Original Paper

Effect of Histone Deacetylase HDAC3 on Cytokines IL-18, IL-12 and TNF- α in Patients with Intrahepatic Cholestasis of Pregnancy

Yong Shao^a Jing Chen^b Jiao Zheng^a Cai-Ru Liu^a

^aDepartment of Obstetrics and Gynecology, the First Affiliated Hospital of Chongqing Medical University, ^bDepartment of Obstetrics and Gynecology, the First People's Hospital of Jiu Long Po District, Chongqing, China

Key Words

Intrahepatic cholestasis of pregnancy • Histone deacetylase 3 • IL-18 • IL-12 • TNF- α

Abstract

Background/aims: The pathogenesis of intrahepatic cholestasis of pregnancy (ICP) is poorly understood. **Objective:** This study aimed to explore the possible effect of HDAC3 (histone deacetylase) on cytokines IL-18, IL-12 and TNF- α in ICP. **Methods:** Serum levels of cytokines IL-18, IL-12 and TNF- α , bile acids and hepatic function parameters were measured. The expression of HDAC3 in the placenta was determined by immunohistochemistry (IHC), western blotting and RT-PCR. **Results:** IL-18, IL-12 and TNF- α serum levels were significantly higher in the severe ICP group than in the mild ICP group and the control group, and the difference between the mild ICP group and control group was not significant. HDAC3 protein expression was identified in the nucleus of the placental trophoblast by IHC. HDAC3 mRNA and protein expression were significantly lower in the ICP groups (mild ICP and severe ICP groups) than in the control groups, and no significant difference was found between the mild ICP and severe ICP groups. **Conclusions:** The low expression of HDAC3 and overexpression of inflammatory cytokines (IL-18, IL-12 and TNF- α) in ICP may be involved in liver cell apoptosis. We suspect that HDAC3 may play an important role in the pathophysiology of ICP.

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Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy specific disease that has a complex etiology and is characterized by unexplained pruritus with increased bile acids and liver transaminases in the third trimester of pregnancy, which quickly resolves after delivery. It is usually associated with spontaneous preterm delivery, fetal hypoxia and sudden intrauterine deaths (IUDs). The incidence of ICP varies widely among different locations and ethnicities, ranging from 0.7-5% [1]. At present, the pathogenesis of ICP has been associated

Yong Shao

Department of Obstetrics and Gynecology, The First Affiliated Hospital of Chongqing Medical University, No. 1 of Youyi Street, Yuzhong District, Chongqing 400016 (China)
Tel. +86 13101280793, Fax +86 023-89011101, E-Mail shaoyong880923@163.com

with reproductive hormones, bile acids, immune imbalance and the genetic variation of bile acid homeostasis-related genes [2-3]. However, the etiology of ICP and the related mechanism underlying fetal hypoxia and IUDs remains poorly understood. Furthermore, the clinical treatment of ICP does not relieve the harm to the fetus. Although high levels of bile acid were found to be associated with fetal distress, the underlying mechanism remains unclear. Therefore, it is very important to explore the molecular mechanisms of pathophysiology in the ICP development process.

Recently, some studies have found that epigenetics may play an important role in the development of ICP. There is a significant decrease in CpG methylation levels in the distal and proximal FXR/NR1H42 promoter sites and in the distal PXR/NR1I221224 site in ICP patients. These changes suggest that disease phenotype is associated with partial demethylation status, which might modulate the development of the disease and bile acid profiling [4]. Epigenetics can regulate gene function, gene expression and the function of heritable change through DNA and histone modification, without changing the genome DNA sequence. The regulatory mechanisms of epigenetics mainly include DNA methylation, histone covalent modification, chromatin remodeling, gene silencing and RNA editing. Acetylation is one kind of histone covalent modification, and the regulation of acetylation level is dependent on histone acetyl transferases (HAT) and histone deacetylase (HDAC). HDACs reverse the regulatory acetylation of histone proteins, influence the nucleosome structure, and alter gene transcription. Histone acetylation is often correlated with gene activation, suggesting that histone deacetylases act to silence genes [5].

