain infertility have focused on morphology according to the criteria of Noyes or measurements of endometrial thickness. Neither of these approaches however has provided any evidence in support of such a factor[38-40].

In contrast, the methods used in this study have shown an altered expression of HB-EGF and its receptor HER4 in endometrium from women with unexplained infertility suggesting an endometrial factor may indeed play a role in infertility

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Legends to figures

Figure 1

Representative photos of immunostaining of HER1, HER4 and AR in the mid-secretory phase endometrium from healthy fertile women are shown. **A**. HER1 **B**. HER4. **C**. Amphiregulin. Amplification x 400

Figure 2.

Box plot of immunostaining of HER1 and HER4 in human endometrium throughout the menstrual cycle is shown. **A**. HER1 in stroma. **B**. HER4 in luminal epithelium. **C**. HER4 in glandular epithelium. P=proliferative phase, E=early secretory phase, M=mid secretory phase and L=late secretory phase. * - $p < 0.05$.

Figure 3

Box plot demonstrates HB-EGF immunostaining in mid-secretory human endometrium from fertile women compared to infertile women with male factor and unexplained infertility. **A**. HB-EGF immunostaining in luminal epithelium. **B**. HB-EGF immunostaining in glandular epithelium. ** - $p < 0.01$, *** - $p<0.001$.

Figure 4

Box plot demonstrates result from real time RT-PCR in relation to GAPDH expression, in mid-secretory endometrium. **A.** Relative expression of HB-EGF mRNA. **B.** Relative expression of HER1 mRNA. **C.** Relative expression of HER4 mRNA. **D.** Relative expression of amphiregullin mRNA. Statistical analysis by Kruskal-Wallis one way analysis of variance on ranks and multiple comparison according to Dunn's method, a is statistically different from b, $p<0,05$.

Table I. Relative expression of HB-EGF, HER1, HER4 and amphiregulin mRNAs in endometrium during different phases of secretory phase

Median and range relative expression are shown.

	Proliferative phase (n=2)	Early secretory phase (n=3)	Mid secretory phase (n=3)	Late secretory phase (n=2)
HB-EGF	7.57 (0.07- 15.07)	0.18 (0.08-1.35)	0.38 (0.18-0.98)	10.34 (10.02-10.66)
HER1	1.37 (1.07-1.69)	0.84 (0.71-1.40)	1.04 (1.01-1.06)	1.49 (1.01-1.98)
HER4	4.68 (0.22-9.13)	1.46 (0.35-1.53)	0.93 (0.54-0.99)	6.37 (6.12-6.63)
amphiregulin	0.95 (0.12-1.79)	0.51 (0.32-1.52)	0.35 (0.09-1.01)	1.11 (0.31-1.92)

Table 2. Results of amphiregulin immunostaining in endometrium from fertile women. LE - luminal epithelium. GE - glandular epithelium. Data presented as median and range. 0 = no staining of cells, 1 = faint staining, 2 = moderate staining and 3 = strong staining

