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# Prenatal and early postnatal ethanol exposure induces hepatic transcriptional factors, gene expression transition and structural changes in the liver of offspring: A three month follow-up

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## Abstract

***Background & Aims***: The aim of the present study was to investigate the effect of maternal pre- and early postnatal ethanol consumption on hepatic transcriptional factors, gene expression transition, liver enzymes alteration, as well as structural changes in the developing rat liver both on postnatal (PN) day 21 and 90.

***Materials & Methods***: Pregnant Wistar rats received ethanol (4.5g/kg BW) once per day from Gestation Day (GD) 7 throughout lactation. The mRNA expression of hepatic Fatty Acid Translocase (FAT)/CD36, Protein Tyrosine Phosphatase 1B (PTP1B) and Hepatocyte Nuclear Factor 4 Alpha (HNF4A) gene expressions, as well as liver structural changes were measured in 21 and 90 day-old offspring of ethanol rats and were then compared to the control rats.

***Results***: Ethanol exposed fetal livers showed a significant up-regulation in FAT / CD36, PTP1B and down-regulation in HNF4A gene expression, as well as an increase in the liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of the offspring on PN 21 and PN 90 compared to the offspring in the control group. In addition, in the ethanol group, cholesterol levels showed a significant increase on PN 21 and PN 90, and triglyceride showed an increase on PN 90, compared to the control group. Histopathological changes, such as ballooning degeneration, feathery degeneration, spotty necrosis, cholestasis, and fibrosis were also observed in the liver of the offspring after 21 and 90 days from birth.

***Conclusion***: In conclusion, results of the current study provide evidence that prenatal ethanol exposure influences liver lipid metabolism through hepatic transcriptional factors, gene expression transition and hepatic enzyme including ALT and AST changes.

***Keywords***: pregnancy, Ethanol, FAT / CD36, PTP1B, lipid

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## Introduction

Alcohol consumption during pregnancy interferes with normal developmental progression of the fetus and results in a spectrum of adverse effects collectively termed Fetal Alcohol Spectrum Disorders (FASD)(1). Although earlier studies have addressed the effects of FASD on neuro developmental changes, other organ systems are also vulnerable to the teratogenic actions of intrauterine ethanol exposure, as demonstrated by recent studies on the heart, lung, and testis (2, 3). Similar to other organs, liver is affected by intrauterine ethanol exposure; however, little is known about the effects of maternal ethanol exposure on the developing liver. Previous studies have demonstrated that ethanol exposure during pregnancy leads to liver fibrosis and fatty degeneration with abnormal liver function tests in children with FASD(4) . Moreover, studies on animal have demonstrated that prenatal exposure to ethanol leads to increased fat deposition in hepatic cells and decreased liver function in the fetus's liver(5) . Furthermore, decreased liver weight and DNA synthesis, hyperbilirubinemia, as well as liver cirrhosis have been reported in newborns exposed to ethanol in gestation period (6, 7). Moreover, several studies have indicated that maternal ethanol consumption during gestation period alters gene expression of factors and hormones associated with liver development including polyamines, growth hormone, insulin-like growth factor-I and II and its related binding proteins in offspring livers (8, 9, 10). All these are main hormones involved in the regulation of lipid metabolism by the liver. Although many studies have addressed the hepatotoxicity associated with FASD, the precise mediating steps between maternal exposure to ethanol and initiation of the cascade of responses leading to liver abnormality in the offspring have not yet been completely clarified. For example, even though it has been reported that ethanol exposure of pregnant females results in liver dysfunction and dyslipidemia in the fetuses(11) , the precise pathogenesis of the disease regarding the specific molecular mediators that could influence liver lipid metabolism after intrauterine ethanol exposure are still not well-known. There is a critical association between the cellular and organismal homeostasis and the regulation of lipid levels and metabolism by liver either in terms of energy utilization and storage, or prevention of potential toxicity(12) . Hence, for the first time, to the best of our knowledge, we investigated the effect of pre and early postnatal ethanol exposure on transcription factors including PTP1B, HNF4α and FAT/CD36 gene expression that are involved in hepatic lipid metabolism in liver of offspring on PN 21 and PN 90. In addition, liver ALT, AST amounts and plasma cholesterol and triglyceride levels were measured in order to evaluate functional alterations of the liver caused by exposure to ethanol during development. Structural alterations of offspring livers were also investigated in the current study by Hematoxylin-Eosin (H&E) staining.