Even though a suppressed mixed lymphocyte reaction was observed in ICP, and its mechanism and role in the disease remains unknown [6,7]. Recent studies have reported that nuclear factor-kappa B (NF- κ B) transcription factor is a critical regulator of immune and inflammatory processes, and plays an important role in regulating Th1 and Th2 immune response [8]. Furthermore, it has been demonstrated that the RelA subunit of NF- κ B is subjected to induce acetylation, and that the acetylated form of RelA interacts weakly with I κ B α , which is an inhibitory protein in the cytoplasm. Acetylated RelA is subsequently deacetylated through a specific interaction with HDAC3. HDAC3-mediated deacetylation is an intranuclear molecular switch that initiates a series of events, which culminate in the termination of NF- κ B transcriptional response [9].

Recent data suggests that natural killer (NK) cells can influence the capability of dendritic cells (DCs) to promote Th1 polarization. This regulatory function is primarily mediated by cytokines released in the microenvironment during inflammatory responses that involve NK cells. Human NK cells promote the distinct pathways of Th1 priming when exposed for short time to interleukin (IL)-12, IL-2, or IL-18. Upon exposure to different cytokines including IL-12, IL-18, IL-4, IFN- α and IL-2, NK cells can exert different regulatory effects on downstream adaptive immune responses *via* different routes. These cytokines are released by various cell types, including both resident and circulating cells recruited at the inflammatory sites in response to chemokine gradients [10]. IL-18 plays a fundamental role in regulating hepatocyte apoptosis, and the overexpression of IL-18 may cause substantial liver injury *in vivo*. IL-18 is produced by Kupffer cells, macrophages, B cells and dendritic cells on lipopolysaccharide (LPS) stimulation [11]. The pleiotropic cytokine IL-18 is a member of the IL-1 family originally described as an inducer of interferon- γ (IFN- γ) production from T cells in the presence of IL-12 (interferon- γ inducing factor). [12] Furthermore, IL-18 is a coinducer of IL-12 driven T helper 1 T cell differentiation by upregulating IL-12 receptor beta-2 chain expression and the transactivation of the IFN- γ promoter *via* transcription factor AP-1 [13]. NK cell activation was indicated by an increased expression of CD69, CD107a, IFN- γ and tumor necrosis factor alpha (TNF- α). Further analysis revealed that the increased activity of both peripheral and hepatic NK cells was positively correlated with liver injury, which was assessed through serum alanine aminotransferase levels (ALT). These functionally activated NK cells are preferentially enriched in the livers of immune clearance (IC) patients, and skew towards cytolytic activity that accelerates liver injury in chronic hepatitis B (CHB) patients. [14]

In the present study, we aimed to measure the expression of HDAC3 in the placental tissue of normal and ICP patients, as well as the serum levels of cytokines IL-18, IL-12 and TNF- α , in order to further investigate the effect of HDAC3 on cytokines IL-18, IL-12 and TNF- α in ICP patients.

Materials and Methods

Reagents

Assay kits for IL-12, IL-18 and TNF- α were purchased from Chongqing Dingguo Biotechnology Co. Ltd. (Dingguo Biotechnology, Chongqing, China). Assay kits for total bile acids (TBA), total bilirubin (TBIL), direct bilirubin (DBIL), cholyglycine (CG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd. (Zhongshan Goldenbridge Biotechnology, Beijing, China) and Beijing Leadman Biotech Co., Ltd. (Leadman Biotech, Beijing, China). The Trizol reagent was a product of Invitrogen (Carlsbad, CA, USA). Taq DNA polymerase and reverse transcriptase were products of Thermo Scientific (Thermo Scientific, USA). The following primers were used for amplification by real-time polymerase chain reaction (RT-PCR): HDAC3 (size: 219bp): forward 5'-ACGTGGGCAACTTCCACTAC-3', reverse 5'-GACTCTTGGTGAAGCCTTGC-3'; β -actin (size: 208bp): forward 5'-CATTAAGGAGAAGCTGTGCT-3', reverse 5'-GTTGAAGGTAGTTTCGTGGA-3'. These primers were synthesized by Shanghai Sangon Gene Company (Sangon Gene Company, Shanghai, China). Commercial kits for the protein extract were supplied by ProMab (Richmond, U.S.). The bicinchoninic acid (BCA) kit for protein concentration was supplied by Beyotime Institute of Biotechnology (Beyotime Institute of Biotechnology, Jiangsu, China). The rabbit monoclonal antibody of HDAC3 was purchased from GeneTex (Irvine, U.S.). All other reagents used in this study were of analytical grade.

