

Reduced expression of follicle stimulating hormone receptor mRNA and protein in pregnancies complicated by pre-eclampsia

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Abstract. Expression and function of the follicle-stimulating hormone receptor (FSHR) are traditionally thought to be limited to the ovary in females. However, recent studies have indicated that the FSHR is also expressed in endothelial cells of the placental vasculature, and that the haploinsufficiency of the feto-placental FSHR impaired the growth of the mouse placenta. The aim of the current study was to investigate the placental expression of FSH and FSHR in pregnancies complicated by pre-eclampsia. Placental tissue samples were collected from 20 pregnancies with pre-eclampsia and 25 normal pregnancies. Placental FSH and FSHR mRNA levels were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), while FSH, FSHR and CD31 protein expression were examined by immunohistochemistry. Additionally, levels of serum FSH were measured by chemical luminescence immunity assay. The results demonstrated that no significant difference was observed in serum FSH levels and expression levels of placental FSH mRNA and protein between normal pregnancy and pre-eclampsia. However, RT-qPCR results indicated that the expression level of FSHR mRNA in pre-eclamptic placental samples was significantly lower than normal pregnancies. Immunostaining results from normal pregnant samples indicated that the FSHR protein was strongly expressed in the endothelial cells of blood vessels in the chorionic villi, moderately expressed in stromal cells of the villus, but not expressed in trophoblast cells of the term placenta. The staining intensity of FSHR-positive area was

significantly lower in the placental villi of pre-eclampsia, when compared with the normal control group. In conclusion, expression levels of placental FSHR mRNA and protein are significantly reduced in pregnancies complicated with pre-eclampsia in the present study. Further studies may investigate whether FSHR could be used as a biomarker for the prediction of pre-eclampsia.

Introduction

Pre-eclampsia is a pregnancy-specific syndrome characterized by hypertension and significant proteinuria developed at or after 20 weeks of pregnancy in a previously normotensive, non-proteinuric patient (1,2). It affects ~5-10% of all pregnancies worldwide and is a major cause of maternal and perinatal morbidity and mortality in developed countries (1,3). Although the etiology of pre-eclampsia is still not fully clear, strong evidence supports the involvement of inadequate trophoblast invasion, failed remodeling of the spiral arteries, imbalance of angiogenic and antiangiogenic factors and endothelial cell dysfunction (1-5). Consequently, elucidating disorder factors of placental angiogenesis is crucial in understanding the pathophysiological process of pre-eclampsia.

Follicle stimulating hormone (FSH), an anterior pituitary gonadotroph-derived heterodimeric glycoprotein which binds G protein-coupled FSH receptor (FSHR), is promoted by hypothalamic decapeptide gonadotrophin-releasing hormone and serves a critical role in hypothalamic-pituitary-gonadal axis (6,7). Traditional considerations manifest the physiological structure and function of the FSH/FSHR system within gonads that stimulates growth of follicles and synthesis of estrogens in the ovary or promotes spermatogenesis in the testes (6,8). However, previous reports revealed that the FSH/FSHR system serves an important role in extragonadal tissues and organs, including prostanoid synthesis of bovine cervix (8,9), electrical activity of the mouse myometrium (10,11), and some organs unrelated to reproduction (12). For instance, FSHR expression of osteoclast is related to bone resorption, which aggravates periodontitis-related alveolar bone loss without estrogen deficiency (12-14). Its relevance to angiogenesis has been also reported well in previous research (15,16). Studies

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indicated that the FSHR was expressed in the endothelial cells of human placental chorionic villi and umbilical vein at term (15). Stilley *et al* (8,15) revealed that FSH promoted the formation of endothelial tubes and other angiogenic processes without increasing secretion of vascular endothelial growth factor (VEGF). Furthermore, the study indicated that the haplo-insufficiency of the feto-placental FSHR impaired the growth of the mouse placenta (8). In addition, Radu *et al* (17) determined that the FSHR was selectively expressed on the endothelium of blood vessels in a wide range of tumors, and endothelial FSHR expression in breast cancer was associated with vascular remodeling at tumor peripheries (18). In light of the evidence pointing to roles of FSHR in the angiogenesis, the authors hypothesized that abnormal expression of the FSH/FSHR system would present in the placentas of women with pre-eclampsia.

