



MnTMPyP mitigates Time-Dependent Molecular Alterations in Ethanol-Induced Hepatic Stress in HepG2 cells

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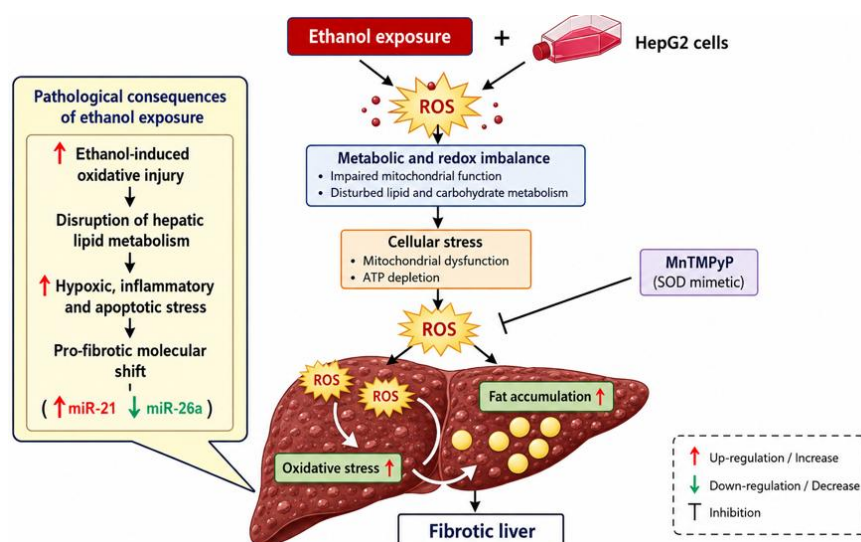
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Abstract

Alcohol associated liver disease (ALD) is a multifactorial disease causes due to persistent intake of alcohol. That entails a complicated interaction among oxidative stress, lipid metabolism, inflammatory pathways, hypoxia regulatory signaling pathways and apoptosis, eventually induces fibrogenesis. The study aims to assess the molecular alteration occurred in ethanol-induced stress associated with biomarkers in ALD pathogenesis and fibrosis in experimental in-vitro model. HepG2 cells in 20 Mm of ethanol concentration were for 1-5 days and the morphology and oxidative stress parameters, lipids levels (TBARS and protein carbonyls), hypoxic markers expression, apoptosis level were estimated 1,3 and 5 days of the experiment. RT-PCR was employed to determine the m-RNA expressions of CYP1A7, PPAR- α , iNOS, LCAT, SREBP-1c, ABCA1, ABCG1, HIF-1 α , HIF-2 α , miR-21, miR-26A respectively. The changes in morphology were observed in time dependent manner by ethanol consumption. The results demonstrated ethanol causes progressive membrane damage, and increase in TBARS levels and protein carbonyl, indicating augmented lipid peroxidation. Whereas, the expression of SREBP-1c, iNOS, HIF-1 α , HIF-2 α , p53, miR-21 and CYP1A7 markedly increased while expression levels of PPAR- γ , ABCA1, ABCG1 and miR-26A levels were significantly suppressed. That suggested the lipogenesis intensification, impaired fatty acid oxidation and cellular cholesterol exportation. Also, inflammatory signaling and hypoxic pathways was triggered, along with the apoptotic and fibrotic process initiation, whereby increased miR-21, while decreased miR-26A expression defines the shift toward pro-fibrotic regulatory axis. Hence, these molecular alterations may constitute valid biological marker in predicting the development of ALD and might also be possible targets for therapeutic management of the disease.

Keywords: Oxidative stress; Lipid metabolism; Alcohol-associated liver disease; miR21; miR-26A; Liver fibrosis.



Ethanol induces progressive oxidative, metabolic and hypoxic stress in HepG2 cells, accompanied by lipid dysregulation, apoptotic activation, and a miRNA shift characterized by increased miR-21 and decreased miR-26A, collectively favoring a pro-fibrotic molecular phenotype.

1. Introduction

Alcohol associated liver disease (ALD) is identified as one of the predominant chronic diseases among all the liver disease globally (Gao and Bataller 2011). Chronic alcohol consumption triggers a spectrum of diseases starting with fat accumulation in hepatocytes to alcohol induced steatosis, followed by hepatitis, cirrhosis and eventually hepatocellular carcinoma (Seitz and Bataller 2021). Liver fibrosis is defined as the deposition of irregular extracellular matrix proteins (ECM) initiated by induction of hepatic satellite cells (HSC) results in altered liver homeostasis (Gouthami et al. 2025). Recent studies emphasize on how signaling is affected in ALD and liver fibrosis offers potential as a path to finding disease markers and therapeutics (Santra et al. 2026).

