The effects of nicotine on microRNA-124 expression in the bile duct ligation-induced liver fibrosis in rats

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Research Article

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Abstract

Background

Smoking causes a variety of adverse effects on organs that have no direct contact with the smoke itself such as the liver. Nicotine as a main compound of smoking may exert its effects by changing the expression of microRNAs (miRNAs). This study was conducted to further investigate the molecular mechanisms of miRNA-dependent effects of nicotine in an animal model of liver fibrosis.

Methods

First, the bile duct ligation (BDL) approach was used in male Wistar rats to create a model of liver fibrosis. Then, the effects of nicotine administration on miRNA-124 expression, as well as fibrosis and inflammation-related genes were investigated using the quantitative Real-Time PCR method. The total bilirubin and liver enzymes activity levels were measured using the colorimetric assay. Also, the effects of nicotine on the process of liver fibrosis were investigated with histological studies.

Results

The development of liver fibrosis in BDL rats leads to a decrease in miRNA-124 expression. Also, a decrease in miRNA-124 expression has been seen in the groups administered nicotine. The decrease in the expression of miRNA-124 is accompanied by the increase in the expression of fibrotic and proinflammatory genes. Also, the significant increase in bilirubin and liver enzymes in fibrotic rats worsens with nicotine administration. The results of histological studies also confirm these results.

Conclusion

Considering that miRNA-124 is an anti-inflammatory miRNA, it can be concluded that the decrease in its expression due to nicotine exposure leads to an increase in inflammatory processes and subsequently to an increase in liver fibrosis.

1. Introduction

The increasing rate of liver fibrosis has become one of the most important health problems in the world [1]. Liver fibrosis or established cirrhosis is seen in a significant percentage (18–27%) of subjects with risk factors for liver disease [2]. Among the known risk factors of liver fibrosis is smoking and the association between smoking and increased severity of liver fibrosis has been shown in previous studies, but the exact mechanism of these effects is not known [3, 4]. Previous studies have shown that nicotine, as a fibrogenic compound in cigarette smoke, is involved in several fibrogenic processes in different types of tissues, including the liver [5, 6]. However, the intracellular mechanism of various effects of nicotine is
not clear and there are mounting studies that emphasize the central role of microRNA (miRNA) in creating these effects [7].

The miRNAs are ~ 20–25 nucleotide sequences that play major roles in modifying mRNA expression [8]. Changes in miRNA expression are seen in liver diseases and they are one of the novel therapeutic targets in the diagnosis and treatment of liver fibrosis [9, 10]. Mounting evidence designated that nicotine leads to alteration in the expression of miRNAs [11]. Among these miRNAs, miRNA-124 is an essential post-transcriptional regulator of inflammatory genes expression [12, 13]. It has been shown in several models of inflammatory diseases that miRNA-124 modulates the expression of their inflammatory target genes and participates in the regulation of inflammatory responses [14–16]. Qin et al. have made a comprehensive review of the role of this miRNA in the immune system and inflammatory diseases, which emphasizes the essential role of this miRNA in regulating immune system activities [17]. In some studies, it has been determined that miRNA-124 can directly target the 3’-untranslated region of signal transducer and activator of transcription 3 (STAT3) [18]. This transcription factor is one of the major signaling pathways converting the cytokine signal into gene expression programs regulating the immune cells [19]. It plays an important role in liver diseases, especially in the process of liver chronic inflammation and resulting fibrosis [20]. Considering that chronic inflammation plays an essential role in the pathogenesis of liver fibrosis, it is rational to investigate the role of this anti-inflammatory miRNA-124 in the pathogenesis of liver fibrosis [21].

On the other hand, there is some evidence that shows that miRNA-124 is one of the influencing factors in the cholinergic anti-inflammatory pathway [22]. The role of inflammatory cytokines and their role in activating hepatic stellate cells and as a result excessive production of extracellular matrix is the main characteristic of liver fibrosis. Therefore, nicotine through the miRNA-124/STAT-3 Pathway may have a possible anti-inflammatory role in liver fibrosis by activating the cholinergic anti-inflammatory pathway. Considering this controversial evidence, this study was conducted to investigate the effects of nicotine in the process of liver fibrosis, focusing on the role of miRNA-124.

