Placenta 83 (2019) 63–71

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Disrupted compensatory response mediated by Wolfram syndrome 1 protein and corticotrophin-releasing hormone family peptides in early-onset intrahepatic cholestasis pregnancy

Tingting Xu[a,b], Zhiyi Zhou[a,b], Na Liu[a,b], Chunyan Deng[a,b], Guiqiong Huang[a,b], Fan Zhou[a,b], Xinghui Liu[a,b], Xiaodong Wang[a,b,*]

[a] Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, China
[b] Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Sichuan, 610041, China

ARTICLE INFO

Keywords:
ICP
Earlyonset
CRH
UCN
WFS1

ABSTRACT

Introduction: The most adverse perinatal outcome of intrahepatic cholestasis of pregnancy (ICP) is sudden fetal death related to acute fetoplacental hypoxia. Corticotrophin-releasing hormone (CRH), urocortin (UCN), and Wolfram syndrome 1 (WFS1) proteins may have a compensatory response to hypoxic stress.

Methods: A total of 108 singleton pregnant women were divided into three groups: control, late-onset ICP, and early-onset ICP. Enzyme-linked immunosorbent assays were used to detected maternal serum CRH, UCN, and WFS1 levels. Western blotting and real-time polymerase chain reaction were conducted to quantify placental protein and mRNA levels of CRH, UCN, and WFS1. Pearson correlation scatterplots and Pearson correlation matrix were employed to testify the correlation.

Results: Placental WFS1 had a positive relation with placental UCN ($r = 0.69$, $P < 0.05$) and serum UCN ($r = 0.36$, $P < 0.05$). Placental CRH was positively correlated with maternal serum CRH ($r = 0.53$, $P < 0.05$). Maternal serum and placental levels of CRH, UCN, and WFS1 significantly increased in the early-onset ICP group compared with the control group ($P < 0.05$). Placental levels of UCN and WFS1 in the early-onset ICP group were significantly elevated and higher in comparison with the late-onset ICP group ($P < 0.05$). However, the transcriptional levels of CRH, UCN, and WFS1 were impaired in the early-onset ICP group.

Discussion: Our study revealed that transcription and translation of WFS1, CRH, and UCN were altered during pregnancies complicated by early-onset ICP. This disrupted compensatory response mediated by WFS1 and CRH family peptides in early-onset ICP may play a significant role in the pathogenesis of sudden fetal death in acute fetal hypoxia.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disease that typically presents in the late second or third trimester [1,2]. The incidence of this disorder fluctuates between 0.2% and 22% and varies widely by population and region [3–6]. In addition, the etiology and pathogenesis of ICP are complex, involve multiple factors, and are yet to be fully elucidated. ICP is characterized by maternal pruritus, icterus, elevated serum total bile acid (TBA), and serum liver aminotransferase activity. It is a benign disease for pregnant women as these changes disappear quickly after birth; however, they often recur in subsequent pregnancies and are associated with several adverse fetal complications including meconium-stained amniotic fluid (MSAF), premature birth, intrauterine fetal distress, and sudden intrauterine fetal death [7–9]. This, in turn, increases the risk of perinatal morbidity and mortality. The most adverse perinatal complication of ICP is sudden intrauterine fetal death.

Early-onset ICP is characterized by early onset, long disease duration, and a higher risk of adverse perinatal outcomes such as preterm birth, fetal distress, MSAF, and low birth weight compared with late-onset ICP [10]. It was reported that hepatitis C seropositivity and *in vitro* fertilization–embryo transfer twin pregnancies may be associated with early-onset ICP [6,11].

The human utero-placental-fetal unit is characterized by high blood flow volume, low vascular resistance, and lack of neural regulation [12,13]. The regulation of the utero-placental-fetal unit is efficient and

* Corresponding author. Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, China.
E-mail address: wangxd_scu@sina.com (X. Wang).

https://doi.org/10.1016/j.placenta.2019.06.378
Received 14 November 2018; Received in revised form 20 March 2019; Accepted 21 June 2019

accurate [14]and mainly depends on vasodilatory factors. Corticotropin-releasing hormone (CRH) family of peptides, mainly including CRH and urocortin (UCN), is one of the most important vasoactive factors that regulate placenta-fetal blood circulation and maintain fetoplacental circulation through a concentration-dependent compensatory vasodilatory effect. Poorly perfused utero-placental-fetal unit blood flow [15] and dysregulation of the vascular resistance balance causes placental insufficiency [16]. The mechanism for sudden intrauterine death in ICP is still unclear, and some researchers believe that it is associated with acute intrauterine fetal hypoxia [17] caused by insufficient utero-placental circulation.

The endoplasmic reticulum (ER) is the major and vital cell organelle for polypeptide biosynthesis. When ER homeostasis is disrupted in ischemia, hypoxia, hyperglycemia, and protein overload, the result is ER stress [18,19]. Wolfram syndrome 1 (WFS1) protein, an ER-resident membrane glycoprotein [20], is highly expressed in placental cells [21] and plays a key role in regulating ER stress to maintain ER homeostasis by affecting both gene transcription and protein translation [22]. Deficiency of WFS1 could activate the ER stress signaling pathway [23]. Besides the CRH family peptides, vasoactive factors and stress-related peptides [24] are also primarily synthesized in the ER of decidua, myometrium, and placental villous syncytiotrophoblasts during pregnancy [12].

The sulfated metabolites of progesterone and estradiol are elevated in pregnant women with ICP. The metabolites of several estrogens and progesterone are able to induce *trans*-inhibition of the bile salt export pump, leading to the accumulation of TBA [25]. This process leads to high levels of bile salts and bile acid from the maternal to the fetal circulation [26,27]. These bile salts and bile acid deposit in the placenta [26,28] and induce vasoconstriction of placental chorionic veins [26].

The objective of the present study is to explore the regulatory mechanism and compensatory response of WFS1 and CRH family peptides and to compare the expression of these proteins between early-onset ICP and late-onset ICP. We hypothesized that bile salts and bile acid deposits act as chronic stimuli that activate chronic ER stress via WFS1, mediating and regulating ER homeostasis and affecting the expression of CRH and UCN to achieve the physiological compensatory response during the pregnancy of ICP patients. However, patients with early-onset ICP have long-term and persist chronic stress of bile salts and bile acid, leading to impaired physiological adaptive responses, which are exacerbated by additional exposure to acute hypoxia, leading to disruption of ER homeostasis. This will ultimately result in cell death [12,29] and adverse perinatal outcomes such as sudden intrauterine fetal death.

2. Materials and methods

This study was approved by the ethical committees at the West China Second University Hospital of Sichuan University. The included population was recruited consecutively in inpatient units and outpatient clinics in our hospital between September 2016 and September 2017.

ICP is diagnosed when the TBA level is higher than 10 μmol/L, based on the guidelines of the Chinese Medical Association of Obstetrics and Gynecology [30]. If fetal hypoxia or adverse events were recognized, a cesarean section was performed immediately. For early-onset ICP, planned cesarean delivery was considered for pregnancy termination to avoid adverse pregnancy outcomes if the pregnant woman was not in labor after 37 weeks of gestation.

2.1. Inclusion criteria

Singleton pregnancies diagnosed as ICP with no other obstetric complications, including gestational hypertension, preeclampsia, gestational diabetes mellitus, virus hepatitis, kidney disease, anemia, and so forth.

2.2. Exclusion criteria

The history of threatened premature delivery, itching with skin disease, abnormal liver function caused by other diseases, and the presence of other pregnancy complications.