Patient Participation and Tissue Collection

Placental tissues were obtained from women who were hospitalized in the Department of Obstetrics of the First Affiliated Hospital of Chongqing Medical University. Prior to surgery, written consent was obtained from each pregnant woman after fully explaining the purpose, nature and risk of all procedures to be performed. This study was approved by Ethical Committee of the First Affiliated Hospital of Chongqing Medical University. This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants. The control group comprised of 20 consecutive normal pregnant women without any complication. Forty pregnant women with ICP (including 20 cases of mild ICP and 20 cases of severe ICP) were enrolled into the study. All patients were singleton pregnancies, who were admitted to the Department of Obstetrics and Gynecology between the 1st of September 2011 and the 1st of September 2012.

According to The Royal College of Obstetricians and Gynaecologists Green-top Guideline, the diagnosis of ICP was based on the following criteria [1]: (1) the presence of pruritus, predominantly located on hands and feet, that resolved within hours or days after delivery; (2) abnormalities in liver-function tests suggestive of ICP; (3) elevated levels of fasting serum TBA of more than 10 mmol/L; (4) no skin lesions caused by systemic diseases that could lead to pruritus, and (5) spontaneous resolution of clinical symptoms and laboratory findings after delivery.

Mild and severe ICP were defined according to the study of Glantz *et al.* [15,16]. The standard reference value established for mild ICP (MICP) and severe ICP (SICP) was TBA <40 μ mol/L and TBA $\geq$ 40 μ mol/L, respectively.

All patients were cleared of hepatitis viruses (HAV, HBV and HCV), preeclampsia and gestational diabetes mellitus. Exclusion criteria included autoimmune diseases, moderate to severe alcohol intake, smoking, biliary obstruction, and drug addiction or alternative medicine therapy known to precipitate cholestasis. Patients with multiple pregnancies were not included in this study. All placental samples were obtained within 15 minutes after caesarean section. Four samples were taken at 0, 3, 6 and 9 o'clock of the middle zone of each placenta after the deciduas and amniotic membranes were removed. Then, 1×1 cm sections of the placenta were dissected between the basal and chorionic plates. Tissues were briefly washed in 0.9% normal saline, dried using clean gauze, placed in sterile tubes and stored in liquid nitrogen at -80°C. All placental samples (20 cases for the control group, 20 cases for the mild ICP group, and 20 cases for the severe ICP group) were stored for immunohistochemistry (IHC), RT-PCR and western blotting.

Blood Sample Collection

Peripheral blood (5 ml) was collected from each participant in EDTA-containing tubes. Samples were centrifuged within two hours (4°C, 35,000 r/min, 10 minutes), and the supernatant was transferred into a sterile 1.5-ml centrifuge tube and stored at -80°C until further use for IL-18, IL-12, TNF-α, TBA, TBIL, DBIL, CG, ALT and AST measurements.

Serum Levels of TBA, TBIL, DBIL, CG, ALT and AST Measurement

Serum levels of TBA, TBIL, DBIL, CG, ALT and AST were determined by routine automated techniques.

Inflammatory Cytokine Assay

The maternal serum levels of cytokines IL-18, IL-12 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions with modifications; and absorbance was detected at 450 nm on an automatic microplate reader (Shanghai Thermoelectric Equipment Co., Ltd., Shanghai, China). Curve fitting and data extrapolation were performed using Soft Max Pro40 software (Molecular Devices, CA, USA).