2. Material and methods

2.1. Chemicals, Animals, and Bile duct ligation

In this experiment, 36 male Wistar rats weighing 220–250 grams (eight weeks old) were purchased from the animal house of the Tabriz University of Medical Sciences. After adaptation, the bile duct ligation (BDL) operation was performed under anesthesia with ketamine and xylazine [23]. In this surgery, the peritoneal cavity was opened and the bile duct was isolated and triply ligated [24]. The sham-operated rats undergo a similar procedure without ligation of the common bile duct. Then, the animals were randomly divided into four groups of six each: 1) Sham + Saline; 2) Sham + Nicotine; 3) BDL + Saline; 4) BDL + Nicotine. Nicotine (Sigma-Aldrich, product number N3876) was administered intraperitoneally at a dose of 10 mg/kg every other day for 3 weeks [25]. Previous evidence demonstrated that nicotine may aggravate the process of liver fibrosis [26–28]. Because of this evidence, we expected to observe the
aggravation of hepatic fibrosis in nicotine-treated groups. Since in previous studies, the severity of liver fibrosis in the BDL model reaches its maximum level after 4 weeks, therefore, we chose a model with the 3-week BDL which exhibits submaximal liver fibrosis and can investigate either protection or exaggeration of hepatic fibrosis in nicotine groups [29]. At the end of the experiments, liver tissue was sampled for histology and gene expression analysis.

2.2. RNA extraction and RT-qPCR

The relative miRNA-124, and mRNA levels of target genes were assessed by reverse transcription quantitative PCR (RT-qPCR) [30]. Total RNA was obtained from nicotine-administrated and nicotine-non-administrated rats after Trizol (Invitrogen, Carlsbad, CA) treatment; this was performed according to the manufacturer’s guidelines. The total RNAs of the liver tissues were obtained and the Prime Script Kit (TaKaRa Bio Inc., Japan) was performed for cDNA synthesis. Then, the purity of extracted RNA was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, USA). The complementary DNA (cDNA) was synthesized by applying the miScript II RT Kit for miRNA-124 (Qiagen, Hilden, Germany) and αSMA, CCL-2, and STAT-3 (Biofact, Korea) based on the manufacturer’s guidelines. Then, using SYBR Green fluorescent-based assay, the expression level of miRNA-124 and mRNA level of target inflammatory genes were determined according to RT-qPCR assay using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). Beta-actin was used as a housekeeping gene for target mRNAs, and the miR-191-5p was used for normalization of miRNA-124 expression. After normalizing, the ratio of expression of each gene was quantified using the $2^{-\Delta\Delta Ct}$ method [31]. The primer sequences of target genes were synthesized and the sequences are available upon request. All experiments were performed in triplicate.

2.3. Serum alanine aminotransferase (ALT, SGPT), aspartate aminotransferase (AST, SGOT), alkaline phosphatase (ALP), and total bilirubin assay

The amount of total bilirubin in the serum and the activity level of liver enzymes including AST, ALT, and ALP were measured using the colorimetric assay. For this purpose, blood was withdrawn from the heart of nicotine and saline received sham and BDL rats for serum liver enzyme assay using an auto-analyzer.

2.4. Histological analysis

The liver tissues were fixed in formalin and sectioned into 5 µm-thick samples. Histological analysis was performed using hematoxylin-eosin (H&E) as well as Masson trichrome staining. The Masson's trichrome staining is performed for the evaluation of collagen fiber deposition. In this staining approach, the collagen fibers will be stained blue, the nuclei will be black and the background will be stained red. In our study, the hepatic inflammation was staged 0–3, with 0 meaning “absent,” 1 meaning “mild,” 2 meaning “moderate,” and 3 meaning “severe [24].”

2.5. Statistical analysis
The data was analyzed by GraphPad Prism 6 software (GraphPad Software, La Jolla California, USA) and expressed as mean ± SEM. One-way analysis of variance was used to compare between the studied groups. P < 0.05 is considered a statistically significant difference.

3. Results

3.1. Confirmation of liver fibrosis development in BDL rats

The induction of liver fibrosis in BDL rats was confirmed by examining the histological H&E (Fig. 1) and Masson's trichrome staining sections (Fig. 2). In addition, the mRNA level of αSMA gene expression as a marker of hepatic stellate cell activation and liver fibrosis was assessed and the results confirmed the development of liver fibrosis in BDL rats (Fig. 3). P < 0.05 in comparison with the corresponding Sham-Saline group. Also, the results of measuring the total bilirubin concentration in the serum and the activity of liver enzymes showed that these enzymes, which are a marker of liver damage, were significantly increased in the group of fibrotic BDL rats (Fig. 4). All these measures related to histology experiments, the upregulated expression of liver fibrosis marker (αSMA), and elevation of liver enzymes activities have a good degree of validity and showed the development of liver fibrosis in rats.