## Materials & Methods

Herein, the animal care and experimental procedure were carried out, according to the Principles of Laboratory Animal Care (NIH publication, no. 85–23, revised 1985), and it was approved by the Animal Ethics Committee of the Urmia University of Medical Sciences. Pregnant Wistar rats were divided into two groups, namely control and ethanol groups. Rats in the ethanol group received ethanol with a dose of 4.5 g/kg body weight (Merck KGaA, Darmstadt, Germany) soluted in tap water (20% w/v) intragastrically by gavage once a day from GD 7 throughout lactation. The control group received an equivalent amount of water in the same time frame. The male offspring from each group were anesthetized by ketamine (10%, 80 mg/kg B.W, IP) and xylazine (2%, 10 mg/kg B.W IP) (n=8 offspring from each group, PN 21 and PN 90). After weighing, the abdominal cavity was opened and blood samples were taken directly from the portal vein, collected in EDTA tubes and centrifuged at 4000× g for 20 min within 30 min of collection and yielded plasma were stored at -80°C without repeated freeze-thaw cycles. Then the liver was isolated for analysis as follows: A part of the liver was fixed immediately in 10% formalin embedded in paraffin, and sectioned at 5 μm for histological examinations. In order to perform

biochemical analysis, another part of the liver was washed with ice-cold physiological saline and then dried on filter papers. For total RNA isolation, 100 mg of liver tissue (the same portion of the liver for all mice) was immersed in 1 ml RiboEX (GeneAll, Seoul, Korea) and stored at -80 ° C up to the time of RNA isolation.

**Biochemistry analysis:**

Plasma total cholesterol (TC) and triglycerides (TGs) levels were carried out by colorimetric and enzymatic methods. The plasma concentration of liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), substances produced by the liver, were measured by colorimetric assay using commercially available kits according to the manufacturer's instructions (Lab test Diagnóstika SNord GmbH, Nordhorn, Germany).

**Isolation of total RNA, amplification primers, and real time polymerase chain reaction (RT-PCR):**

For assay gene expression of PTP1B, HNF4A and FAT/CD36, the total RNA was obtained from 100 mg of the frozen liver tissue by using a kit (Gene All, South Korea, Cat no 305-101) in accordance with the kit instructions. The RNA concentration was verified by spectrophotometric measurement of the absorbance at 260–280 nm and determined by TAE-agarose gel electrophoresis. Reverse transcriptase (RT) was performed using Hyperscript™ Reverse Transcriptase (Gene All, SouthKorea). Moreover, RT-PCR was carried out using an amplification reagent kit (Ampliqon, Denmark) by the XP-Cycler instrument (TCXPD, Bioer, USA) with PTP1B, HNF4A and FAT/CD36, and the rats' glyceraldehydes- 3-phosphate dehydrogenase (GAPDH) primers. In order to amplify the cDNA, the 5' and' primer sequences (forward and reverse) of the PTP1B, HNF4α and FAT/CD36, designed via the Gene Bank, revealed that the primers were gene specific. Furthermore, all the primers were verified using the Gene Runner software. The primers (forward and reverse) were then synthesized to amplify the cDNA encoding GAPDH as a housekeeping gene.

**Gene Expression Analysis:**

Real-time quantification of the target genes was performed using a Real-Time PCR Master Mix Green kit (Ampliqon, Denmark) in a total volume of 25 µl and in accordance with the instructions offered by the manufacturer. In addition, the gene expressions were analyzed using an iQ5 real-time PCR detection system (Bio-Rad, CA, USA).Then, the reactions were made for 10 min at 95 °C in a 96-well optimal plate followed by 40 cycles of 20s at 59 °C. In order to confirm the specificity of the amplification reactions, a melting curve was recorded. Each sample was repeated three times. The relative expression of each mRNA was calculated performing the $2^{-\Delta\Delta Ct}$ method (where Ct is cycle threshold). Then, the calculated levels were normalized to GAPDH.