RNA Extraction and RT-PCR

According to manufacturer's instructions, total RNA was prepared from placenta tissues using Trizol reagent. Quantitative PCR was performed on an FTC 2000 Light Cycler system (Funglyn Biotech Inc., Toronto, Canada). PCR conditions were as follows: 95°C for five minutes; 94°C for 20 seconds, 58.5°C for 20 seconds, and 72°C for 20 seconds (40 cycles). For analysis, quantitative amounts of HDAC3 gene expression were standardized against the housekeeping gene β-actin. PCR products from placental tissues were purified and confirmed by sequencing. RNA levels were expressed as a ratio using the "2^{-ΔΔCt} method" for comparing relative expression results between different groups in real-time PCR.

Protein Extraction and Western Blotting

Protein was extracted from placentas using the commercial kit, according to manufacturer's instructions; and protein concentration was determined using a BCA kit. The samples were separated on SDS-polyacrylamide gels, subjected to electrophoresis, and transferred to nitrocellulose membranes. Then, the membranes were blocked with 5% non-fat dried milk at room temperature for two hours and incubated with the primary antibodies for HDAC3 (1:1,000 dilution) and β-actin (1:1,000 dilution) at 4°C overnight. The membranes were washed three times with TBS-T and incubated with secondary antibodies at room temperature for two hours. In order to control for sampling errors, the ratio of band intensities to β-actin was obtained to quantify relative protein expression levels.

Immunohistochemistry

Placental tissues were fixed in PBS with 10% formalin for 24 hours at 4°C, dehydrated in a graded series of ethanol, and embedded in paraffin. Deparaffinized placental sections were used for HDAC3 labeling. After the inhibition of endogenous peroxidases with 3% H₂O₂, the sections were incubated for 30 minutes at room temperature with 10% rabbit serum to block non-specific background signals. Then, serial sections were incubated with specific primary antibodies against human HDAC3 (1:50) overnight at 4°C. After several washes in 50 mM of Tris/HCl, the slides were incubated with biotinylated goat anti-rabbit antibody (1:200) for one hour at room temperature. Negative controls were performed by omitting the primary antibody. The sections were examined by conventional light microscopy.

Statistical Analysis

Statistical analysis was performed with commercially available software (SPSS for Windows, version 11.0; SPSS Inc., Chicago, IL, USA). When data were normally distributed, the data were presented as mean ± standard deviation (SD); and data of intergroup comparisons were tested using Student's *t*-test and ANOVA. For non-normally distributed data, Spearman correlations and Kruskal-Wallis H tests were used. For all statistical analyses, a two-tailed *P*-value <0.05 was considered statistically significant.

Results

Clinical Findings

Table 1 presents the clinical characteristics of women with ICP (mild ICP and severe ICP) and controls. Maternal age, weight, gravidity and Apgar score ≤ 7 at five minutes were not significantly different between patients with ICP (mild ICP or severe ICP) and controls. Delivery week and birth weight were significantly lower in the severe ICP group than in the mild ICP and control groups, and no significant difference was found between the mild ICP and control groups. Cases of meconium-stained amniotic fluid were significantly higher in severe ICP group than in the mild ICP and control groups, and no difference was found between the mild ICP and control groups (Table 1).

Serum Biochemical Characteristics of Patients

Serum biochemical characteristics are shown in Table 2. ALT, AST, CG and TBA serum levels were significantly higher in the severe ICP group than in the mild ICP group; while these levels were significantly higher in mild ICP group than in the control group. Furthermore, TBIL and DBIL serum levels were significantly higher in the severe ICP group than in the mild ICP and controls, and no difference was found between the mild ICP and control groups (Table 2).

Serum Cytokine Levels of Patients

Inflammatory cytokines IL-12, IL-18 and TNF- α were assessed by ELISA. IL-12, IL-18 and TNF- α serum levels were significantly higher in the severe ICP group than in the mild

Table 1. Clinical characteristics of ICP and control group. The data are expressed as the mean $\pm$ S.D., * $p < 0.05$ vs. control group. Chi-square test was used to evaluate the comparisons of the rate