2.3. Sample collection

Maternal blood was collected before cesarean delivery and centrifuged to obtain serum, then stored at −80 °C.

Placental samples were collected within 15 min of the cesarean section. After briefly washing with sterile phosphate-buffered saline and dried with clean gauze, the placental tissues were immediately frozen with liquid nitrogen.

2.4. Serum biochemical analysis

Maternal serum levels of alanine transaminase (ALT), aspartate transaminase (AST), TBA, total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IDIL), γ-glutamyltransferase (GGT), albumin (ALB), globulin (GLB), albumin/globulin (A/G), total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), prealbumin (PA), fasting plasma glucose (FPG),apolipoproteins A1 (apo A1), apolipoproteins B (apo B), and homocysteine (HCY) were detected by Siemens-advia® chemistry kits using a fully automatic biochemical analyzer (Siemens-advia® 2400, Germany) by a medical laboratory at West China Second University Hospital of Sichuan University.

2.5. Enzyme-linked immunosorbent assay (ELISA) analysis

Maternal serum CRH, UCN, and WFS1 levels were tested using a specific commercially available ELISA kit (DL-develop, Cat. No.: DL-WFS1-Hu, DL-CRH-Hu, DL-UCN-Hu, Canada-China) according to the manufacturer's instructions.

2.6. RNA isolation, reverse transcription, and quantitative real-time PCR (RT-PCR)

Individual placenta chunks (100 μg) from the included patients were used for RNA isolation. TaKaRa RNAiso Plus (Trizol) was used to isolate total RNA, which was subsequently subjected to reverse-transcription into cDNA by the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). RT-PCR analyses of target mRNA were performed in two stages: Stage 1: initial denaturation 95 °C for 30s and Stage 2: PCR reaction in a 40-cycle. The PCR cycling programs were as follows: 95 °C for 5 s, 60 °C for 30 s (β-actin), 57 °C for 30s (CRH), 60 °C for 30s (WFS1), and 57 °C for 30s (UCN) with the Premix Ex Taq™ (Probe qPCR) master mix reagent (Takara, Japan) by the BioRad CFX96 qPCR system.

The probe and primer sequences used for RT-PCR were synthesized by Ruijie Biotechnology (Shanghai, China).

1 Human CRH: forward primer 5′- AGGCACCGGAGAGAGAAAGG-3′, probe: 5′-TCCGAGGAGCCTCCCATCTCCC-3′, reverse primer: 5′-CCTGGCCATTTCCAAGACTTC-3′
2 Human UCN: forward primer:5′-ACCCTTCTCTGTCCATTGA-3′, probe: 5′-TTTCACCTGCTGCGGACCCTGC-3′, reverse primer: 5′-CCACCGAGTCGAATATGATG-3′
3 Human WFS1: forward primer:5′-TACCAGGAGCCGGGAAAGA-3′, probe:5′- CAGACGGCACCGGGCCTACAAAG-3′, reverse primer: 5′-AGTGCTTCCCCACCTCAGTCT-3′
4 Human β-actin: forward primer:5′- CTACCTCATGAAGATCCTCAC CGA-3′, probe: 5′- CGGCTACAGCTTCACCACCACGGC-3′, reverse primer: 5′- TTCTCCTTAATGTCACGCACGATT-3′.

Target gene expression was normalized relative to β-actin.

2.7. Western blotting

Protein concentrations of placental tissue were tested using a Pierce® BCA Protein Assay Kit (NCI3225CH, Thermo Scientific). Total proteins (30 μg) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% milk (dissolved inTris-buffered saline buffer containing 0.05% Tween 20 [TBS-T]) for 1 h at room temperature, then incubated with the corresponding primary antibody overnight at 4 °C. Primary antibodies employed in this study were as follows: CRH (Abcam, No: ab8901, rabbit polyclonal, 1:1000), UCN (Abcam, No: ab80358, chicken polyclonal, 1:500), WFS1 (Abcam, No: ab135893, rabbit polyclonal, 1:1000), and GAPDH antibody (Thermo Fisher Scientific, No: PA1-987, rabbit polyclonal, 1:2000). Membranes were washed with TBS-T buffer (three times, 15 min each). Corresponding horseradish peroxidase-conjugated secondary antibodies were incubated at room temperature for 1 h. Blots were visualized using enhanced chemiluminescence and imaged on X-ray film. The ratio of the target band intensities to GAPDH was obtained to quantify the relative protein expression level.

2.8. Statistical analysis

All analyses were conducted using R software (version 3.5.0; R Foundation for Statistical Computing, Vienna, Austria). Pauta criterion (data greater than $\mu + 3\sigma$ or less than $\mu - 3\sigma$ will be rejected) was used to define and reject outliers. For continuous variables, mean ± standard deviation or standard error of the mean (SEM) were calculated for each group; however, for categorical variables, relative frequencies were calculated by the chi-squared test. According to the distributions of variables, One-way analysis of variance and the least significant difference test was used to establish group comparisons. Pearson parametric correlation test was used to build the correlation matrix. The probability values of correlation matrices were corrected for multiple measures and adjusted using the Holm correction. Pearson correlation scatterplots were built to test the correlation in the individual groups. All statistical tests were two-tailed, and P-values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical characteristics

One hundred eight singleton pregnant women who received regular prenatal care were enrolled in our study. These subjects were divided into three groups: control (54 cases), late-onset ICP (37cases), and early-onset ICP (17 cases). The normal pregnancy category had the same inclusion and exclusion criteria, except for the diagnosis of ICP. All included participants delivered by cesarean section, because spontaneous vaginal deliveries may influence the expression of hypoxia stress-related hormones.

There was no significant difference in age, maternal weight, gravidity, parity, or 10-min Apgar score among these three groups. Similarly, no significant difference in the rate of perinatal death, MSAF, history of ICP, family history of ICP, and transfer to the neonatal intensive care unit was found. However, there were significant differences in gestational days, fetal weight, the rate of preterm birth, and the rate of intrauterine fetal stress among these three groups ($P < 0.05$). In addition, early-onset ICP was associated with a higher risk of preterm birth, low birth weight, and intrauterine fetal stress (Table 1).

3.2. Maternal serum biochemical parameters

There was a significant difference in maternal serum ALT (U/L), AST (U/L), TBA (umol/L), TBIL (umol/L), DBIL (umol/L), IDIL (umol/L), GGT (U/L), GLB (g/L), apo A1 (g/L), and A/G levels among control, early-onset ICP, and late-onset ICP groups ($P < 0.05$). The levels of TBA (86.19 vs. 30.24) and DBIL (6.86 vs. 4.92) in early-onset ICP were much higher than those in late-onset ICP. However, no significant difference was found in the levels of maternal serum TC (mmol/L), TG (mmol/L), HDL (mmol/L), LDL (mmol/L), ALP (U/L), LDH (U/L), PA (mg/L), FPG (mmol/L), apo B (g/L), and HCY (umol/L) among these three groups (Table 2).

3.3. Maternal serum expression of CRH and UCN increased in early-onset ICP

Serum CRH levels in the early-onset ICP group were significantly higher than those in the control group ($P < 0.05$). Similarly, maternal serum CRH levels in the late-onset ICP group were significantly higher than those in the control group ($P < 0.05$). However, no significant differences were observed between early-onset ICP and late-onset ICP (Fig. 1A).

Serum expression of UCN in the early-onset ICP group was significantly higher than that in the control group ($P < 0.05$), although no other difference was found between the late-onset ICP group and control group or between the early-onset ICP group and late-onset ICP group (Fig. 1B).