**Histopathological examinations:**

Histopathological staining was done using tissue sections of 5 –µm thickness of paraffin containing liver tissue. To assess general histological changes, Hematoxylin and Eosin stained (H & E) were used.

**Data and Statistical analysis:**

Normal distributions of data within each group were verified applying the Kolmogorov-Smirnov test. Statistical differences between the groups were tested conducting an independent samples T- test. In each test, the data were expressed as the mean ± S.E., and p = < 0.05 was considered to be statistically significant.

## Results

**Liver enzyme alteration, plasma cholesterol and triglyceride levels:**

Plasma lipid profile and the amounts of liver enzymes are shown in table-1. Plasma total cholesterol levels showed significant increases in ethanol-treated pups compared to the control pups on PN 21 and PN 90 ($p<0.005$). The amount of plasma triglyceride, however, showed a significant increase on PN 90 in the offspring from the ethanol group compared to those from the control group ($P<0.01$). Moreover, the plasma ALT content was significantly increased on both PN 21 and PN 90 in the ethanol group rats

compared with control rats. In addition, plasma AST level showed a significant increase on PN 90 and remained unchanged on PN 21, as compared to the control group.

**Gene expression (PTP1B, HNF4A and FAT/CD36) in liver tissue:**

Table 2 shows the treatment of pregnant mothers with ethanol on the gene expression alteration of HNF4A, FAT/CD36, and PTP1B involved in the hepatic lipid metabolism. Ethanol administration significantly increased the expression of liver FAT/CD36 and PTP1B mRNA level on PN 21 and PN 90 compared to those in the control group ($p<0.05$). In the liver obtained from the ethanol offspring, HNF4A mRNA levels were significantly lower than the same gene expression in the control offspring on PN 21 and PN 90 ($p<0.05$).

The histhopatological changes in the liver sections of the control and ethanol groups are shown in figure 1. After ethanol treatment with doses of 4.5 g/kg body weight in gestation throughout feeding revealed several histopathological changes in the liver sections including spotty necrosis, cholatestasis, hepatocyte cell ballooning, feathery degeneration, inflammation, and fibrosis on both PN 21 and PN 90 (fig-1).

**Table 1.** Effect prenatal and postnatal ethanol consumption on changes of lipid profile and liver enzymes in offspring on PN21 and PN90

| | Control | Ethanol |
|---|---|---|
| TG-21(mg/dl) | 103± 5.1 | 88.5±10 |
| TG-90(mg/dl) | 41±5 | 55±2.1* |
| Choles-21(mg/dl) | 81.4±6.9 | 100±4.6* |
| Choles-90(mg/dl) | 60±1.3 | 68±3.5* |
| (ALT)SGPT-21 | 30.9±3 | 47.6±3.7* |
| (ALT)SGPT-90 | 60.3±2.4 | 70.6±3.9* |
| (AST)SGOT-21 | 126.4±4.4 | 139±8.6 |
| (AST)SGOT-90 | 91.25±2.1 | 120.4±4* |

Values are mean ±SE for 8 rats per group
*Denotes significant difference compared to the control
† Denotes significant difference compared to the ethanol group

**Table 2.** Effect prenatal and postnatal ethanol consumption on changes of CD36, HNF4α, and PTPB1 gene expression in offspring liver on PN21 and PN90

| | Control-21 | Ethanol-21 | Control-90 | Ethanol-90 |
|---|---|---|---|---|
| CD36(Fold) | 1±0.09 | 17.9±4.4* | 1.1±0.049 | 3.7±1.4* |
| HNF4α(Fold) | 1.04±0.19 | 0.31±0.07* | 1.03±0.16 | 0.17±0.008* |
| PTPB1(Fold) | 0.99±0.003 | 5.62±3.66* | 1±0.06 | 3.73±0.02* |