Exposure of ethanol tends to accumulation of reactive oxygen species (ROS) in hepatocytes, which acts as a major hallmark in ALD. Lipid peroxidation caused by ROS is commonly assessed by measuring thiobarbituric acid reactive substances (TBARS), which serve as prominent indicators of oxidative damage and membrane lipid degradation (Mackowiak et al. 2024; Wu et al. 2023). The combination of impaired mitochondria and persistent oxidative stress causes hepatocyte injury, increasing inflammation and fibrogenesis (Chacko and Reinus 2016). Alcohol-induced alterations in lipid metabolism further aggravate disease progression through dysregulation of key metabolic regulators, such as sterol regulatory element-binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor- γ (PPAR- γ). SREBP-1c is one the primary inducer of hepatic lipid synthesis, which is increased in response to chronic alcohol exposure (Meng et al. 2020). Whereas, PPAR- γ is key facilitator of fatty acid oxidation that is typically suppressed during ethanol metabolism, resulting in excessive triglyceride accumulation and hepatic steatosis (Namachivayam and Gopalakrishnan 2021). In addition, impairment of cholesterol transporters such as ATP-binding cassette transporter G1 (ABCG1) and ATP-binding cassette transporter A1 (ABCA1) disrupts cholesterol efflux and reverse cholesterol transport in hepatocytes, that enhances lipid accumulation, oxidative stress, and inflammatory responses, alters liver homeostasis (Wang et al. 2024, Wang et al. 2024).

Oxidative stress and more lipid accumulation create hypoxia which stabilizes hypoxia inducible factor-1 α (HIF-1 α) and hypoxia inducible factor-2 α (HIF -2 α) which are involved in transcription of various genes involving angiogenesis, glycolysis, lipid metabolism, and cell survival (Ariyeloje et al. 2024). In response to stress, inducible nitric oxide synthase (iNOS) activates, which results in the release of excess nitric oxide leading to formation of peroxynitrite that in turn exaggerates the hepatocyte injury and stimulation of inflammatory cytokines (Zhang et al. 2025). The tumor suppressor gene p53 regulates apoptosis and arrests cell-cycle in response to oxidative damage although sustained activation may further contribute to hepatocyte loss and fibrosis (Zeng et al. 2025; Zhang et al. 2026).

Recent studies demonstrated the crosstalk between ALD pathogenesis and epigenetic regulation including the role of micro RNAs. MicroRNA-21 (miR-21) is considered as pro-fibrotic micro-RNA which enhances activation of HSC and collagen synthesis through interaction with tumor growth factor- β (TGF- β), whereas, miR-26A acts antagonistically to ALD through its anti-fibrotic role (Zeng et al. 2025; Zhang et al. 2026). Moreover, cytochrome P450 family 7 subfamily A member 1 (CYP1A7), the mitochondrial cholesterol side-chain cleavage enzyme acts on cholesterol at the first rate-limiting step of all steroid synthesis pathways has recently gained considerable attention in studies (Arab et al. 2025; Zhu et al. 2025). Our study investigates the metabolic role and potential impact on hepatic inflammatory process and oxidative stress associated with the chronic alcohol exposure. Together these pathways generate a regulatory web which orchestrates oxidative stress, lipid metabolism, inflammation, hypoxic signaling pathways, apoptosis and fibrogenesis pathways. Hence, the investigation of specific transcriptional factors including miR-21 and miR-26A provide valuable insights to develop biomarkers in progression of ALD pathogenesis and management.

2. Methodology

Cell culture conditions and Cell treatment

Primary hepatocyte cells will be grown in complete growth medium (William's E Medium (GlutaMAX Supplement) with 10% FBS and 1% antibiotic-antimycotic solution), cells will be maintained in 95% atmospheric air and 5% CO₂ at 37°C. Cells will be treated with and without ethanol (10 and 100mM) in the presence of 20 μ M MnTMPyP (Manganese (III) tetrakis (1-methyl 4- pyridyl) porphyrin) which is a superoxide dismutase mimetic and cell permeable, acts as a ROS scavenger. After treatment cells will be washed with cold 1X PBS and will be used for various biochemical estimations (Singal and Mathurin 2021; Maity et al. 2026).