There was no significant difference in maternal serum WFS1 expression among these three groups (Fig. 1C).

3.4. Placental CRH, UCN, and WFS1 protein expression increased in early-onset ICP

CRH expression in the placentae of the early-onset ICP group was significantly higher than that in healthy controls ($P < 0.05$). Similarly, CRH levels in the late-onset ICP group were significantly higher than that in control group ($P < 0.05$; Fig. 2A).

Placental UCN expression in the early-onset ICP group was significantly higher than that in control and late-onset ICP group ($P < 0.05$). In addition, UCN expression in the late-onset ICP group was also significantly higher than that in the control group ($P < 0.05$; Fig. 2B).

WFS1 levels in pregnant women with early-onset ICP were significantly higher than that in the control group and late-onset ICP group ($P < 0.05$; Fig. 2C).

3.5. Placental UCN and WFS1 mRNA expression was impaired in early-onset ICP

Placental CRH mRNA levels were unchanged and had no significant difference among these three groups. However, CRH mRNA expression in the early-onset ICP group was lower than that in the late-onset ICP group (Fig. 3A).

In the early-onset ICP group, the expression of placental UCN mRNA was significantly lower than that in the late-onset ICP and control groups ($P < 0.05$; Fig. 3B).

Placental WFS1 mRNA levels in the early-onset ICP group were significantly lower than those in the late-onset ICP group ($P < 0.05$). Similarly, the expression of WFS1 mRNA in the early-onset ICP group was lower than that in the control group. However, WFS1 mRNA levels in the late-onset ICP group were significantly higher than that in the control group ($P < 0.05$; Fig. 3C).

3.6. Pearson correlation scatterplots of stress factors

Data of maternal serum and placental stress factors were used to map scatterplots with correlation. The corresponding scatters diagram showed a strong positive correlation between placental WFS1 and UCN in the control group ($r = 0.96$, $P < 0.05$) and late-onset ICP group

Table 1
Clinical characteristics among control, early-onset ICP, and late-onset ICP groups.

Group	Control	Late-onset ICP	Early-onset ICP	F	P
Number	54	37	17		
Age (years)	31.5 ± 0.69	31.11 ± 1.11	31.29 ± 0.83	0.12	0.89
Maternal weight (Kg)	65.74 ± 1.86	66.21 ± 2.01	65.31 ± 1.87	0.12	0.89
Gravidity	0.5 ± 0.12	0.32 ± 0.12	0.76 ± 0.47	0.96	0.39
Parity	2.44 ± 0.28	2.19 ± 0.32	2.53 ± 0.52	0.33	0.72
GD (day)	274.09 ± 2.26	262.92 ± 1.8	253 ± 2.4	28.71	< 0.05
Fetal weight (g)	3258.44 ± 82.11	2972.57 ± 109.46	2667.06 ± 70.12	12.28	< 0.05
10min Apgar score	10 ± 0	10 ± 0	10 ± 0	0.84	0.44
History of ICP	0% (0/54)	0% (0/37)	0% (0/17)		
Famil history of ICP	0% (0/54)	0% (0/37)	0% (0/17)		
Preterm birth	11.11% (6/54)	27.03% (10/37)	64.71% (11/17)		< 0.05
Intrauterine fetal stress	0% (0/54)	2.70% (1/37)	11.76% (2/17)		< 0.05
MSAF	0% (0/54)	0% (0/37)	5.88% (1/17)		0.07
NICU	0% (0/54)	0% (0/37)	0% (0/17)		
Perinatal death	0% (0/54)	0% (0/37)	0% (0/17)		

ICP: intrahepatic cholestasis of pregnancy; GD: gestational day; MSAF: meconium-stained amniotic fluid.

Table 2
Biochemical indicators of included study population.

Group	Control	Late-onset ICP	Early-onset ICP	F	P
Number	54	37	17		
ALT (U/L)	20.75 ± 2.39	223.43 ± 40.46	219.29 ± 44.18	23.01	< 0.05
AST (U/L)	24.19 ± 2.00	147.38 ± 26.69	164.00 ± 35.22	18.86	< 0.05
TBA (umol/L)	2.60 ± 0.32	30.24 ± 6.94	86.19 ± 22.09	18.38	< 0.05
TBIL (umol/L)	9.69 ± 0.52	12.15 ± 1.09	12.88 ± 1.54	4.29	< 0.05
DBIL (umol/L)	1.23 ± 0.20	4.92 ± 0.93	6.86 ± 1.46	16.56	< 0.05
IDIL (umol/L)	8.35 ± 0.45	6.64 ± 0.53	6.02 ± 0.41	9.04	< 0.05
GGT (U/L)	18.78 ± 3.22	67.27 ± 17.14	58.75 ± 19.35	6.45	< 0.05
ALB (g/L)	36.82 ± 0.45	36.85 ± 0.93	37.11 ± 0.88	0.01	0.99
GLB (g/L)	28.89 ± 0.85	29.72 ± 0.96	26.24 ± 0.45	5.73	< 0.05
A/G	1.30 ± 0.04	1.26 ± 0.04	1.49 ± 0.08	6.72	< 0.05
TC (mmol/L)	5.76 ± 0.25	6.69 ± 0.31	6.28 ± 0.32	1.95	0.16
TG (mmol/L)	3.59 ± 0.30	4.59 ± 0.44	3.73 ± 0.27	2.02	0.15
HDL (mmol/L)	1.69 ± 0.05	1.50 ± 0.15	1.49 ± 0.10	0.91	0.41
LDL (mmol/L)	3.42 ± 0.26	4.1 ± 0.27	3.47 ± 0.30	1.50	0.24
ALP (U/L)	179.86 ± 11.82	299.50 ± 19.12	225.06 ± 26.14	2.16	0.15
LDH (U/L)	238.13 ± 13.10	302.13 ± 27.07	235.47 ± 19.48	0.01	0.94
PA (mg/L)	215.00 ± 5.30	189.69 ± 9.77	204.41 ± 15.29	0.49	0.49
FPG (mmol/L)	18.95 ± 0.99	14.92 ± 1.19	18.18 ± 2.34	0.45	0.50
apo A1 (g/L)	23.88 ± 0.82	19.14 ± 2.29	14.15 ± 2.10	6.58	< 0.05
apo B (g/L)	16.81 ± 1.21	20.00 ± 1.61	19.23 ± 2.37	0.62	0.44
HCY (umol/L)	16.00 ± 0.85	17.71 ± 1.87	19.08 ± 2.23	3.51	0.07

N: number; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBA: total bile acid; TBIL: total bilirubin; DBIL: direct bilirubin; IDIL: indirect bilirubin; GGT: γ-glutamyltransferase; ALB: albumin; GLB: globulin; A/G: albumin/globulin; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; PA: prealbumin; FPG: fasting plasma glucose; apo A1: apolipoproteins (apo) A1; apo B: apolipoproteins (apo) B; HCY: homocysteine.