3.2. Effect of nicotine administration on the miRNA-124 expression in BDL rats

The obtained results showed that the induction of biliary fibrosis in BDL rats leads to a decrease in miRNA-124 expression (Fig. 5). Nicotine administration for 3 weeks in healthy rats leads to a significant decrease in the expression of this miRNA. The important results of our study were obtained when the group of BDL rats was administered nicotine. The results showed that the administration of nicotine in the BDL group leads to a further decrease in the expression of miRNA-124 (P < 0.05) (Fig. 5). Therefore, since miRNA-124 is an anti-inflammatory miRNA, in the continuation of the study, we investigated the effects of nicotine administration and reduced expression of miRNA-124 during the development of liver fibrosis.

3.3. Effect of nicotine administration on the infiltration of inflammatory cells and the expression of inflammatory genes

Chronic liver injury leads to the secretion of cytokines such as chemokine ligand 2 (CCL-2) which are responsible for the infiltration of inflammatory cells in the liver [32, 33]. The effect of nicotine administration on the expression of CCL2 in rat liver was assessed by using RT-qPCR. The results showed that CCL-2 expression was increased significantly in BDL rats in comparison with sham-operated rats. Nicotine administration further induced the CCL-2 expression in BDL rats (Fig. 6A). It seems that CCL-2 upregulation is associated with nicotine-induced liver fibrosis in BDL rats. The CCL-2, also known as
monocyte chemoattractant peptide-1 (MCP-1), acts as a cytokine that leads to an influx of inflammatory cells to the injured tissues [33]. Our histological studies confirmed that there is an infiltration of inflammatory cells in the fibrotic BDL rats, which is increased in the liver of rats receiving nicotine (Table 1). On the other hand, given that previous studies have shown that STAT-3 is one of the specific targets of miRNA-124, therefore we investigated the changes in the expression of STAT-3 in the liver of fibrotic rats receiving nicotine. The results showed that the expression of this gene increases during the development of liver fibrosis, and this increase is even further in the group of nicotine-received fibrotic rats (Fig. 6B).

### Table 1

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<thead>
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<th>Sham + Saline (n = 6)</th>
<th>Sham + Nicotine (n = 6)</th>
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#### 3.4. Effect of nicotine administration on the liver enzymes in BDL rats

Serum bilirubin levels as well as AST, ALT, and ALP activity of BDL rats are shown in Fig. 4. BDL was associated with hyperbilirubinemia and increased ALT, AST, and ALP activity (P < 0.0001). These increased liver enzymes worsened when nicotine was administered and indicated more liver damage in the nicotine group (Fig. 4).

#### 3.5. Effect of nicotine exposure on liver fibrosis

The histology of liver fibrosis was studied using H&E and Masson's trichrome staining (Fig. 1 and Fig. 2). BDL was associated with bile duct proliferation (Fig. 1) and extensive bridging fibrosis (portal to portal and portal to central linkage with fibrotic bands) (Fig. 2). As shown in these figures nicotine has a significant effect on hepatic fibrosis and portal expansion in BDL rats. Likewise, nicotine in sham-operated rats has altered the histological structure of the liver as assessed by H&E and trichrome staining. Also, the increased level of the liver fibrosis marker (αSMA) in the fibrotic groups is increased when nicotine is administrated, which indicates higher levels of activation of hepatic stellate cells and more production of extracellular matrix (Fig. 3). All these results indicate the adverse effects of nicotine on the severity of liver fibrosis development. To date, there was no study investigating the effects of
nicotine administration on the development of liver fibrosis in the BDL model, and the results of this study clearly showed that nicotine has a wide range of effects in both liver damage and liver inflammation, as well as the development of liver fibrosis.

4. Discussion

Today, the change in lifestyle and the use of fast foods and high-fat foods has increased the prevalence of liver diseases worldwide [34]. According to global statistics, two million people die each year due to chronic liver disorders and these conditions are responsible for 4% of all deaths [35]. Genetic background, consumption of a high-fat diet, viral infection, and autoimmune diseases are mentioned as the most common causes of chronic liver diseases. Among these risk factors, some studies have mentioned smoking [36]. Wijarnpreecha et al. found that smoking is associated with a significantly higher risk of advanced liver fibrosis among patients with primary biliary cholangitis [3]. The association between smoking and increased severity of liver fibrosis has been reported in some other studies [37–39]. Even though various studies have been conducted for many years in the field of understanding the mechanism of liver tissue damage in liver disorders, unfortunately, the main mechanism of liver tissue damage in liver disorders, especially liver fibrosis, has not been fully understood yet. Undoubtedly, knowing the exact mechanism of this damage can be effective in treating and reducing the liver complications of people with liver fibrosis. Many studies emphasize the pivotal role of miRNAs in the pathogenesis of liver fibrosis, and therapeutic approaches based on restoring the expression of altered miRNAs are introduced as new methods of treating liver fibrosis [40, 41]. One of the important miRNAs that anti-inflammatory activities are shown in previous studies is miRNA-124. Considering these anti-inflammatory features, changes in the expression level of this miRNA during the process of liver fibrosis can have many consequences. On the other hand, nicotine has been shown in many studies that can exert its effects through miRNA pathways. The results of this study showed that nicotine seems to stimulate the activity of the STAT-3 signaling pathway through miRNA-124-related effects. Similar to nicotine, which in this study was associated with an increase in the severity of liver fibrosis, in another study, it was shown that the use of heroin was also associated with an increase in liver fibrosis [42]. We have previously shown that selective hepatic vagotomy leads to a decrease in the AST activity in the serum of BDL rats [24]. In this present study, instead of inhibiting the activity of nicotinic acetylcholine receptors, we used the stimulation of these receptors by injecting nicotine. The results we obtained are in line with our previous study and show that the stimulation of nicotinic acetylcholine receptors leads to increased damage to the liver, which performs these effects to some extent by reducing the expression of miRNA-124. Therefore, exposure to nicotine or increasing the activity of nicotinic receptors through endogenous or exogenous ligands can lead to increased liver damage and subsequent liver fibrosis.