To verify the hypothesis, the authors examined placental mRNA and protein expression and localization of FSH and FSHR by RT-qPCR and immunohistochemistry methods in normotensive control and pre-eclamptic women. Additionally, serum levels of maternal FSH were also tested using chemiluminescence immunoassay. The results indicated that FSHR mRNA and protein levels were significantly decreased in the placentas of women with pre-eclampsia.

Materials and methods

Patients. The present study was approved by the Ethical Committee of Nanchang University (Nanchang, China) and Jiangxi Province People's Hospital (Nanchang, China), and informed consent was obtained from each participant. Placentas were collected from pregnancies with: (1) Normal pregnancy (maternal blood pressure <140/90 mmHg, absence of proteinuria and no medical complications), (2) severe pre-eclampsia (new-onset hypertension, defined as systolic blood pressure of >160 mmHg or diastolic blood pressure of >110 mmHg, with at least two measurements, accompanying by significant proteinuria >5 g/24 h or 3+ by dipstick in two random samples collected at >4 h interval after 20 weeks of gestation). Those who developed renal disease, gestational diabetes, spontaneous abortion, transient hypertension of pregnancy, intrauterine fetal death, fetal chromosomal abnormalities, congenital abnormalities, pregnancies conceived by fertility treatment, or had hereditary history and smoking and alcohol the history were not included in the study. The clinical characteristics of patients and controls are presented in Table I. Tissues were collected from the villous tree within 1 h following delivery. To minimize blood contamination, each piece of tissue was intensively washed in Dulbecco's phosphate-buffered saline (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tissue samples were then snap-frozen and stored at -80°C.

RNA isolation and RT-qPCR. Total RNA was extracted from placental tissues with the RNAiso Plus solution (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RNA (2 µg) samples were reverse-transcribed into single-stranded cDNA in a 25 µl reaction mixture, containing 4 µl 5X reaction buffer, 1 µl RNase inhibitor, 2 µl 10 mM dNTP, 1 µl reverse transcriptase and 1 µl 10 µM primers (Takara Biotechnology Co., Ltd.). RT-qPCR

was then performed in a 20 µl reaction volume containing 10 µl 2X Brilliant SYBR Green Mix (Takara Biotechnology Co., Ltd.), 2 µl template cDNA, 0.5 µM primers, and 300 nM reference dyes using the ABI thermal cycler 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions were 95°C for 30 sec, followed by 40 cycles at 94°C for 5 sec, 60°C for 34 sec. Melting curve analysis and agarose gel electrophoresis were conducted following the RT-qPCR assays to monitor PCR product purity. The results were analyzed using ABI Prism 7500 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). 18S rRNA was used for normalization (19). The following primers were used: FSH sense, 5'-CCACTTGGTGTGCTGGCTACT-3', and anti-sense, 5'-GGCCTGGCTGGGTCCCTTATA-3'; FSHR sense, 5'-GCCATGCTGCCAGTGTCAT-3', and antisense, 5'-GAG GGCAGCTGCAAAAGC-3'; 18S sense, 5'-GCTGAGAAG ACGGTGCAACT-3', and antisense, 5'-TTAATGATCCTT CCGCAGGT-3'.

Immunohistochemistry. Tissues were fixed in Bouin's solution, dehydrated, and embedded in paraffin. Tissue sections were deparaffinized, and rehydrated in a graded series of ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in PBS for 10 min. Nonspecific binding was blocked with 5% BSA in PBS for 30 min. Then, the sections were incubated in rabbit anti-FSHR (1:200; ab113421; Abcam, Cambridge, UK), rabbit anti-CD31 (1:300; ZM-0044; ZSGB-Bio, Beijing, China) and mouse anti-FSH monoclonal antibody (1:150; ZA-0264; ZSGB-Bio) overnight at 4°C. Following washing in PBS, the sections were incubated with a horseradish peroxidase-conjugated secondary antibody (1:200; ZM-0003; ZSGB-Bio) for 50 min at 37°C. The primary antibody was visualized with fresh diaminobenzidine solution, together with counter-staining with Harris' hematoxylin. In some sections, the primary antibodies were omitted or replaced with rabbit or mouse pre-immune IgG as a negative control.