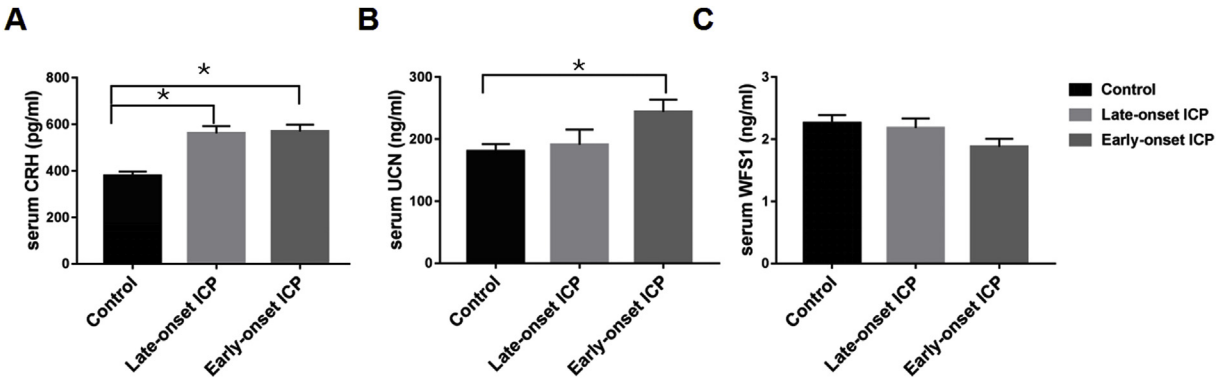

Fig. 1. Serum expression of CRH, UCN, and WFS1. (A) Serum levels of CRH in pregnant women (overall $F = 17.77$, $P < 0.05$). (B)Serum expression of UCN among the three groups ($F = 5.48$, $P < 0.05$). (C) Serum levels of WFS1 among the three groups ($F = 1.16$, $P = 0.32$). Control, n = 24; late-onset ICP, n = 16; and early-onset ICP, n = 10. *$P < 0.05$. Data are shown as M ± SEM.

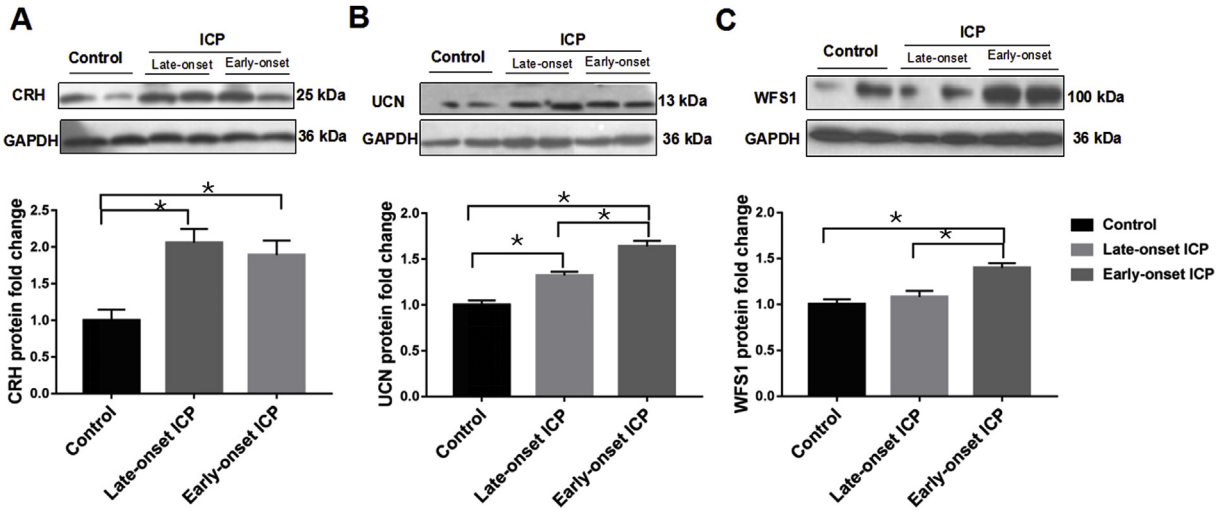

Fig. 2. CRH, UCN, and WFS1 protein levels. (A) Representative western blots of placental levels of CRH and GAPDH protein and densitometric analysis of CRH expression normalized to GAPDH ($F = 16.34$, $P < 0.05$). (B) Representative western blots of placental levels of UCN and GAPDH protein and densitometric analysis of UCN protein expression normalized to GAPDH ($F = 15.7$, $P < 0.05$). (C) Representative western blots of placental levels of UCN and GAPDH protein and densitometric analysis of placental WFS1 protein expression normalized to GAPDH ($F = 11.69$, $P < 0.05$). Control, n = 8; late-onset ICP, n = 9; and early-onset ICP, n = 9. *$P < 0.05$. Data are shown as M ± SEM.

($r = 0.86$, $P < 0.05$). Conversely, a negative correlation was found between maternal serum WFS1 and CRH in the early-onset group ($r = -0.73$, $P < 0.05$). However, no significant correlation of other stress factors was found among these three groups (Fig. 4).

3.7. Pearson correlation matrix

All maternal serum biochemical indicators (hepatic and biliary parameters), stress factors, and placental stress factors were used in the Pearson parametric correlation test for building a correlation matrix (Fig. 5).

Our data showed that maternal serum ALT ($r = 0.32$, $P < 0.05$), AST ($r = 0.36$, $P < 0.05$), and TBA ($r = 0.34$, $P < 0.05$) had a significant positive correlation with placental WFS1 protein. Maternal serum TC levels ($r = 0.57$, $P < 0.05$) significantly and positively correlated with placental UCN protein.

Besides, maternal serum ALT ($r = 0.53$, $P < 0.05$), AST ($r = 0.50$, $P < 0.05$), GGT ($r = 0.41$, $P < 0.05$), ALP ($r = 0.35$, $P < 0.05$), LDH ($r = 0.29$, $P < 0.05$), and HCY($r = 0.49$, $P < 0.05$) levels had a significant and positive relationship with placental CRH protein. Maternal serum GGT ($r = 0.35$, $P < 0.05$) positively correlated with placental CRH mRNA. Furthermore, maternal serum ALT ($r = 0.46$, $P < 0.05$), TBIL ($r = 0.31$, $P < 0.05$), DBIL ($r = 0.32$, $P < 0.05$), ALP ($r = 0.26$, $P < 0.05$) and HCY ($r = 0.55$, $P < 0.05$) significantly correlated positively with maternal serum CRH.

Similarly, placental WFS1 protein was positively correlated with placental UCN ($r = 0.69$, $P < 0.05$) and serum UCN ($r = 0.36$, $P < 0.05$). Placental CRH had a positive correlation with maternal serum CRH ($r = 0.53$, $P < 0.05$) and placental UCN ($r = 0.74$, $P < 0.05$). Placental UCN protein was positively correlated with maternal serum CRH ($r = 0.60$, $P < 0.05$).

4. Discussion

Our results indicate that WFS1 may regulate the expression of CRH and UCN. In early-onset ICP, maternal serum and placental CRH, UCN, and WFS1 protein expression were elevated, while the transcription levels of these proteins were aberrant. This altered transcription and translation of CRH, UCN, and WFS1 in pregnant women with early-onset ICP may be the reason for the increased risk of sudden fetal death.