Values are mean ±SE for 8 rats per group
*Denotes significant difference compared to the control
† Denotes significant difference compared to the ethanol group

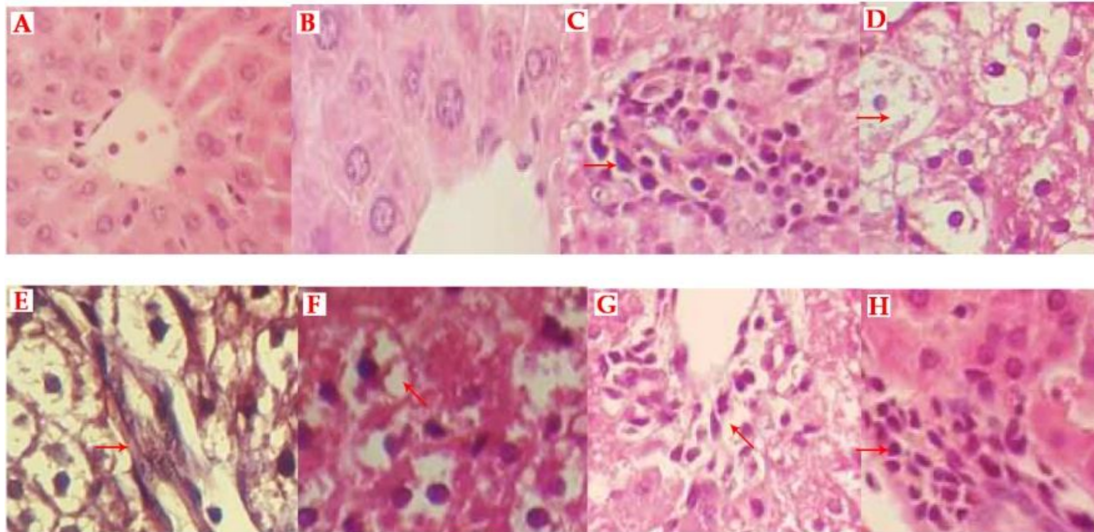


**Figure1.** photomicrographs of haematoxylin and eosin stained sections of liver tissues from each group are shown. A and B, control; C, D, E , F , G, and H, ethanol PN. Histological changes are shown by arrows(→) as follow: C, Spotty necrosis; D, Ballooning degeneration; E, Choleostasis; F, Feathery degeneration; G, lymphocytic inflammation; H , PMN

## Discussion

The main findings of the present study could be summarized as follows: 1) pre- and early postnatal ethanol exposure led to an increase in liver ALT and AST amounts, plasma cholesterol and triglyceride of offspring on PN 21 and PN 90; 2) Maternal ethanol exposure markedly increased FAT / CD36, PTP1B and decreased HNF4A gene expression in the ethanol group compared to the control group; 3) Structural alterations in the liver tissue such as spotty necrosis, cholatestasis, hepatocyte cell ballooning, feathery degeneration, inflammation, and fibrosis were also found in the ethanol-treated group litters' liver compared with the control group in both stages.

Several lines of evidence indicate that the intrauterine environment enhances predisposition of adults to disease via epigenetic regulation, which is due to chromatin structure and function alteration (13, 14) . Ethanol ingestion during pregnancy-induced genotoxic damage and gene expression alteration in liver, blood, and the brain have been reported by previous studies in animal models (15, 16, 17) . In the current study, we investigated the effect of pre- and early postnatal ethanol ingestion on hepatic genes involved in liver lipid turnover. The data obtained by the current study showed that heavy alcohol consumption by pregnant mothers decreased expression of HNF4α in liver tissue of offspring on PN 21 and PN 90. HNF4α regulates transcription of numerous genes involved in lipid metabolism and transport such as Apo-A, Apo-B, microsomal triglyceride transfer protein (MTP), and cholesterol 7α-hydroxylase (CYP7A) (18, 19, 20, 21). Mechanistically, HNF4α contributes to lipid metabolism by transporting fatty acids into mitochondria, and it increases their beta-oxidation (22). In addition, since HNF4α establishes the expression of the hepatocyte-specific gene during embryonic period, stabilizes the hepatic transcription factor network and maintains hepatocyte function, it is accepted as the master manager of hepatocyte differentiation (23, 24, 25). It has also been reported that HNF4α can trigger gene transcription in the absence of exogenous ligands (26) . Due to the pivotal rule of HNF4α in lipid metabolism and its transcriptional properties, however, , as previously reported in diabetes, obesity, non-alcoholic fatty liver, and high-fat dietary supplementation, disruption of HNF4α gene results in increased free fatty acids and cholesterol (27, 28). Our