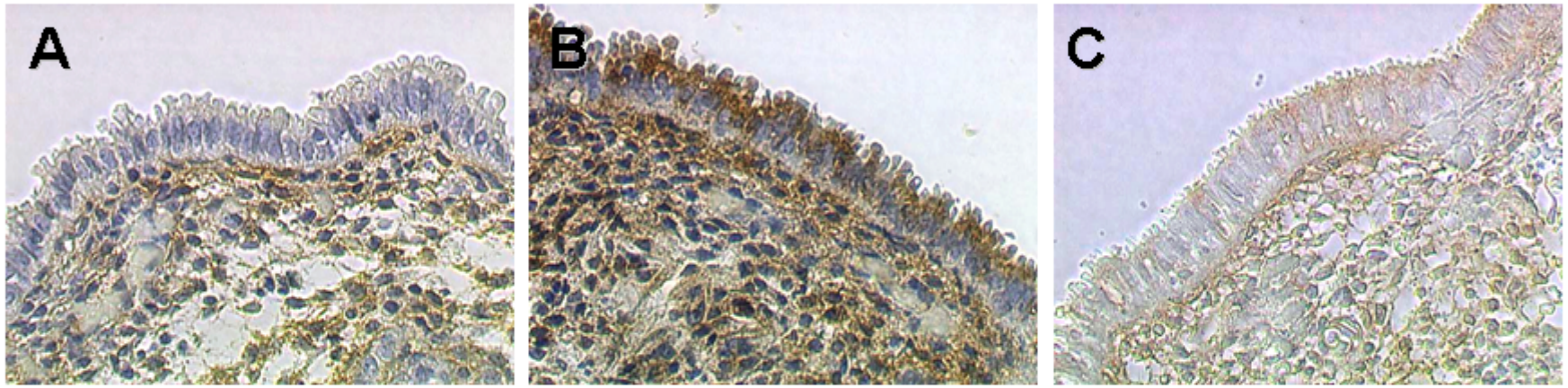
Phase	LE cytoplasm	LE nucleus	GE cytoplasm	GE nucleus	Stroma cytoplasm	Stroma nucleus
Proliferative phase n=5	2.0 (0.5—2.0)	1 .0 (0.0-2.0)	1.0 (0.0-0.5)	0.0 (0.0-0.0)	0.5 (0.5-0.5)	1.75 (0.5-3.0)
Early secretory phase n=6	1.0 (0.0-1.5)	0.0 (0.0-2.0)	1.0 (0.5-1.0)	1.0 (0.0-2.0)	0.75 (0.0-2.0)	1.25 (0.5-2.0)
Mid secretory phase n=14	1.0 (0.0-3.0)	0.5 (0.0-2.0)	1.0 (0.0-2.5)	0.25 (0.0-2.0)	1.0 (0.0-2.5)	1.0 (0.0-2.5)
Late secretory phase n=8	0.5 (0.5-1.5)	0.5 (0.0-1.5)	1.0 (0.0-1.5)	0.75 (0.0-2.0)	1.0 (0.5-2.5)	1.0 (0.5-3.0)

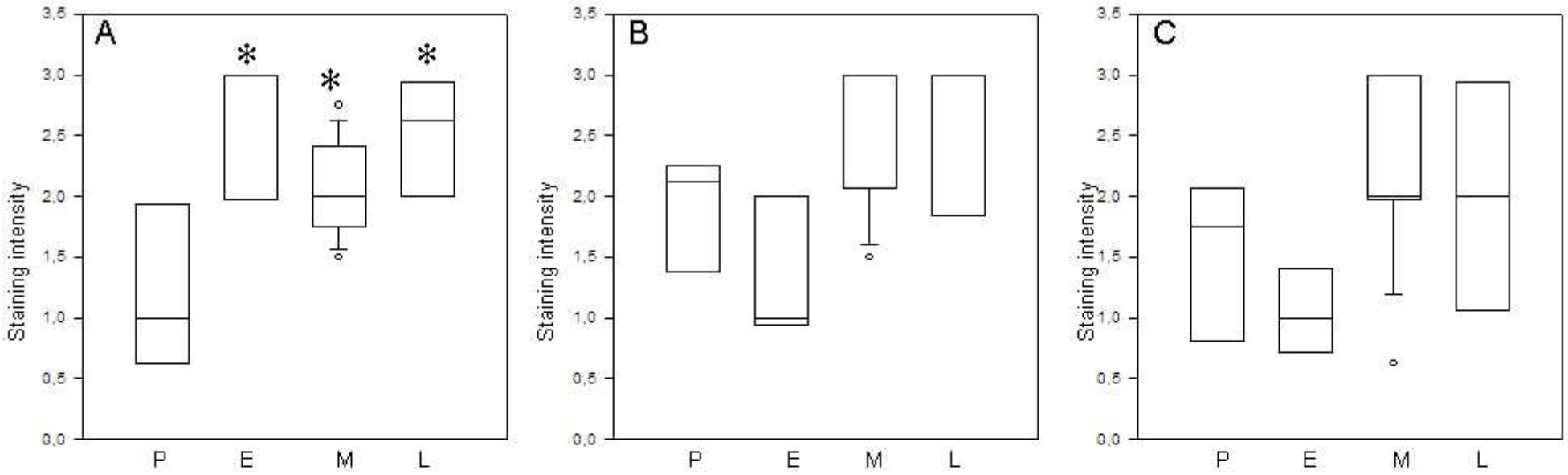
Table III. Immunostaining of HER1 and HER4 in mid-secretory endometrium from fertile women compared to infertile women. 0 = no staining of cells, 1 = faint staining, 2 = moderate staining and 3 = strong staining