In 1995, Kirkinen, P [31]. first reported an unusual case of ICP occurring as early as 13 weeks of gestation. Since then, other studies have reported that some ICP were diagnosed during the first or early second trimester [32–35]. Most scholars believe that the perinatal

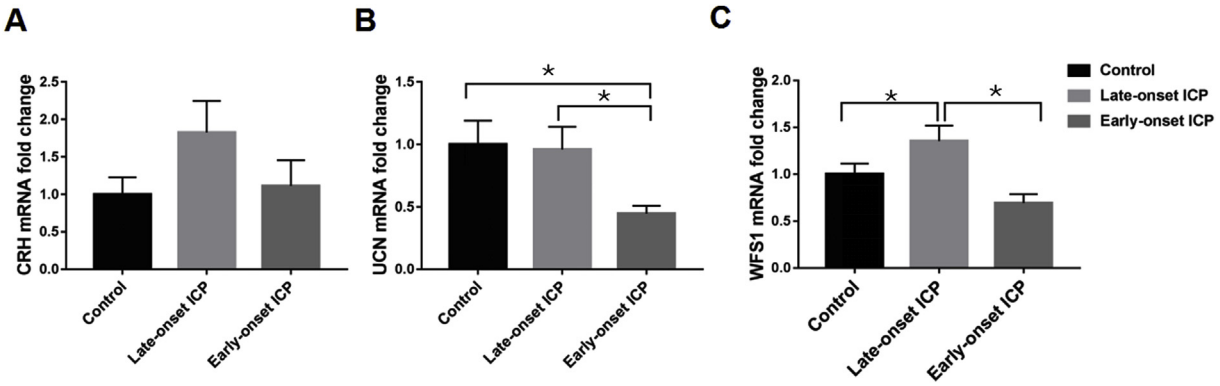

Fig. 3. CRH, UCN, and WFS1 mRNA expression in human placentae. (A) Quantitative analysis of CRH mRNA expression normalized to β-actin among control, late-onset ICP, and early-onset ICP groups (overall $F = 0.40$, $P = 0.68$). (B) Quantitative analysis of UCN mRNA expression among these three groups (overall $F = 10.48$, $P < 0.05$). (C) Quantitative analysis of WFS1 mRNA expression among the three groups (overall $F = 7.63$, $P < 0.05$). Control, n = 10; late-onset ICP, n = 8; late-onset ICP, n = 10. Data are shown as M ± SEM.

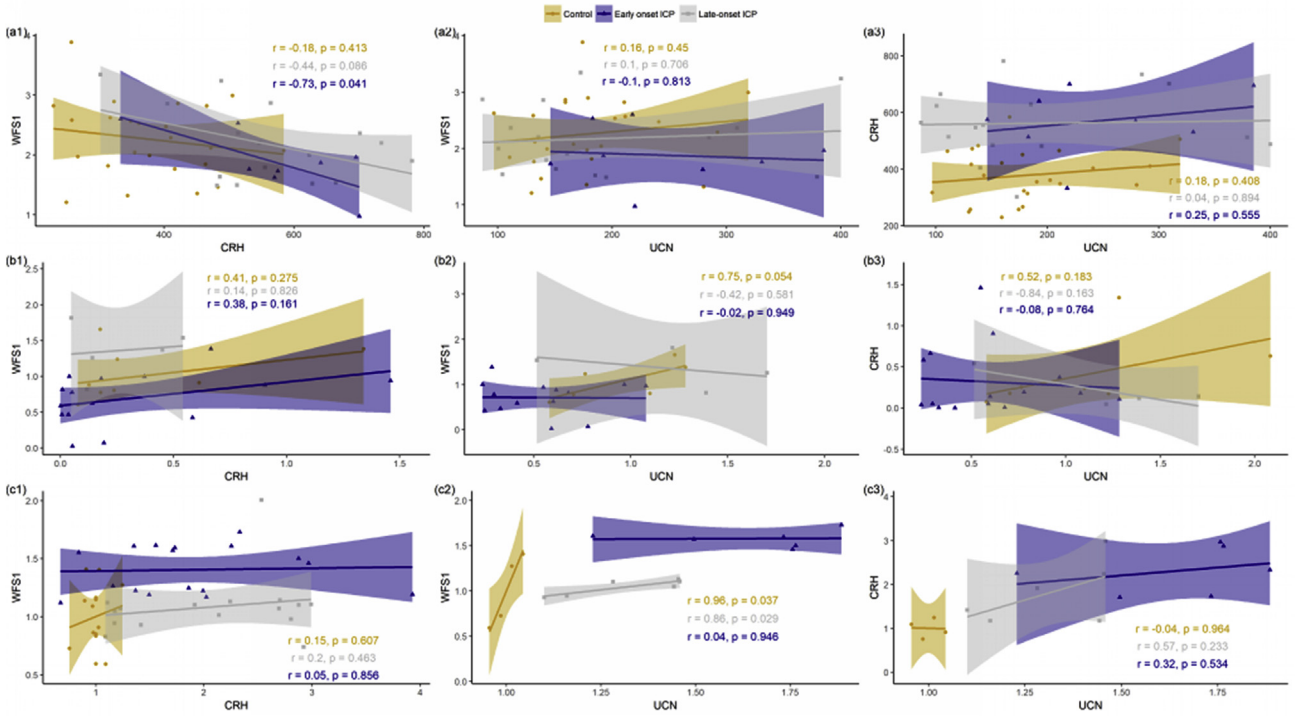

Fig. 4. Pearson correlation scatterplots of maternal serum and placental stress factors. Scatter plots containing the correlation coefficients and paired *P*-values between each variable, in which the orange, blue, and gray dots represent variables consistent with control, early-onset ICP, and late-onset ICP groups, respectively. Figure a1, a2, and a3 represent maternal serum variables; b1, b2, b3 represent placental mRNA variables; and c1, c2, c3 represent placental protein variables. Correlations with *P*-value < 0.05 are considered significant.

outcome of early-onset ICP is poor. Hence, the concept of early-onset ICP has been gradually proposed, and some obstetricians are curious about the clinical features and the prognosis of infants in early-onset ICP. Currently, there is still no consensus regarding uniform diagnostic criteria for early-onset ICP. Thus, the definition of gestational age for the onset of early-onset ICP is still inconclusive. Some researchers consider the 28th gestational week to be the cutoff between early-onset and late-onset ICP [11,36–39], while others use 32nd [40] or 34th [10] weeks as the cutoff gestational age.

Zhou L et al. [36] found that TBA levels and perinatal outcomes of ICP patients between 28 and 31^{+6} gestational weeks and ≥32 weeks were similar, with no significant difference. Hence, the authors propose that it is much more reasonable to diagnose early-onset ICP before 28 weeks. Uyar I et al. [40] classified pregnant women who were diagnosed before 32 weeks as early-onset ICP. Lin J et al. [10] conducted a retrospective clinical study and reported that 34 weeks was the most appropriate cutoff gestational age for diagnosing early-onset ICP.

In our study, we diagnosed early-onset ICP before 28 gestational weeks based on our own clinical experience and data published by other scholars. Overall, our results indicated that early-onset ICP is associated with a higher risk of preterm birth, low birth weight, and intrauterine fetal stress, which is consistent with the work of other researchers who considered the 28th gestational week as the cutoff for diagnosing early-onset ICP [11,36–39].

ER stress is either acute or chronic in nature and is manifested as distinct cell survival or death outcomes [41,42]. Chronic ER stress can persist for several days to years and is often a prosurvival, adaptive mechanism [41]. However, when ER stress persists, cellular survival mechanisms are overcome by ER stress-mediated apoptotic cell death [23]. From our results, we found that maternal serum ALT, AST, and TBA levels were positively correlated with placental WFS1 protein, whereas levels of placental WFS1in the late-onset group and the early-onset group were higher than in the control group. In addition, placental WFS1 was positively correlated with placental UCN and serum UCN. Besides, we also found some hepatic and biliary parameters including ALT, AST, TBIL, DBIL, GGT, ALP, LDH, HCY were positively correlated with maternal serum CRH, placental CRH protein or CRH mRNA. Furthermore, maternal serum TC had a significant positive correlation with placental UCN protein. We speculate that ER stress was likely activated in ICP pregnant women and that WFS1 can regulate the expression of CRH family peptides. The future work of our team will precisely examine molecular activation of the ER stress response in these women.