Determination of TBARS and protein carbonyls

To measure the extent of lipid peroxidation (LPO) on erythrocyte membranes induced by alcohol, concentration of malondialdehyde (MDA) formed was determined. 2 mL of thiobarbituric acid reagent has added with the samples as described by the previous papers (Damodara et al. 2010). Determination of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH), was carried out according to the above cited protocol (Padmavathi et al. 2010).

The genes expressing in lipid metabolism was examined in HepG2 cell lines against the control. Total mRNA was extracted using Trizol method and c-DNA was synthesized as μg of total RNA. qRT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, CA, USA) and Qiagen Rotor-Gene 6000 real-time PCR apparatus. As a reference drug β - Actin was used. **Table 1** contains a list of the oligonucleotide primers selected in the target gene amplification. The fold change was computed using the $2^{-\Delta\Delta\text{Ct}}$ technique (Narayanaswamy *et al.* 2026).

Table 1 Oligonucleotide primers used for gene expression analysis

Name of the gene	Name of the species	Primers used	
		Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
CYP1A7	Human	TGGCATCCTCTACAGACTCTG	CTTCAGGTTGCGTGCCATCTCA
PPAR- α	Human	TCGGCGAGGATAGTTCTGGAAG	GACCACAGGATAAGTCACCGAG
INOS	Human	TAAATTGTCCCCAGCATCAA	TCTGTGTGTTGGGAAATTCA
L-CAT	Human	TGTGGGTGTGCTCTATGAGG	ATTGATGTGCTCCAGGGTCA
SREBP-1c	Human	ACACAGCAACCAGAACTCAAG	AGTGTGTCCTCGACCTCAGTC
ABCA1	Human	CAGGCTACTACCTGACCTTGGT	CTGCTCTGAGAAACACTGTCCTC
ABCG1	Human	GAGGGATTTGGGTCTGAAGTGC	TCTACCAGCCGACTGTTCTGA
HIF-1 α	Human	TGCCACTTCAGCTTCTTTTCT	GGCAGTGGTGGCATTAGC
HIF-2 α	Human	CTGACCTGAGCTTGGGTTTG	CCTGGGTCTTCTGGCTGAT
miR-26A	Human	GCCGAGTTCAAGTAATCCAGG	GTGCAGGGTCCGAGGT
miR-21	Human	TGCCATACCAGAGTACAGTA	AAATCTCTCCCACCAACATG

Statistical analysis

The results have been statistically analyzed and data were normally distributed and performed one way ANOVA followed by Tukeys test, the data presented in figures mean $\pm$ SEM of three independent experiments; the student t-test was used to compare two groups and values of statistical significance at the 5% level of confidence ($p < 0.001$ and $p < 0.05$) were stated (Reddy *et al.* 2017).

3. Results

Microscopic evaluation of HepG2 cells revealed morphological changes after ethanol treatment as concentration changes. The control group was composed of cells with morphology characterized by normal, homogeneously distributed biconcave discs and the membrane structure was conserved. Slight changes in HepG2 cells morphology are detected upon treatment with 5mM of ethanol, whereas in the presence of 10mM of ethanol, obvious change in the integrity of membrane and shape alteration is observed. Further increase in ethanol concentration up to 20 mM resulted in severe morphological deformity of HepG2 cells, involving severe membrane damage, swelling of cells and more heterogeneity of cells structures (**Fig. 1**).