data demonstrated prenatal ethanol exposure increased the mRNA expression of FAT / CD36 gene in the liver of liters on PN 21 and PN 90. Moreover, as a trans membrane glycoprotein receptor, CD36 plays an important role in energy metabolism in different tissues including adipocytes, pancreatic beta cells, and skeletal myocytes. It also has a fundamental role in hepatic fatty acids transport (29, 30, 31) . In addition, this multifunctional receptor facilitates the uptake of long-chain fatty acids and oxidized low-density lipoproteins in the liver (32). Consequently, the superabundant accumulation of lipids in hepatocytes could exceed the oxidative facility of metabolism, leading to the production of reactive oxygen species and oxidative stress, and it is possible for CD36 to participate in this process (33). It has been previously reported that the overexpression of CD36 magnifies fatty acid uptake and TG storage in human and animal livers(34, 35) . Interestingly, in the current study, parallel with overexpression of CD36 in liver tissue of offspring exposed to ethanol during the development period, increased TG and cholesterol, along with some features of liver steatosis were also found in the ethanol group litters.

In the present study, the expression of liver PTP1B in the ethanol group was increased from fetus to adulthood. PTP1B play an important role in the regulation and improvement of metabolic parameters including hepatic insulin sensitivity, decline in liver triglyceride, and lower serum and hepatic cholesterol levels. They also protect against endoplasmic reticulum stress induced by high-fat diets(36, 37) . It has been recently reported that disruption of PTP1B expression in liver as well as reduction of PTP1B result in a decrease in liver fat depots and triglyceride levels and lead to an increased hepatic insulin signaling and reduced serum and hepatic TG and cholesterol levels(36, 38) . In addition, the association between over-expression of PTP1B and insulin resistance and development and progression of liver steatosis has been discussed in the animal model of obesity and non-alcohol-fatty liver(39, 40) . Another important result of the current study is that pre- and early postnatal ethanol exposure lead to liver injury, evidenced by enhanced blood liver enzymes including ALT and AST. In general, in most types of liver injury, the ALT activity is higher than AST activity; however, in cases of alcoholic liver injury, AST activity shows a higher elevation than ALT activity. There are some explanations for the higher level of AST activity in alcoholic liver injury. Among different forms of hepatitis, only alcohol increases mitochondrial AST activity(41) . In alcoholic liver disease, the deficiency of Pyridoxine, which is a co-factor for the enzymatic activity of ALT, is reported by a previous study(42) . Moreover, AST released from mitochondrial due to alcohol, has a longer half-life compared to AST released from cells without visible cell damage(43) . Liver injury in the current study was also confirmed by histological alterations such as spotty necrosis, cholatestasis, hepatocyte cell ballooning, feathery degeneration, inflammation, and fibrosis in the liver of ethanolic group compared to those of the control group. In fact, all these changes are characteristic of alcoholic hepatitis, as documented by previous studies (44, 45). In conclusion, results of our study demonstrate that intrauterine ethanol exposure induces the dyslipidemia and liver damage manifested by an increase in ALT, AST and histological alteration of the liver. Furthermore, we have uncovered, for the first time, the molecular mechanism including HNF4A, FAT/CD36, and PTP1B involved in the hepatic lipid metabolism, which could be used to acquire novel targets for the prevention and treatment of hepatic injury and may provide a new window to investigators studying FASD.

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**Conflict of interest**: The authors declare that they have no conflict of interest.

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