Diagnosis	Fertile n=14	Male factor n=10	Unexplained infertility n=14
HER1 Stroma	2.5 (1.5 – 3.0)	2.0 (1.5 – 2.5)	2.0 (1.0 – 2.5)
HER4 Luminal epithelium	3.0 (1.5 – 3.0)	2.5 (1.0 – 3.0)	2.5 (1.5 – 3.0)
HER4 Glandular epithelium	2.0 (0.5 – 3.0)	2.0 (0.5 – 3.0)	2.0 (1.0 – 3.0)

Table IV. Staining intensity of amphiregullin in mid-secretory endometrium from fertile and infertile women LE - luminal epithelium, GE - glandular epithelium. Data presented as median and range. 0 = no staining of cells, 1 = faint staining, 2 = moderate staining and 3 = strong staining

Diagnosis	LE cytoplasm	LE nucleus	GE cytoplasm	GE nucleus	Stroma cytoplasm	Stroma nucleus
Fertile (n=14)	1.0 (0.0-3.0)	0.5 (0.0-2.0)	1.0 (0.0-2.5)	0.25 (0.0-2.0)	1.0 (0.0-2.5)	1.0 (0.0-2.5)
Male factor (n=14)	1.0 (0.0-2.5)	1.0 (0.0-2.0)	1.0 (0.0-2.5)	1.0 (0.0-2.0)	1.5 (1.0-2.5)	1.0 (0.0-3-0)
Unexplained infertility (n=10)	1.0 (0.0-2.5)	0.5 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (0.5-2.0)	0.5 (0.0-2.5)


A
B
C


A
B
C
Staining intensity
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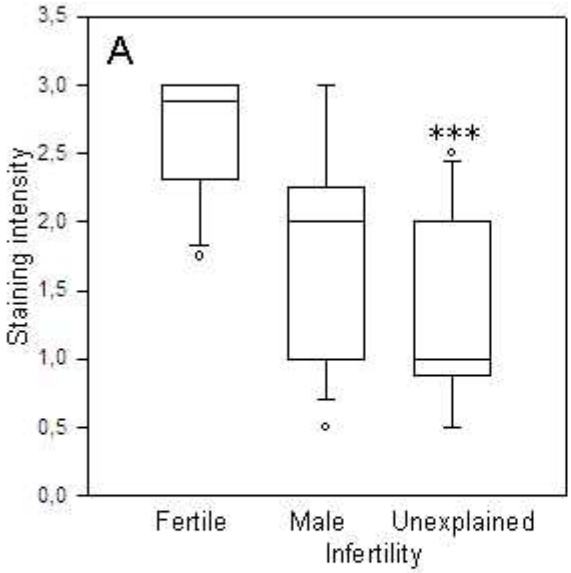
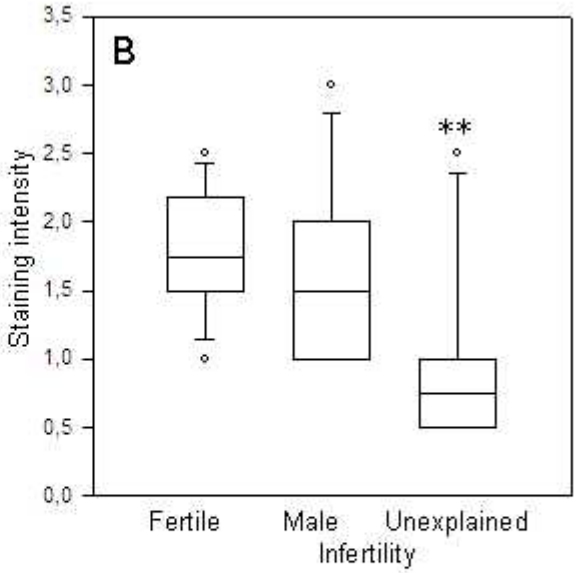
3,0
2,5
2,0
1,5
1,0
0,5
0,0
P
E
M
L
*