The CRH family peptides is vital to maintain efficient fetoplacental circulation and exert a critical role in several physiological as well as pathological obstetric conditions [43]. These peptides mainly participate in regulating utero-placental-fetal unit blood flow to maintain its physical protection role when the fetoplacental unit encounters hypoxic stress. Moreover, as stress-related peptides, they also play a pivotal role in adapting to stress via activation of signaling cascades [44,45] that are involved in decidualization, embryo implantation, and maintenance of pregnancy [46,47]. Research has shown that concentrations of CRH are increased in cases of intrauterine growth restriction and preterm birth [46] and that placental CRH levels are increased in maternal blood under stress [48–50]. In addition, our results showed that placental and maternal serum CRH and UCN levels increased in early-onset ICP and late-onset ICP, which is consistent with the results of the above studies.

In physiological conditions, in response to stress, the CRH family peptides are released by the hypothalamus, while the placenta mainly produces and secretes CRH family peptides into maternal-fetal circulation during pregnancy [46]. In our study, the trend of expression of maternal serum and placental CRH family peptides was similar, and placental CRH had a positive correlation with maternal serum CRH. This suggests that the human placenta is a major organ that produces the CRH family peptides, and some of these peptides can be released from the placenta into circulation to play a role in the vasodilatory effects and adaptation to stress.

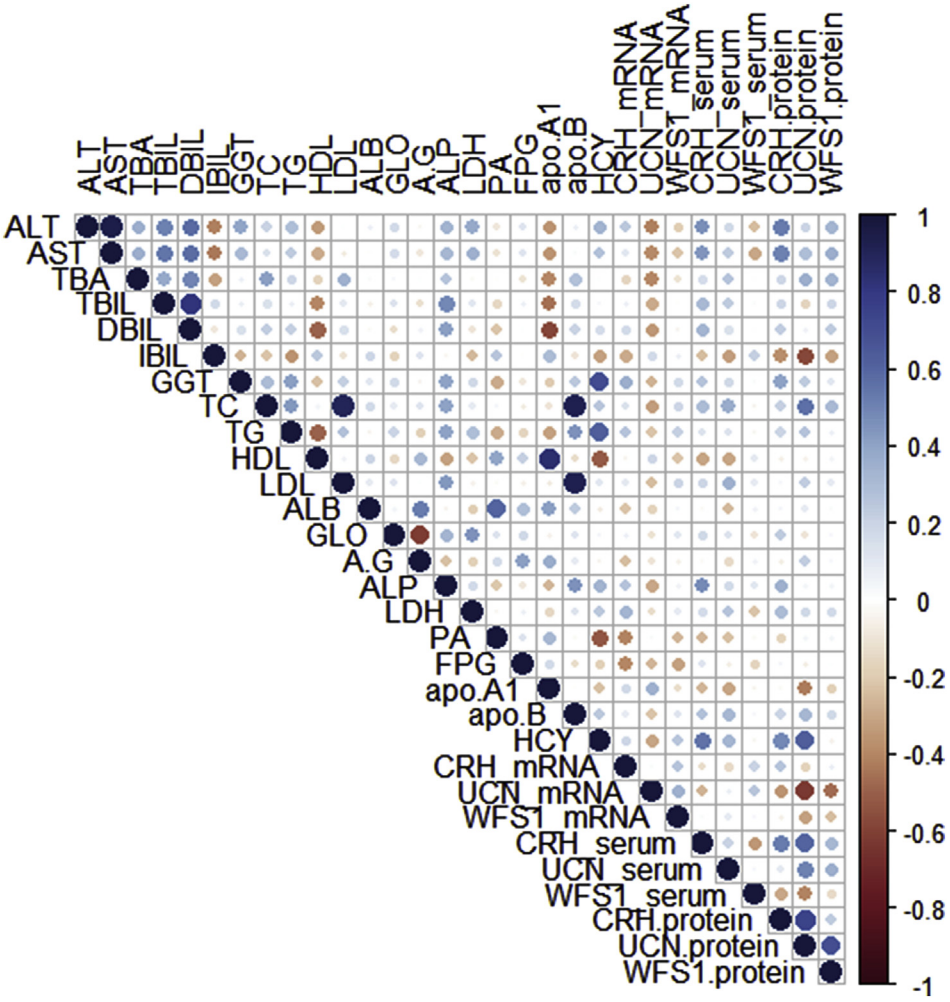

Fig. 5. Pearson correlation matrix of maternal serum biochemical indicators, stress factors, and placental stress factors. Pearson correlation matrix was performed by R package "corrgram": Plot a Correlogram (Version 1.13). The value of the Pearson correlation is color coded using the color map. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. On the right side of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. In the above plot, correlations with a *P*-value < 0.05 are considered significant.

It has been reported that CRH vasodilatory effects are 53 times more potent than those of PGI_2 [51], and UCN is approximately 30-fold higher potency than that of CRH [52]. CRH and UCN are complexly interrelated; however, UCN seems to play a much more important role in stress adaptation [46]. In the current study, we found that placental CRH had a positive correlation with placental UCN protein, and placental UCN protein was positively correlated with maternal serum CRH. This implies that CRH and UCN interact with their own expression. What's more, placental CRH levels in late-onset ICP were higher than those in early-onset ICP; however, placental UCN levels in early-onset ICP were elevated and higher than in late-onset ICP. This is indicative of UCN's playing a more significant role in early-onset ICP than CRH. Conversely, CRH may be more involved in the pathogenesis of late-onset ICP. Considering the fact that early-onset ICP involves long-term bile salt and bile acid stimulation and an increased risk of adverse perinatal outcomes, we believe that CRH and UCN exist in an efficient feedback and compensatory loop in utero-placental-fetal unit vasodilation to maintain its physiologic protection role.

Besides its canonical functions, WFS1 has been implicated in regulating ER stress to maintain ER homeostasis by affecting both gene transcription and protein translation. Research has shown that the CRH family of peptides as stress peptides are potent modulators of cell physiology by their influence on gene transcription [53]. In the control group and late-onset ICP group, placental WFS1 and UCN had a strong positive correlation. This strong positive correlation may be a physiological control mechanism to achieve ER homeostasis. Conversely, maternal serum WFS1 and CRH negatively correlated with one another, and the gene transcription of WFS1, CRH, and UCN was impaired in the early-onset group. Hence, we hypothesize that the compensatory responses mediated by the WFS1 and CRH family peptides may be impaired in early-onset ICP induced by long-term and persistent chronic stress, which leads to poor fetoplacental vascular perfusion. In the event of sudden fetal death, this disrupted compensatory mechanism is likely to be exacerbated by additional exposure to acute hypoxia leading to disruption of ER homeostasis, cell death, and adverse perinatal outcomes. This may explain why patients with early-onset ICP often have an increased rate of sudden fetal demise.

Several studies have addressed the role of ER stress in obstetric complications [54,55]. The research of WFS1 mainly focuses on Wolfram syndrome, including diabetes insipidus [56], diabetes mellitus [57], optic atrophy [58], and deafness [59]. However, there is only one published paper that focuses on WFS1 protein and obstetric complications. Lucariello A et al. [21] found that although WFS1 protein had a high level during the first trimester and a moderate level in the third trimester, it was strongly reduced in the third trimester in diabetic women. It seems that WFS1 may participate in maintaining glucose homeostasis. There are no reports on the dysfunction of WFS1 and CRH family peptides in ICP pathophysiology. To the best of our knowledge,

we are the first to report WFS1 and CRH family peptides and their dysregulation in ICP under ER stress. Our results testify that the compensatory response mediated by WFS1 and CRH family peptides is disrupted in early-onset ICP. We believe our results shed light on the contribution of these critical factors in mediating stress responses during pregnancy, with implications for the pathogenesis of utero-placental-fetal hypoxia.