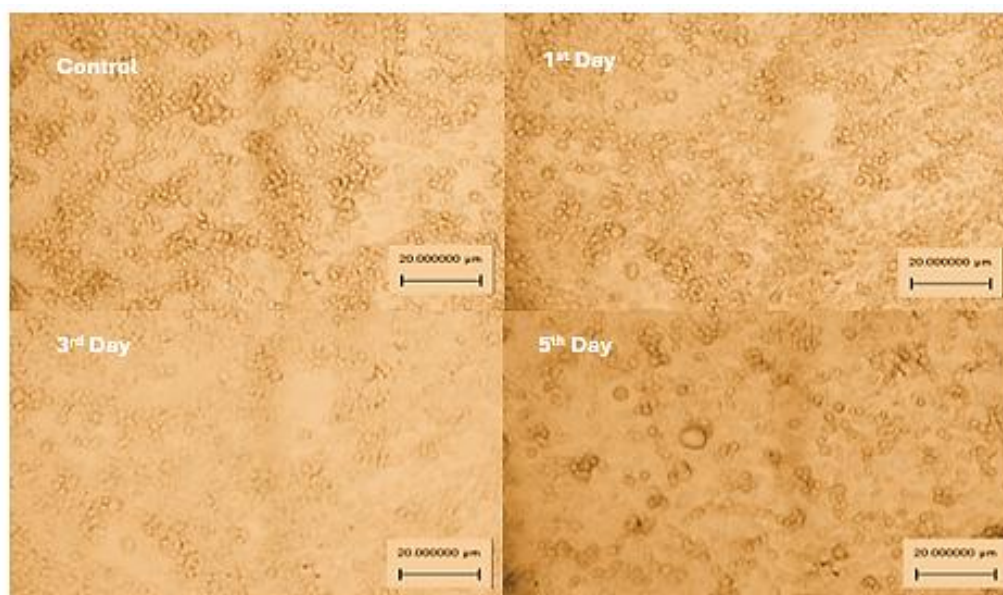


Fig. 1. Time-dependent response of hepatocytes to ethanol exposure

Fig. 2. shows the ethanol treatment in HepG2 cell line has significantly increased the TBARS concentrations at all three time points compared to control rats (**Fig. 2A**). TBARS accumulation from Day 1 to Day 5 shows time-dependent accumulation. The elevated TBARS levels caused by ethanol treatment were significantly decreased when MnTMPyP was administered. These results shown in Day 3 and 5 demonstrate the potency of MnTMPyP to counter chronic alcohol-mediated increase of lipid peroxidation.

Protein carbonyl levels were also found to be significantly increased in animals treated with ethanol at Days 1, 3, and 5 (**Fig. 2B**).

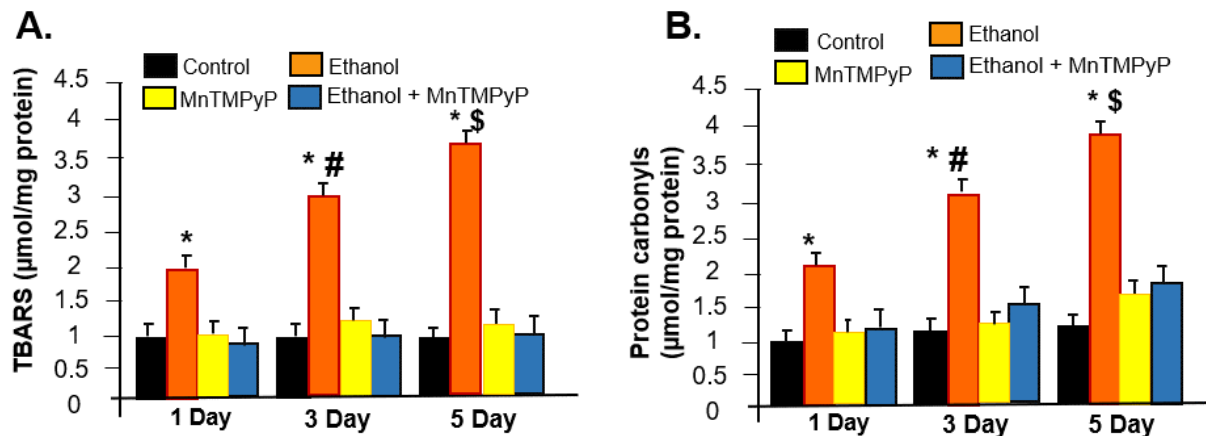


Fig. 2. Effect of ethanol and MnTMPyP treatment on lipid peroxidation. TBARS and protein carbonyls levels were measured in control, ethanol-treated, MnTMPyP-treated, and ethanol+ MnTMPyP-treated groups after 1, 3, and 5 days of exposure. The significance between groups with $p < 0.05$ was compared, * indicates the significant difference from the controls. *# Indicates the significant difference from control and ethanol treated cells. *\$ indicates highly significant difference from control and ethanol treated cell.

Different expression levels of the genes were studied in time-dependent manner where, some genes have up-regulated during ethanol treatment. Ethanol-treated HepG2 cells comparing to the MnTMPyP treated HepG2 cells specifically altered on Day 5 including iNOS, p53, and CYP1a7. The result indicated an accelerated activation of oxidative stress, inflammation in ethanol treated cells (**Fig. 3**).