Analyses of immunohistochemical staining. Images were captured in three sections per sample using digital camera head DS-Fi1 (Nikon Corporation, Tokyo, Japan), and analyses of immunohistochemical staining were taken using NIS-ELEMENTF analysis system (Nikon Corporation). Under the same magnification (x100) and light intensity, each slide was measured in randomly selected eight fields. Mean values of optical density for positive cells were calculated using sections from normal pregnancy and severe pre-eclampsia.

Hormone measurements. Blood samples were collected into clotting tubes between 7:30 a.m. and 8:30 a.m. from the cubital vein during the routine visits at the end of gestation. Blood was centrifuged at 2,000 x g for 20 min at 4°C and then stored at -80°C until the assay. The serum concentration of FSH was measured using a chemiluminescence immunoassay kit (BD-2003; DPC Biermann GmbH, Bad Nauheim, Germany). The intra- and interassay coefficients of variation did not exceed 10%. The cross-reactivities with other peptides and steroid hormones did not exceed 4%. The detection limitation of the FSH kit is 0.2 mIU/ml.

Table I. Clinical characteristics of study population.

Clinical characteristic	Normal pregnancy	Pre-eclampsia	P-value
Case (n)	25	20	
Maternal age (mean \pm SD, years)	27.3 \pm 4.2	29.3 \pm 3.9	0.37
Gestational age at delivery (mean \pm SD, weeks)	37.7 \pm 2.4	34.7 \pm 2.6	0.013
Birth weight (mean \pm SD, g)	3347.6 \pm 311.6	2806.5 \pm 665.3	<0.001
Maternal height (cm)	163.1 \pm 7.8	160.9 \pm 6.4	0.314
Maternal weight (kg)	60.2 \pm 4.5	62.1 \pm 5.0	0.175
Body mass index	22.2 \pm 1.4	23.0 \pm 2.1	0.133
Systolic blood pressure (mean \pm SD, mmHg)	106.3 \pm 11.3	165.4 \pm 11.9	<0.001
Diastolic blood pressure (mean \pm SD, mmHg)	69.3 \pm 10.2	109.1 \pm 13.4	<0.001
Fetal sex (male/female)	14/11	9/11	>0.05
Serum FSH (mean \pm SD, mIU/ml)	2.92 \pm 0.95	2.79 \pm 0.63	0.61

SD, standard deviation; FSH, follicle stimulating hormone.

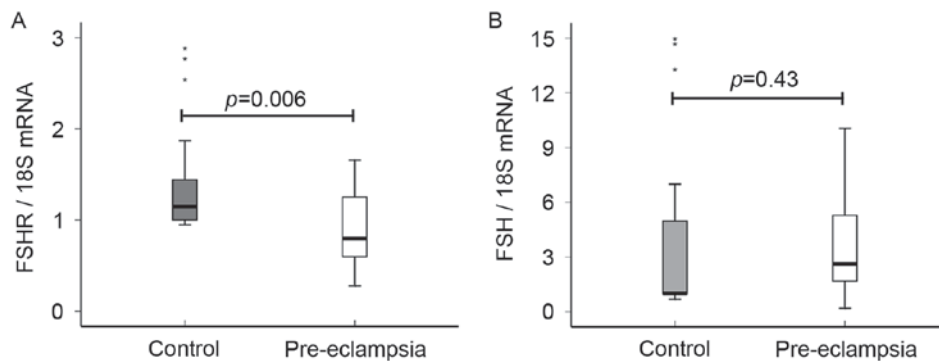


Figure 1. Expression levels of placental FSHR and FSH mRNA in normal controls and pregnancies with pre-eclampsia. (A) Box and whisker plots depicting relative expression levels of FSHR mRNA. (B) Box and whisker plots depicting relative expression levels of FSH mRNA. mRNAs levels were quantified using reverse transcription-quantitative polymerase chain reaction and normalized to 18S rRNA. *denotes outliers. FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor.