	Control	Mild ICP	Severe ICP
n	20	20	20
Maternal Age (years)	29.6 $\pm$ 3.5	29.3 $\pm$ 4.6	28.3 $\pm$ 4.7
Maternal weight (kg)	69.0 $\pm$ 6.7	67.9 $\pm$ 6.7	64.9 $\pm$ 7.0
Gravidity (times)	2.1 $\pm$ 1.3	2.2 $\pm$ 1.1	2.2 $\pm$ 1.7
Parity (times)	1.5 $\pm$ 0.3	1.6 $\pm$ 0.2	1.6 $\pm$ 0.3
Delivery week (weeks)	39.6 $\pm$ 0.7	39.5 $\pm$ 0.8	36.5 $\pm$ 0.9*
Birth weight (g)	3380 $\pm$ 340	3320 $\pm$ 310	2900 $\pm$ 350*
Apgar score ≤ 7 at 5 min (cases)	0	1(5%,1/20)	1(5%,1/20)
Meconium-stained amniotic fluid (cases)	1(5%,1/20)	1(5%,1/20)	6(30%,6/20)*

Table 2. Serum biochemical characteristics of ICP and control group. The data are expressed as the mean $\pm$ S.D., * $p < 0.05$ vs. mild ICP or control

	Control	Mild ICP	Severe ICP
n	20	20	20
ALT (U/L)	16.40 $\pm$ 2.27	117.95 $\pm$ 26.30*	323.62 $\pm$ 34.33*
AST (U/L)	21.85 $\pm$ 1.96	89.50 $\pm$ 11.30*	294.69 $\pm$ 32.08*
CG (mg/dL)	3.51 $\pm$ 1.34	30.75 $\pm$ 3.76*	53.99 $\pm$ 5.46*
TBA (umol/L)	3.57 $\pm$ 1.02	26.70 $\pm$ 3.35*	48.05 $\pm$ 6.61*
TBIL (mmol/L)	6.97 $\pm$ 1.56	7.09 $\pm$ 3.11*	15.26 $\pm$ 5.67*
DBIL (mmol/L)	2.86 $\pm$ 0.87	3.60 $\pm$ 2.10*	10.51 $\pm$ 3.44*
IL-12 (ng/L)	11.6 $\pm$ 2.0	18.1 $\pm$ 2.3	50.3 $\pm$ 4.6*
IL-18 (ng/L)	44.2 $\pm$ 3.7	48.5 $\pm$ 2.8	81.3 $\pm$ 4.2*
TNF- α (ng/L)	33.6 $\pm$ 2.9	39.3 $\pm$ 3.8	51.7 $\pm$ 4.0*

Fig. 1. HDAC3 protein expression in the nucleus of placental trophoblast ($\times 400$). The expression of HDAC3 in control group (Fig. 1-1) was higher than mild ICP (Fig. 1-2) and severe ICP (Fig. 1-3).

Fig. 2. Expression of HDAC3 protein in placenta of ICP and control. (A) Western blotting analysis of placental HDAC3 protein expression in control, mild ICP and severe ICP groups. (B) Graphical summary of data on the expression of HDAC3 protein. The data are expressed as the mean $\pm$ S.D., * $p < 0.05$ vs. control group.

Fig. 3. Expression of HDAC3 mRNA in placenta of ICP and control. Graphical summary of data on the expression of HDAC3 mRNA. The data are expressed as the mean $\pm$ S.D., ** $p < 0.01$ vs. control group.

ICP and control groups, and there were no significant difference between the mild ICP and control groups (Table 2).

Localization of HDAC3 in Human Placenta

HDAC3 protein expression was stained in the nucleus of the placental trophoblast by IHC. The expression of HDAC3 in the control group was higher than in the mild ICP and severe ICP groups (Fig. 1).

HDAC3 mRNA and Protein Expression in Human Placenta

RT-PCR and western blotting were used to determine HDAC3 mRNA and protein expression in the placentas. HDAC3 mRNA and protein expression were significantly lower in the ICP group (mild ICP and severe ICP) than in the control group, and no significant difference was found between the mild ICP and severe ICP groups (Fig. 2 and 3).