5. Conclusion

Smoking cessation and avoiding exposure to nicotine should be taken seriously in people who are at high risk of developing liver fibrosis. All the results of this study clearly show that exposure to nicotine leads to
an increase in the severity of liver fibrosis. Based on these findings, it is important to investigate the role of anti-inflammatory miRNA-124 expression in the processes of liver fibrosis. Restoring miRNA-124 expression in liver fibrosis can be considered a therapeutic approach.

**Declarations**

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**Acknowledgments**

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**Conflicts of interest/Competing interests**

The authors have no conflicts of interest to declare.

**Availability of data and material**

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

**Authors' contributions**

KH and MRA provided biological materials and reagents. KH performed the experiments, and wrote the initial draft of the manuscript and performed data analysis. PS, EKS, and MRA participated in the design of the work and reviewed and edited the manuscript. All of the authors have read and approved the final version submitted.

**Ethics approval**

All experiments were performed by the ethical principles registered internationally and were approved by the Ethics Committee of Tabriz University of Medical Sciences. The experiments were performed in compliance with the ethical principles of Tabriz University of Medical Sciences and approved by the Regional Medical Research Ethics Committee (Ethical code: IR.TBZMED.AEC.1402.054).

**Consent to participate**

The animal study has been approved by a research ethics committee of laboratory animals at Tabriz University of Medical Sciences (Ethical code: IR.TBZMED.AEC.1402.054).

**Consent for publication**
Not Applicable.

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16. Li X, Ye S, Lu Y. Long non-coding RNA NEAT1 overexpression associates with increased exacerbation risk, severity, and inflammation, as well as decreased lung function through the interaction with microRNA-124 in asthma. J Clin Lab Anal. 2020;34.


**Figures**
Figure 1

The H&E-stained liver tissues of sham-operated and BDL rats. The histological structure of sham groups was normal and ductular proliferation was absent. The livers of nicotine-treated rats had some degree of portal tract expansion. Portal expansion with the portal-to-portal linkage was the distinctive feature of the BDL group. Livers of nicotine-treated BDL rats displayed enhanced degrees of pathological damage.
Figure 2

The deposition of collagen fibers was assessed in sham and BDL rats with and without nicotine. 3 weeks after the surgical procedure, liver tissues were obtained and collagen fibers were stained with Masson's trichrome. BDL was associated with extensive bridging fibrosis (portal to portal and portal to central linkage with fibrotic bands). The deposition of fibrotic tissue has increased in the rats of the BDL group that received nicotine injections.
Figure 3

The expression of αSMA in sham and BDL rats about beta-actin as an internal standard. BDL has increased this marker of liver fibrosis. *P < 0.05 in comparison with the sham-operated group. Nicotine administration caused a further increase in its expression. ** P < 0.01 in comparison with the BDL-Saline group. Data are shown as Mean ± SEM.
Figure 4

The effect of 3-week nicotine administration on ALT, AST, and ALP activities and total bilirubin level in sham-operated or BDL rats. * P<0.05, *** P<0.001, **** P<0.0001 in comparison with corresponding sham-operated group.
Sham and BDL rats were administrated with nicotine (10 mg/kg) for 3 weeks, and then the expression level of miRNA-124 was evaluated using RT-qPCR assay. * P<0.05, *** P<0.001, **** P<0.0001 in comparison with corresponding sham-operated group. Results are expressed as the Mean ± SEM of three experiments (triplicate).
The nicotine or saline were treated in sham or BDL rats and then the expression level of CCL-2 and STAT-3 were evaluated using RT-qPCR assay. * P<0.05, ** P<0.01, *** P<0.001 in comparison with corresponding sham-operated group.

Figure 6