Statistical analysis. Data were presented as means \pm standard deviation. Statistical analysis was performed by independent-samples t-test for parametric and Wilcoxon test for nonparametric data to determine the significance of the differences. Additionally, the chi-squared test was used to examine fetal sex. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software (version, 13.0; SPSS, Inc., Chicago, IL, USA).

Results

Clinical data analysis. Compared with normal pregnancy, the gestational age of women with pre-eclampsia was 3 weeks shorter at delivery ($P = 0.013$), and gained less weight during their pregnancies ($P < 0.001$). However, there was no significant difference in serum concentrations of FSH between normal pregnancy and pre-eclampsia ($P = 0.61$; Table I).

FSHR expression in human placentas. RT-qPCR results indicated that expression level of placental FSHR mRNA in pre-eclamptic samples was significantly lower than that of the normal sample (1.27 ± 0.56 , 0.92 ± 0.42 ; $P = 0.006$; Fig. 1A). The

authors then analyzed the spatiotemporal expression of the FSHR protein in the placental tissues by immunohistochemistry method (Fig. 2). Immunostaining results from normal pregnant samples demonstrated that the FSHR protein was strongly expressed in endothelial cells of blood vessels in the chorionic villi (confirmed by CD31 staining, Fig. 2A and B), moderately expressed in the chorionic stromal cells, but not expressed in trophoblast cells of term placenta (Fig. 2D and E). Compared to the normal control group, the staining intensity of the FSHR-positive area was significantly lower in the placental villi of pre-eclampsia ($P = 0.0018$; Fig. 3A), in accordance with the RT-qPCR results.

FSH expression in human placentas. RT-qPCR analysis revealed that no significant difference was observed in the expression levels of placental FSH mRNA between normal pregnancy and pre-eclampsia (3.22 ± 2.93 , 3.88 ± 2.95 ; $P = 0.43$; Fig. 1B). Furthermore, immunohistochemical analysis also verified the above results. Immunostaining results indicated that expression level of the FSH protein was generally low in the cytotrophoblasts and syncytiotrophoblasts, blood vessel and stroma of placental villi (Fig. 4A-F). Compared

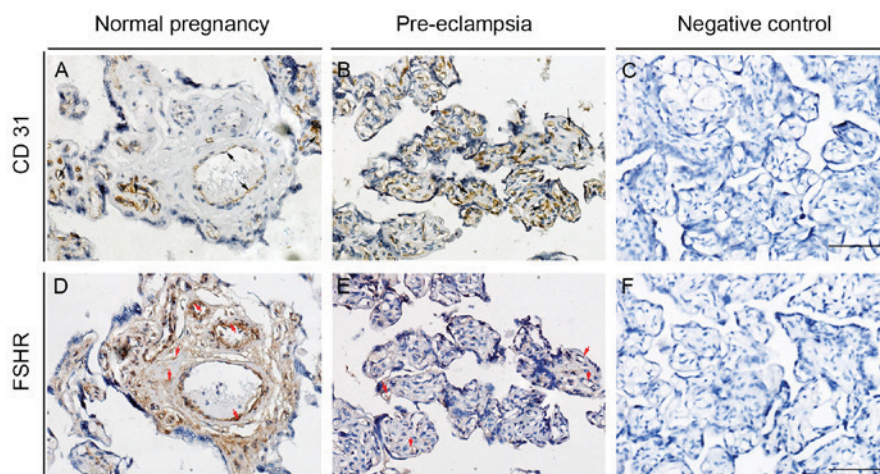


Figure 2. Expression of the FSHR protein in normal controls and pregnancies with pre-eclampsia. Serial sections of human placental tissues from (A and D) normal controls and (B and E) pre-eclampsia were stained with (A and B) CD31, (D and E) FSHR antibodies or (C and F) without primary antibodies. CD31 staining was confirmed endothelial cells identity of placental blood vessels. Arrows indicate positive cells (brownish yellow) of CD31 (black arrow) or FSHR (red arrow) staining. Scale bar, 50 μ m. FSHR, follicle-stimulating hormone receptor.