Our study is observational, and we found that the observed alterations in the markers of ER stress are the underlying mechanisms in the pathology condition. However, it is possible that these observed alterations could result from the underlying pathology, rather than cause it, and so hypotheses of the mechanism still need to be proved in the future. Hence, future lines of investigation will expand on the differential regulation of transcription and translation of these stress factors and the role of ER stress in order to explore the molecular and signaling pathways that are affected by this disease. The number of early-onset ICP patients in our study was small, so it is difficult for us to perform a clinically relevant analysis, including primiparae, gestational length, and early delivery. A large-scale, multicenter cohort study of ICP, especially of early-onset ICP, is very necessary in the future to investigate alternative diagnostic and therapeutical interventions in ICP disease.

In summary, our study confirms that WFS1 can regulate the expression of CRH and UCN and that the compensatory response of these stress factors is impaired in early-onset ICP. This attenuated phenomenon in early-onset ICP might impair the blood flow regulation of the utero-placental-fetal unit, resulting in poor fetoplacental vascular perfusion and adverse pregnancy outcomes, which are of important clinical interest.

Funding

This work was supported by National Natural Science Foundation of China (Project No. 81571446).

Declarations of interest

The authors declare that there is no conflict of interest.

Acknowledgments

Tingting Xu and Xiaodong Wang conceived the study. Tingting Xu and Zhiyi Zhou performed experiments. Tingting Xu and Na Liu analyzed data. Chunyan Deng and Guiqiong Huang contributed to the preparation of tables and figures. Xinghui Liu provided technical assistance. Tingting Xu drafted the manuscript. Fan Zhou revised the manuscript. Xiaodong Wang supervised the whole study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.06.378.

References

[1] Y. Bacq, Liver diseases unique to pregnancy: a 2010 update, Clin. Res. Hepatol. Gastroenterol. 35 (3) (2011) 182–193.
[2] C. Williamson, V. Geenes, Intrahepatic cholestasis of pregnancy, Obstet. Gynecol. 124 (1) (2014) 120–133 Jul.
[3] B. Berg, G. Helm, L. Petersohn, et al., Cholestasis of pregnancy. Clinical and laboratory studies, Acta Obstet. Gynecol. Scand. 65 (2) (1986) 107–113.
[4] L.M. Glynn, C.A. Sandman, Evaluation of the association between placental corticotrophin-releasing hormone and postpartum depressive symptoms, Psychosom. Med. 76 (5) (2014) 355–362.
[5] M. Vatish, H.S. Randeva, D.K. Grammatopoulos, Hormonal regulation of placental nitric oxide and pathogenesis of pre-eclampsia, Trends Mol. Med. 12 (5) (2006) 223–233.
[6] V. Geenes, C. Williamson, Intrahepatic cholestasis of pregnancy, World J. Gastroenterol. 15 (17) (2009) 2049–2066 May 7.
[7] H. Reyes, What have we learned about intrahepatic cholestasis of pregnancy? Hepatology 63 (1) (2016) 4–8.
[8] V. Geenes, L.C. Chappell, P.T. Seed, et al., Association of severe intrahepatic cholestasis of pregnancy with adverse pregnancy outcomes: a prospective population-based case-control study, Hepatology 59 (4) (2014) 1482–1491.
[9] X.D. Wang, Q. Yao, B. Peng, et al., A clinical of intrahepatic cholestasis of pregnancy in 1241 cases(in Chinese), Chin. J. Hepatol. 15 (4) (2007) 291–293.
[10] J. Lin, W. Gu, Y. Hou, Diagnosis and prognosis of early-onset intrahepatic cholestasis of pregnancy: a prospective study, J. Matern. Fetal Neonatal Med. (2017) 1–7 Nov 7.
[11] C. Feng, W.J. Li, R.H. He, et al., Impacts of different methods of conception on the perinatal outcome of intrahepatic cholestasis of pregnancy in twin pregnancies, Sci. Rep. 8 (1) (2018) 3985.
[12] F. Petraglia, A. Imperatore, J.R. Challis, Neuroendocrine mechanisms in pregnancy and parturition, Endocr. Rev. 31 (6) (2010) 783–816.
[13] A.L. Boura, W.A. Walters, M.A. Read, et al., Autacoids and control of human placental blood flow, Clin. Exp. Pharmacol. Physiol. 21 (10) (1994) 737–748.
[14] V. Hansen, S. Maigaard, J. Allen, et al., Effects of vasoactive intestinal polypeptide and substance P on human intramyometrial arteries and stem villous arteries in term pregnancy, Placenta 9 (5) (1988) 501–506.
[15] Y. Zhang, S. Liu, X. Wang, Study on fetal hypoxia in intrahepatic cholestasis of pregnancy (in Chinese), Zhonghua Fu Chan Ke Za Zhi 35 (10) (2000) 113–114.
[16] M. He, Z. Liu, X. Wang, Decreased volume of placental lobular villi vessels in patients with intrahepatic cholestasis of pregnancy (in Chinese), J Sichuan Univ(Med Sci Edi) 42 (6) (2011) 797–801.
[17] S. Ozkan, Y. Ceylan, O.V. Ozkan, et al., Review of a challenging clinical issue: intrahepatic cholestasis of pregnancy, World J. Gastroenterol. 21 (23) (2015) 7134–7141 Jun 21.
[18] B. Jiang, Q.C. Fang, W.P. Jia, The research of WFS1 and endoplasmic reticulum stress in the pathogenesis of diabetes mellitus (in Chinese), Natl. Med. J. China (Peking) 91 (8) (2011) 568–571.
[19] I. Kim, W. Xu, J.C. Reed, Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities, Nat. Rev. Drug Discov. 7 (12) (2008) 1013–1030.
[20] K. Takeda, H. Inoue, Y. Tanizawa, et al., WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain, Hum. Mol. Genet. 10 (5) (2001) 477–484.
[21] A. Lucariello, A. Perna, C. Sellitto, et al., Modulation of wolframin expression in human placenta during pregnancy: comparison among physiological and pathological states, BioMed Res. Int. (2014) 985478 2014.
[22] P. Walter, D. Ron, The unfolded protein response: from stress pathway to homeostatic regulation, Science 334 (6059) (2011) 1081–1086.
[23] T. Yamada, H. Ishihara, A. Tamura, et al., WFS1-deficiency increases endoplasmic reticulum stress, impairs cell cycle progression and triggers the apoptotic pathway specifically in pancreatic beta-cells, Hum. Mol. Genet. 15 (10) (2006) 1600–1609.
[24] D. Grammatopoulos, The family of corticotropin-releasing hormone (CRH) peptides: important regulators of adipocyte function, Endocrine (2008) 16.
[25] M. Vallejo, O. Briz, M.A. Serrano, et al., Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy, J. Hepatol. 44 (6) (2006) 1150–1157.
[26] I. Lata, Hepatobiliary diseases during pregnancy and their management: an update, Int. J. Crit. Illn. Inj. Sci. 3 (3) (2013) 175–182.
[27] W.M. Ataalla, D.H. Ziada, R. Gaber, et al., The impact of total bile acid levels on fetal cardiac function in intrahepatic cholestasis of pregnancy using fetal echocardiography: a tissue Doppler imaging study, J. Matern. Fetal Neonatal Med. 29 (9) (2016) 1445–1450.
[28] V.L. Geenes, Y.H. Lim, N. Bowman, et al., A placental phenotype for intrahepatic cholestasis of pregnancy, Placenta 32 (12) (2011) 1026–1032.
[29] X.D. Wang, H.Y. Yu, M.M. He, et al., Action of corticotropin-releasing hormone on its own receptor type-1 in human placenta (in Chinese), J Sichuan Univ(Med Sci Edi) 38 (1) (2007) 101–104.
[30] Guidelines of the Chinese medical association of obstetrics and Gynecology, Chin. J. Obstet. Gynecol. 31 (7) (2015) 481–485.
[31] P. Kirkinen, M. Ryynanen, First-trimester manifestation of intrahepatic cholestasis of pregnancy and high fetoplacental hormone production in a triploid fetus. A case report, J. Reprod. Med. 40 (6) (1995) 471–473.
[32] D. Brites, C.M. Rodrigues, C. Cardoso Mda, et al., Unusual case of severe cholestasis of pregnancy with early onset, improved by ursodeoxycholic acid administration, Eur. J. Obstet. Gynecol. Reprod. Biol. 76 (2) (1998) 165–168.
[33] T.T. Chao, J.S. Sheffield, Primary dermatologic findings with early-onset intrahepatic cholestasis of pregnancy, Obstet. Gynecol. 117 (2 Pt 2) (2011) 456–458.
[34] A.M. Zamah, Y.Y. El-Sayed, et al., Two cases of cholestasis in the first trimester of pregnancy after ovarian hyperstimulation, Fertil. Steril. 90 (4) (2008) 1202.e7-10.
[35] R. Smolarczyk, M. Grymowicz, J. Sienko, et al., Successful perinatal outcome in an early onset intrahepatic cholestasis of pregnancy with extremely high serum hepatic function tests, Gynecol. Endocrinol. 25 (7) (2009) 475–476.
[36] L. Zhou, H.B. Qi, X. Luo, Analysis of clinical characteristics and pednatal outcome of early-onset intrahepatic cholestasis of pregnancy, Chin. J. Obstet. Gynecol. 48 (1) (2013) 20–24.
[37] X. Ge, Y.Q. Xu, S.H. Huang, et al., Intrahepafic cholestasis of pregnancy and fetal outcomes: a prospective birth cohort study, Chin. J. Epidemiol. 37 (2) (2016) 187–191.
[38] J. Jin, S.L. Pan, L.P. Huang, et al., Risk factors for adverse fetal outcomes among women with early- versus late-onset intrahepatic cholestasis of pregnancy, Int. J. Gynaecol. Obstet. 128 (3) (2015) 236–240.
[39] J.W. Li, J.M. Niu, P. Zhou, et al., Study of characteristic of Glucolipid Metabolism in