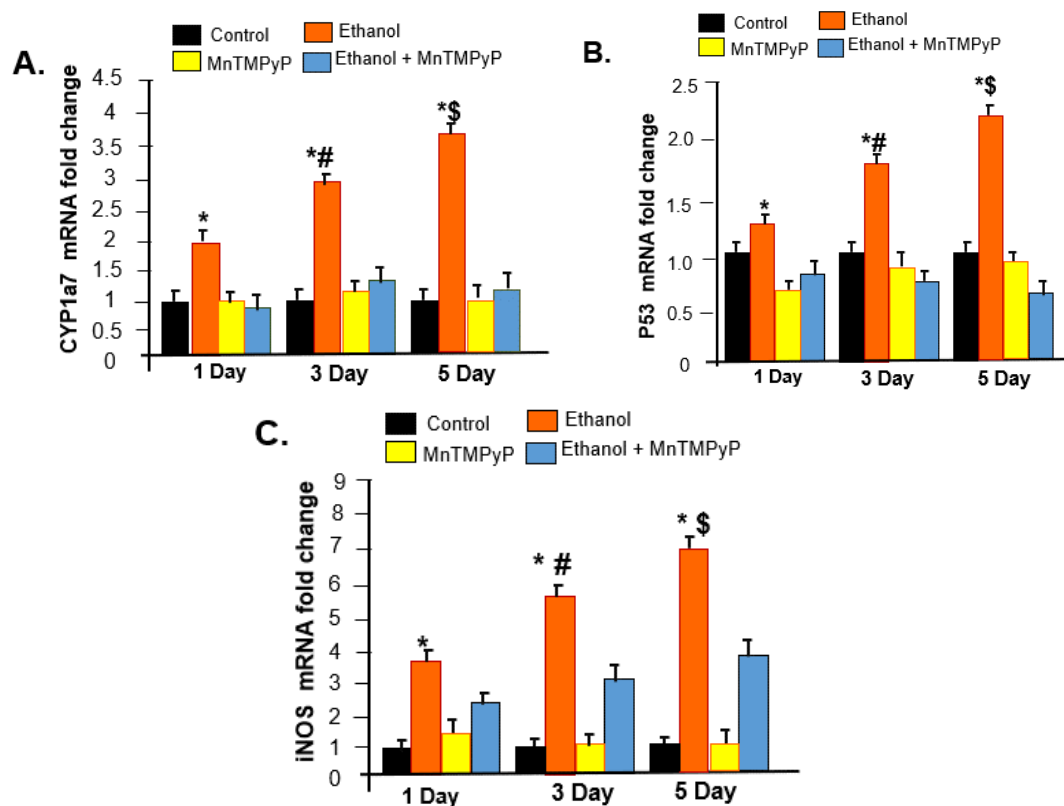


Fig. 3. Time-dependent alterations in CYP1a7, p53 and iNOS gene expression in ethanol treated HepG2 cell lines. The significance between groups with $p < 0.05$ was compared, * indicates the significant difference from the controls. *# Indicates

the significant difference from control and ethanol treated cell. *^s indicates highly significant difference from control and ethanol treated cell

The gene expression level of PPAR- γ , ABCA-1, ABCG-1 were significantly increased throughout the ethanol intoxication whereas LCAT and SREBP1c expression has decreased. The result indicated an accelerated activation of oxidative stress, inflammation in ethanol treatment. In addition with MnTMPyP ethanol treated cells have shown significant decrease of gene expression compared to ethanol treated cells (Fig. 4.).