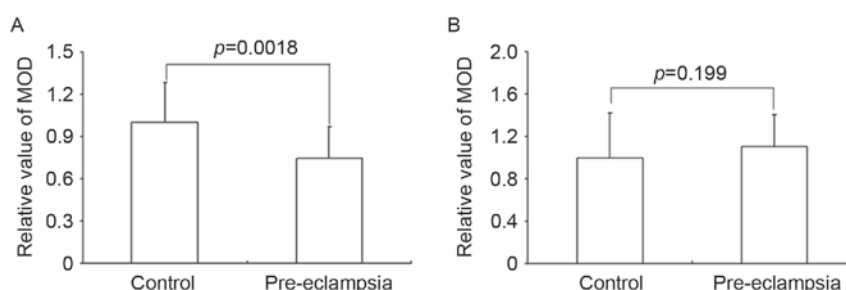


Figure 3. Relative optical density values of FSHR and FSH protein in the placental villi of normal controls and pregnancies with pre-eclampsia. (A) MOD of FSHR protein. (B) MOD of FSH protein. The results are normalized to control group and represented as the mean \pm standard deviation. FSHR, follicle-stimulating hormone receptor; FSH, follicle-stimulating hormone; MOD, mean values of optical density.

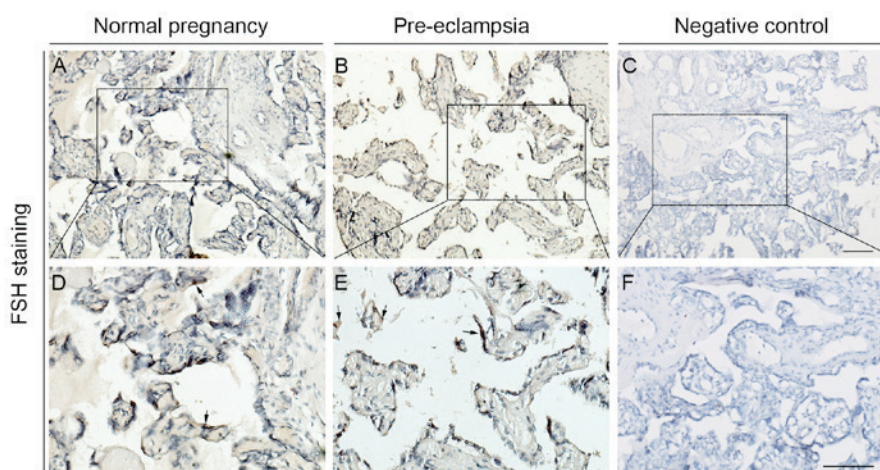


Figure 4. Expression of FSH protein in normal controls and pregnancies with pre-eclampsia. (A and D) Immunohistochemical staining of FSH in normal control placentas. (B and E) Immunohistochemical staining of FSH in pre-eclamptic placentas. (C and F) Negative control, the primary antibody was omitted or replaced by normal rabbit serum. Squared areas in A-C (magnification, $\times 100$) were magnified in the bottom with higher magnification (magnification, $\times 200$), respectively. Arrows indicate positive cells of FSH staining (brownish yellow). Scale bar, 50 μ m. FSH, follicle-stimulating hormone.

to the normal control group, the staining intensity of the FSH-positive area was a little stronger in the placental villi of pre-eclampsia, but no significant difference was identified ($P=0.199$; Fig. 3B).

Discussion

The hypothesis that was investigated in the present study involved whether placental dysfunction associated with

pre-eclampsia correlates with altered FSH and FSHR mRNA and protein expressions. The current results indicated that decreased FSHR mRNA and protein levels in placental tissues derived from pre-eclamptic women compared to those with uncomplicated pregnancies. No significant difference was demonstrated in serum FSH levels and expression levels of placental FSH mRNA and protein between normal pregnancy and pre-eclampsia.