Discussion

The molecular mechanism of the pathophysiology of ICP remains unknown [2, 17]. It has been demonstrated that epigenetics may play a role in the development of ICP. IHC, western blotting and RT-PCR experiments were used to demonstrate the mRNA and protein expression of HDAC3 in human placenta in our study. HDAC3 protein was predominantly stained in the nucleus of syncytiotrophoblasts. HDAC3 protein and mRNA were significantly down regulated in ICP placentas, while serum levels of cytokines IL-12, IL-18 and TNF- α were significantly increased in ICP patients. These results suggest that a lower HDAC3 expression may upregulate the serum levels of cytokines IL-12, IL-18 and TNF- α , which might disturb the balance of the immune system and maternal liver function.

In recent years, a number of studies have confirmed that IL-18 is involved in the process of liver dysfunction. IL-18 can activate NK cells in different ways, which may cause liver cells apoptosis and liver dysfunction. IL-12 can enhance the effect of IL-18, and promote NK cell proliferation and toxicity [9-10]. These findings suggest that cytokines play an important role in liver dysfunction in hepatitis patients. Normal pregnancy is considered to be associated with the balance of Th1/Th2 cytokine. There is an imbalance of Th1/Th2 cytokines to Th1 type in ICP patients. It has been demonstrated that TNF- α , IFN- γ , IL-12, IL-18 and IL-6 serum levels were significantly higher in ICP patients, while IL-4 and IL-10 serum levels were lower than in normal pregnant woman [6,18-20]. Placental TNF- α mRNA and protein expression were also higher and positively correlated with serum bile acid concentration in ICP patients [21].

In this study, we found that the serum levels of IL-12, IL-18 and TNF- α were significantly higher in ICP patients when compared with controls. TNF- α can directly damage liver cells and cause liver cell peroxidation by stimulating mitochondria to produce more oxygen-free radicals. At the same time, it can also induce the apoptosis of placental trophoblasts, impair placental function, and cause harm to the fetus [22]. Cases of meconium-stained amniotic fluid and premature birth were significantly higher in the severe ICP group than in the mild ICP and control groups. Furthermore, there was no difference in cases of Apgar score ≤ 7 at five minutes among the three groups; and the possible reason may be that the active clinical treatment to ICP, especially for severe ICP. The overexpression of IL-12, IL-18 and TNF- α in pregnant women with ICP may be closely related to liver dysfunction, and may result to fetal adverse outcomes. Recent studies have shown that HDAC3 was involved in regulating the expression of inflammatory cytokines. HDAC3 has been found to specifically deacetylate RelA, which can regulate the diverse functions of NF- κ B including DNA binding activity, transcriptional activity and its ability to associate with I κ B α ; and plays important roles in NF- κ B-mediated inflammatory response [23]. Many molecules including TNF- α , IL-1 β , IL-2, IL-6, IL-8, IL-12, iNOS, COX2, chemotactic factor, adhesion molecules and colony-stimulating factor are involved in every stage of the inflammatory reaction and in the early stage of immune response; and these molecules are modulated by NF- κ B, according to Mahlknecht [24]. HDAC3 acts as a regulator of TNF gene expression in LPS-stimulated cells, especially in mononuclear phagocytes. It has been demonstrated that p300, which is a kind of histone acetylation enzyme activity of transcription cofactors, can strengthen transcription factor c-Fos to induce IL-18 gene transcription activity. HDAC3 can decrease the transcription of IL-18 by inhibiting the p1 promoter of IL-18 [25, 26]. These findings indicate that HDAC3 can increase the expression of IL-12, IL-18 and TNF- α directly or indirectly.

This study is the first to reveal that the protein and mRNA expression of HDAC3 decreased in the mild and severe ICP groups than in the control group. The low expression of HDAC3 in ICP placental tissues may activate the inflammatory pathway. Then, inflammatory cytokines (IL-12, IL-18 and TNF- α) overexpressed in the placental local site may be released into the maternal blood, induces liver cell apoptosis, and impairs the liver function of pregnant women with ICP. This may start a new insight on the pathogenesis of ICP. In conclusion, we suspect that HDAC3 might play an important role in the pathophysiology of ICP. More research in this area may present a new way to treat ICP.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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