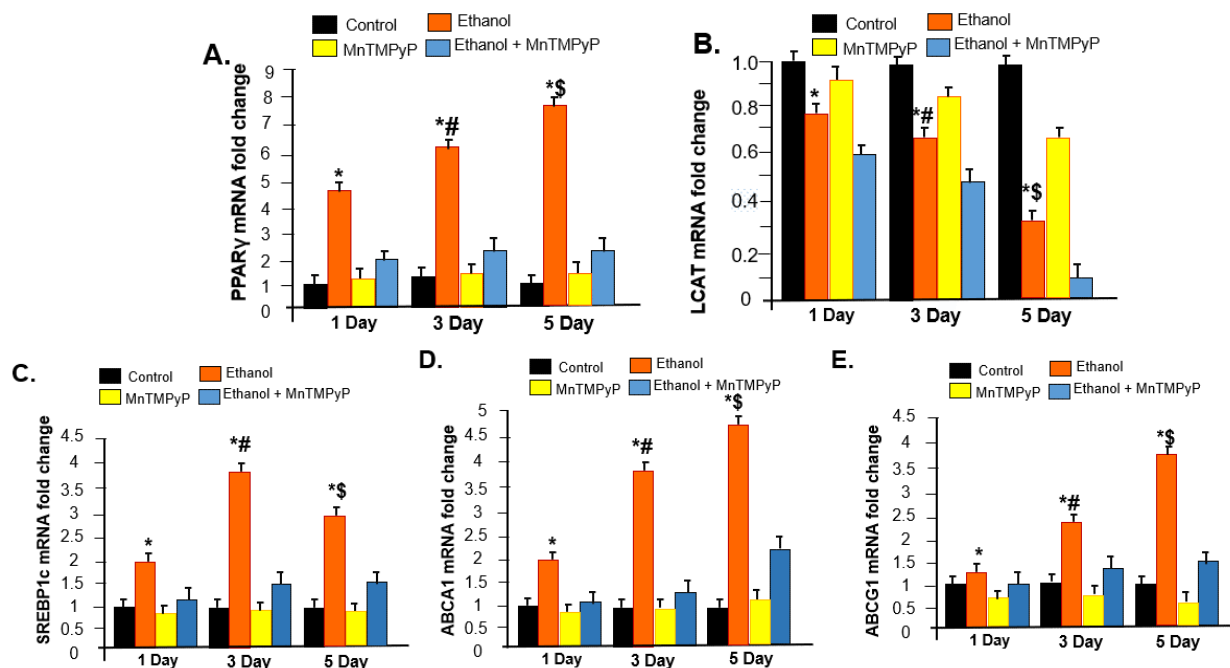


Fig. 4. Expression of PPAR γ , LCAT, SREBP1c, ABCA1, ABCG1 mRNA in ethanol treated HepG2 cell lines in 1,3 and 5 days with and without MnTMPyP. The significance between groups with $p < 0.05$ was compared, * indicates the significant difference from the controls. *# Indicates the significant difference from control and ethanol treated cell. *^s indicates highly significant difference from control and ethanol treated cell

The upregulation of HIF-2 α and HIF-1 α in Fig. 5 indicates the activation of hypoxic pathway, while in presence of the pharmacological indicator MnTMPyP it has observed a significant reduction in gene expressions of HIFs.

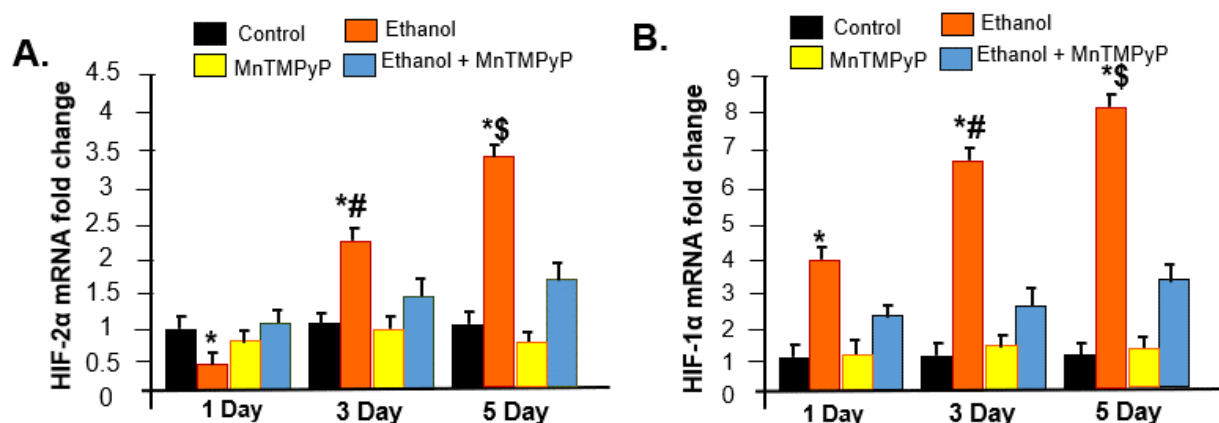


Fig. 5. HIF-2 α and HIF-1 α mRNA expression in ethanol treated HepG2 cell lines in time-dependent manner. The significance between groups with $p < 0.05$ was compared, * indicates the significant difference from the controls. *# Indicates the significant difference from control and ethanol treated cell. *^s indicates highly significant difference from control and ethanol treated cell.