pregnent women with Intrahepatic Cholestasis and analysis of the relation of disease and pregnancy outcomes, Chin. J. Fam. Plan. 23 (12) (2015) 815–818.
[40] I. Uyar, I. Gulhan, D. Oztekin, et al., Intrahepatic cholestasis of pregnancy may lead to low birth weight, Turk. J. Med. Sci. 45 (3) (2015) 723–728.
[41] D.T. Rutkowski, R.J. Kaufman, That which does not kill me makes me stronger: adapting to chronic ER stress, Trends Biochem. Sci. 32 (10) (2007) 469–476.
[42] Y. Liu, J.R. Connor, From adaption to death: endoplasmic reticulum stress as a novel target of selective neurodegeneration? Neural Regen. Res. 10 (9) (2015) 1397–1398.
[43] S.G. Vitale, A.S. Lagana, A.M. Rapisarda, et al., Role of urocortin in pregnancy: an update and future perspectives, World J. Clin. Cases 4 (7) (2016) 165–171.
[44] S. Lachize, E.M. Apostolakis, S. van der Laan, et al., Steroid receptor coactivator-1 is necessary for regulation of corticotropin-releasing hormone by chronic stress and glucocorticoids, Proc. Natl. Acad. Sci. U. S. A. 106 (19) (2009) 8038–8042.
[45] J.M. Deussing, W. Wurst, Dissecting the genetic effect of the CRH system on anxiety and stress-related behaviour, C. R. Biol. 328 (2) (2005) 199–212.
[46] P. La Marca-Ghaemmaghami, S.M. Dainese, G. Stalla, M. Haller, R. Zimmermann, Second-trimester amniotic fluid corticotropin-releasing hormone and urocortin in relation to maternal stress and fetal growth in human pregnancy, Stress 20 (3) (2017) 231–240.
[47] A. Vergetaki, U. Jeschke, T. Vrekoussis, et al., Differential expression of CRH, UCN, CRHR1 and CRHR2 in eutopic and ectopic endometrium of women with endometriosis, PLoS One 8 (4) (2013) e62313.
[48] V. Ruth, M. Hallman, T. Laatikainen, Corticotropin-releasing hormone and cortisol in cord plasma in relation to gestational age, labor, and fetal distress, Am. J. Perinatol. 10 (2) (1993) 115–118.
[49] C.A. Sandman, L.M. Glynn, Corticotropin-releasing hormone (CRH) programs the fetal and maternal brain, Future Neurol. 4 (3) (2009) 257–261.
[50] M. Thomson, The physiological roles of placental corticotropin releasing hormone in pregnancy and childbirth, J. Physiol. Biochem. 69 (3) (2013) 559–573.
[51] V.L. Clifton, M.A. Read, I.M. Leitch, et al., Corticotropin-releasing hormone-induced vasodilatation in the human fetal placental circulation, J. Clin. Endocrinol. Metab. 79 (2) (1994) 666–669.
[52] F. Zhou, L. Zhang, Q. Sun, et al., Expression of urocortin and corticotrophin-releasing hormone receptor-2 in patients with intrahepatic cholestasis of pregnancy, Placenta 35 (11) (2014) 962–968.
[53] E. Fuchs, G. Flugge, Chronic social stress: effects on limbic brain structures, Physiol. Behav. 79 (3) (2003) 417–427.
[54] X.T. Ma, L.Z. Sun, Relationship between the Endoplasmic Reticulum Stress in Placenta Tissues and the Pathogenesis of Preeclampsia (In Chinese). Nan Jing. Nanjing Medical University, (2011), pp. 1–51.
[55] J.H. Veerbeek, M.C. Tissot Van Patot, G.J. Burton, et al., Endoplasmic reticulum stress is induced in the human placenta during labour, Placenta 36 (1) (2015) 88–92.
[56] K. Cryns, T.A. Sivakumaran, J.M. Van den Ouweland, et al., Mutational spectrum of the WFS1 gene in Wolfram syndrome, nonsyndromic hearing impairment, diabetes mellitus, and psychiatric disease, Hum. Mutat. 22 (4) (2003) 275–287.
[57] H. Inoue, Y. Tanizawa, J. Wasson, et al., A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome), Nat. Genet. 20 (2) (1998) 143–148.
[58] H. Eiberg, L. Hansen, B. Kjer, et al., Autosomal dominant optic atrophy associated with hearing impairment and impaired glucose regulation caused by a missense mutation in the WFS1 gene, J. Med. Genet. 43 (5) (2006) 435–440.
[59] T.L. Young, E. Ives, E. Lynch, et al., Non-syndromic progressive hearing loss DFNA38 is caused by heterozygous missense mutation in the Wolfram syndrome gene WFS1, Hum. Mol. Genet. 10 (22) (2001) 2509–2514.