A
Staining intensity
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
***
Fertile
Male
Unexplained
Infertility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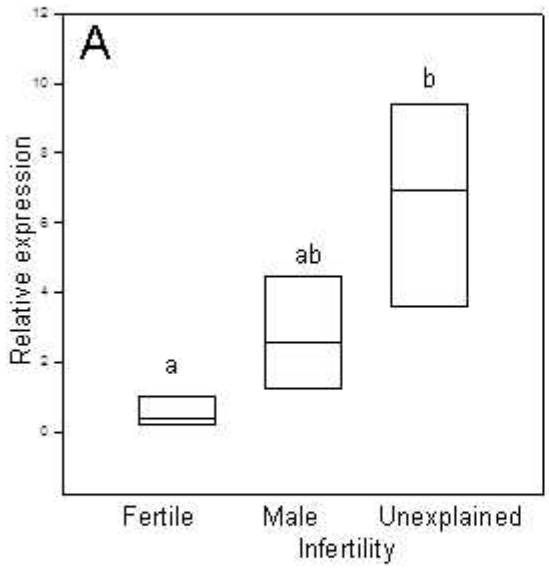
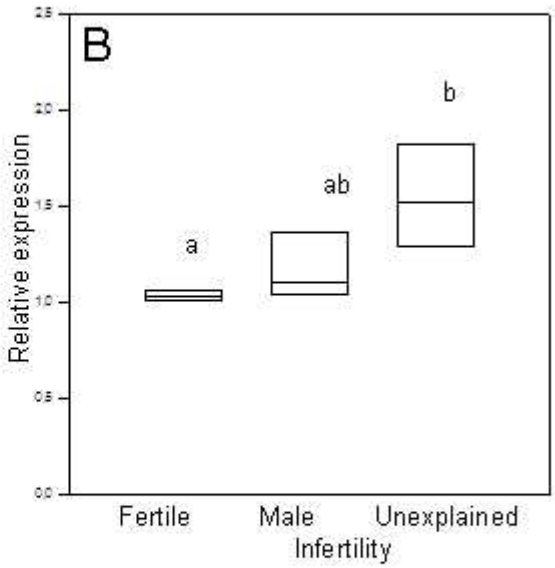
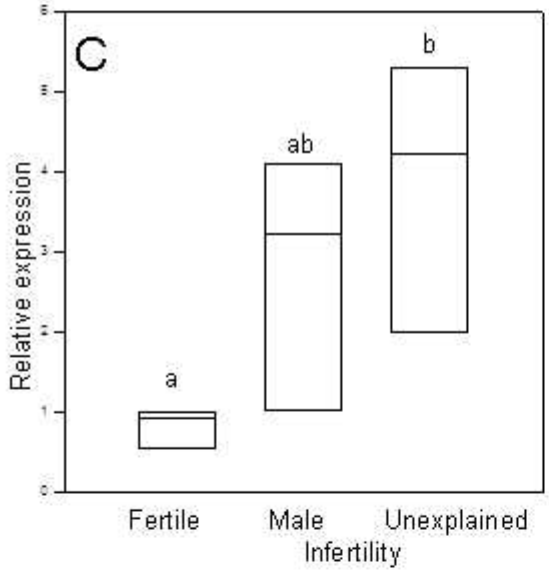
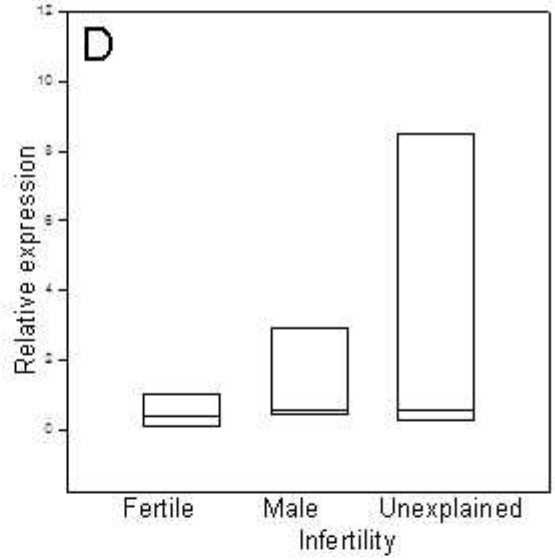
Staining intensity
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
Fertile
Male
Unexplained
Infertility


A
Relative expression
a
ab
b
Fertile
Male
Unexplained
Infertility


B
Relative expression
a
ab
b
Fertile
Male
Unexplained
Infertility


C
Relative expression
a
ab
b
Fertile
Male
Unexplained
Infertility


D
Relative expression
Fertile
Male
Unexplained
Infertility