The significant upregulation of miR-21 and downregulation of miR-26A in Fig. 6 reflect their important roles in epigenetic regulation of hepatic stellate cell (HSC) activation, transcriptional responses, and collagen deposition during liver fibrosis, lipid accumulation as well as apoptosis while attenuated lipid oxidation and cholesterol efflux, suggesting that chronic ethanol mediates a series of changes in downstream pathways contributing to ALD in a time dependent manner.

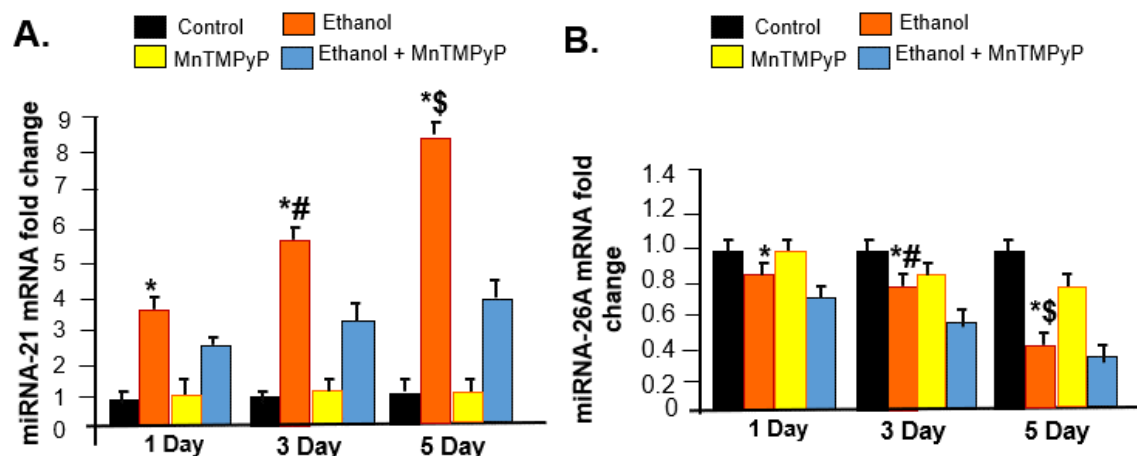


Fig. 6. Time-dependent alterations in miRNA-21 and miRNA-26A mRNA expression in ethanol treated HepG2 cell lines. The significance between groups with $p < 0.05$ was compared, * indicates the significant difference from the controls. *# Indicates the significant difference from control and ethanol treated cell. *\$ indicates highly significant difference from control and ethanol treated cell