In women, FSH serves an important role during the growth and development of ovarian follicles, including granulosa cell function and production of estrogens from androgen substrates (6,7). It is generally believed that level of pituitary FSH in the peripheral blood is suppressed during pregnancy (20). Faiman *et al* (20) observed only low levels of radio-immunoassayable serum FSH throughout human pregnancy, and its concentrations averaged 0.39 mIU/ml. The results identified that the level of serum FSH averaged 2.92 mIU/ml during the third trimester, which is consistent with Penny, Olambiwonnu and Frasier's results (21,22). However, Jaffe, Lee and Midgley (23) indicated that 76% of 45 pregnant subjects displayed values >6 mIU/ml and only 4% of subject's values <3 mIU/ml (22,23). The difference between these results may have attributed to the different sampling times and numbers, assay methods and FSH antibody used in these studies. In addition, the current data indicated that no significant differences were identified in levels of serum FSH between normal pregnancies and pre-eclampsia, suggesting that locally produced FSH at the maternal-fetal interface may exert its physiological effects through paracrine ways. Stilley *et al* (8) revealed that both FSHB mRNA (encoding the FSH β subunit) and CGA mRNA (encoding the common FSH α subunit) are present in the placental tissue, uterine deciduas and myometrium (24-26). The present RT-qPCR and immunostaining results also indicated that FSH was expressed in term placental tissues, but no significant difference was observed in the expression levels of placental FSH mRNA and protein between normal pregnancy and pre-eclampsia.

Previous studies have indicated that the FSHR is expressed in endothelial cells of placental blood vessels and FSH could promote angiogenesis of human umbilical vein endothelial cells through the FSHR (8,15,27). The results indicated that expression levels of placental FSHR were significantly reduced in pregnancies complicated by pre-eclampsia. It suggests that decreased FSHR expression could contribute to aberrant angiogenesis and trophoblast development associated with pre-eclampsia. FSH stimulates angiogenesis possibly via a different mechanism (26). Fatima *et al* (28) demonstrated that FSH could upregulate mRNA and proteins of VEGF, fibroblast growth factors 2, and their receptors *in vitro* and *in vivo* in luteal cells of buffaloes. High/mid-dose FSH significantly stimulated VEGF secretion in the slow-growing follicles at 5% O₂ environments (16). However, recombinant human FSH directly stimulates angiogenesis without VEGF secretion in FSHR-expressing endothelial cells by the PI3 K/AKT signaling pathway (15). In addition, the FSHR is selectively expressed on the surface of the blood vessels of a wide range of tumors (17,18,29-31). FSHR expression of endothelial cells may be involved in the proliferation of tumor tissues in this particular location, and could promote angiogenesis by inducing VEGF and VEGF receptor 2 signaling in tumor endothelial cells (17,30).

Interestingly, relatively recent genetic studies identified an association of single nucleotide polymorphisms in the FSHR gene to preterm birth, polycystic ovary syndrome and premature ovarian failure (32-35). However, no significant association was identified in the comparison of genotypes and allele of the FSHR gene, rs1394205, with pre-eclampsia in a Chinese population with a small sample size (~100) reported (32). To confirm the association of FSHR gene polymorphisms with pre-eclampsia, further genetic studies in other populations with larger sample sizes and denser markers are required for further investigation.

It should be noted that the present study has potential limitations. Firstly, the number of patients studied was relatively small, so further studies employing large numbers of samples are required to confirm the findings. Secondly, the authors did not completely assess all of the factors related to placental angiogenesis. Some known confounding factors, such as soluble Fms-like tyrosine kinase-1, soluble endoglin and VEGF were not included (36,37). Finally, the design of the present study does not allow us to define if altered levels of placental FSHR mRNA and protein represent a response to abnormal placentation or its cause. Also, these events may be a component of an adaptation to placental hypoxia that incorporates other angiogenic factors as well.

Overall, the current findings indicated that the expression levels of placental FSHR mRNA and protein were significantly decreased in pregnancies complicated by pre-eclampsia. These results indicated that decreased FSHR expression could contribute to aberrant angiogenesis and trophoblast development associated with pre-eclampsia. In order to determine whether similar differences antedate the clinical onset of the disease, future longitudinal studies are needed to trace the mRNA and protein expression of FSHR in first and second trimester placenta and determine whether the results are the cause or effect.

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