4. Discussion

ALD is characterized by interconnected molecular alterations involving oxidative stress, impaired lipid metabolism, inflammatory responses, apoptosis, and fibrogenesis, ultimately leading to hepatic dysfunction (Tan *et al.* 2020). The present study demonstrates that the integrity of the HepG2 was affected by ethanol by damaging its membrane through activating oxidative stress pathway that further alters lipid peroxidation. After 5 days of treatment with ethanol leads to the production of more ROS which eventually causes oxidation of membrane protein and phospholipids with a reduction in fluidity of cell membrane causing change in cell structure. Chronic ethanol treatment causes progressive molecular changes that mediates ALD (Michalak *et al.* 2021). The increased expression of CYP1a7 indicates elevation in ethanol metabolism and overproduction of ROS. The increased CYP1a7 therefore dysregulates the cholesterol homeostasis and steroidogenic processes which are affected by alcohol-induced metabolic anomalies. That further causes oxidative stress leading to hepatocellular injury (Liu *et al.* 2021; Wu *et al.* 2023). The significant increase in TBARS, indicates the rate of lipid peroxidation and membrane injury. The progressive increase in protein carbonyls indicates increasing oxidative modification of cellular proteins during prolonged ethanol exposure. It has been observed the expression iNOS up-regulates enormously leading to increased nitric oxide production and subsequently peroxynitrite formation, amplifying the oxidative stress. The elevated expression of SREBP-1c along with reduced PPAR- γ expression implies a shift from β -oxidation to lipogenesis, accumulation of triglycerides in the hepatocytes that initiates steatosis. Moreover, down-regulation of ABCA1 and ABCG1 leads to impaired cholesterol efflux. Intracellular accumulation of cholesterol activates other inflammatory cytokines and creates hypoxia in cells (Shen *et al.* 2025; Kuruvalli *et al.* 2026). That further stimulates the expression of HIF-1 α and HIF-2 α , implies induction of hypoxia-responsive signaling pathway and activation of HSCs, increased angiogenesis and extracellular matrix synthesis in liver fibrosis. The elevation in p53 expression suggests the increased rate of apoptosis and the DNA damage response pathways (Park and Seo 2025), those are being induced in hepatocytes treated with oxidative stress induced by ethanol. In addition, ethanol significantly upregulated miR-21, a well-established profibrotic microRNA that enhances TGF- β signaling and collagen synthesis. Conversely, the suppression of miR-26A indicating the loss of its protective anti-fibrotic effects on hepatic stellate cells (Hong *et al.* 2024). The significant changes from day 1 to day 5 implies that ethanol induced cellular oxidative stress is the key signaling entity mediating effects on inflammatory processes, hypoxic signals and lipid profiles, apoptosis, and fibrogenesis. These interconnected molecular events collectively promote hepatic injury and accelerate the progression of alcoholic liver disease toward fibrosis, therefore, modulation of these biomarkers may represent promising therapeutic targets for preventing or slowing the progression of alcohol-induced liver fibrosis (Mackowiak *et al.* 2024; Mullish and Thursz 2024).

5. Conclusion and future prospectives

ALD is an etiologically and pathogenically diverse liver disorder, characterized by continuous structural and biochemical damage of hepatocytes during persistent use of alcohol. In the present study, chronic alcohol intake induced remarkable molecular changes in oxidative stress, lipid metabolism, inflammation, hypoxia, apoptosis, and epigenetic regulation, leading to the pathogenesis of hepatic fibrosis development and progress. Severe hepatocytes damages induced by oxidative stress caused up-regulation of hepatic iNOS which exaggerated inflammations through overproduction of nitric oxide and peroxynitrite. Meanwhile, over expression of SREBP-1c coupled with repression of PPAR- γ in hepatic tissues caused significant intra-hepatic fat accumulation via shift of fatty acid metabolism from oxidation toward synthesis. Impaired cholesterol transport and reverse cholesterol from increased reduction of ABCA-1 and ABCG-1 accumulation of intracellular

lipids and increased inflammatory cytokines, which leads increased inflammation. Moreover, induction of expression of both HIF-1 α and HIF-2 α indicates the enhancement of those activated hypoxia mediate pathways which lead to stimulation of HSC activation, deposition of extracellular matrix accumulation through activated transcription factor. Increased expression of p53 is interpreted to increased apoptosis and DNA damage owing to excessive oxidative stress during long-period chronic. The significant alteration of the expression of miR-21 upregulation and miR-26A downregulation, reflect the key role in epigenetic mediation of HSC transcription activation and collagen deposition and development.

Therefore, the study strongly suggests that in alcoholic liver pathogenesis oxidative stress may function as a central molecule to mediate other biochemical processes including metabolic disequilibrium, inflammation, hypoxic response, and apoptosis which lead to hepatic fibrosis through activation of epigenetic regulation mechanisms including up-regulation of miR-21 and down-regulation of miR-26A in liver tissues. The studied biomarkers may be helpful for understanding ALD and potential target of treatment. These findings would pave the way towards developing therapeutic interventions such as targeting PPAR- γ , HIF signaling pathway and epigenetic mechanisms that play role in progression from inflammation to fibrosis in ALD progression that should also lead the novel markers to assess and treat ALD effectively. This could also give rise to further research that targeting such pathways could prevent and regress hepatic fibrosis or progression into cirrhosis from alcoholic hepatitis. This would significantly develop effective diagnose markers and therapeutic targets for a proper management of ALD in clinics.

CRedit authorship contribution statement

Subhashish Maity, Ayantika Santra: Writing – review & editing, **Saraladevi Selvam:** Writing – review & editing, **Kuruvalli Gouthami & Challaraj Emmanuel ES:** Writing – review & editing, **Vaddi Damodara Reddy:** Supervision, Conceptualization.

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Conflict of Interest statement: None

Patient consent: NA

Ethical approval: NA

Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

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