Protective role of trans-chalcone against the progression from simple steatosis to non-alcoholic steatohepatitis: Regulation of miR-122, 21, 34a, and 451

Abstract
Purpose: Non-alcoholic steatohepatitis (NASH) is an inflammatory disorder and an aggressive form of fatty liver disease. Certain microRNAs, including miR-122, 21, 34a, and 451, are involved in the transition from steatosis to NASH. This study examined how trans-chalcone (the core of chalcone derivatives) affects NAFLD progression by regulating miRNAs.
Methods: Male rats were divided into three groups (n = 7/group) as follows: control, rats were gavaged with 10% tween 80 (for two weeks); NASH, rats were gavaged with a high-fat liquid diet (HFD; for six weeks) and 10% tween 80 (for two weeks); NASH + Chal, rats were gavaged with the HFD (for six weeks) and trans-chalcone (for two weeks). Hepatic expression levels of miR-122, 21, 34a, and 451 were determined.
Results: trans-Chalcone reversed histological abnormalities, reduced liver injury markers, and attenuated insulin resistance in HFD-fed rats. In the liver, HFD-induced NASH increased the expression level of miR-34a and decreased expression levels of miR-122, 21, and 451. However, trans-chalcone inhibited HFD-induced changes in expression levels of these miRNAs.
Conclusion: *trans*-Chalcone could inhibit the transition from steatosis to NASH through the modulation of miR-122, 21, 34a, and 451 expression levels in the liver. **Keywords:** non-alcoholic steatohepatitis; *trans*-chalcone; miRNAs; liver; rat

Introduction
Nowadays, non-alcoholic fatty liver disease (NAFLD) is a well-known cause of chronic liver disease.\(^1\)\(^-\)\(^3\) The increasing prevalence of NAFLD has been linked to a combination of a sedentary lifestyle and excess calorie intake.\(^4\)\(^-\)\(^6\) This common liver disease ranges from simple steatosis (lipid accumulation in more than 5-10 percent of hepatocytes) to non-alcoholic steatohepatitis (NASH; the inflammatory and more aggressive stage), leading to liver cirrhosis and -only in a small number of patients- liver cancer.\(^7\)\(^-\)\(^10\) It has been known that inflammation, fat accumulation in the liver tissue, hepatocellular damage, and fibrosis are characteristics of NASH.\(^8\)

MicroRNAs (miRNAs) are extremely conserved, endogenous, small, and non-coding RNAs involved in regulating gene expression using the post-transcriptional mechanisms.\(^11\)\(^-\)\(^14\) It has been known that dysregulation of specific microRNAs contributes to NAFLD progression.\(^13\),\(^15\) In particular, dysregulation of miR-122, 21, 34a, and 451 is responsible for transitioning from simple steatosis to NASH.\(^16\) Therefore, these non-coding RNAs are possible therapeutic targets for NAFLD/NASH treatment.\(^15\)

Lack of FDA approved anti-NASH drugs creates an urgent need to develop effective and safe medicines to treat this liver disease.\(^17\) Chalcone derivatives have protective effects on NAFLD and liver cancer through several molecular mechanisms.\(^18\) In this regard, it has been shown that *trans*-chalcone, the core structure of chalcones, protects high-fat diet (HFD)-fed rats against liver inflammation and insulin resistance by miR-34a, 451, and 33a-dependent mechanisms.\(^19\) Furthermore, co-administration of this chalcone with HFD improved hepatic lipid metabolism and protected rats against NASH induction.\(^20\) The possible effect of *trans*-chalcone on the transition from simple steatosis to NASH and its impact on expression levels of miR-122 and 21, critically involved in the NAFLD progression, is still unknown. The present study investigated how orally administered *trans*-chalcone affects NAFLD progression by regulating hepatic miR-122, 21, 34a, and 451 levels.

Material and methods

Animal experiments
Male Wistar rats (210–250 grams) were obtained and kept under standard conditions (22 ± 2 °C and 12 hours light: 12 hours dark cycle). Animals had free access to food (standard rat diet) and water.

After acclimatization period (one week), twenty-one rats were divided randomly into three experimental groups and kept for six weeks (\(n = 7\)/group) as follows: (1) control group, received 10% tween 80 (2 ml);\(^19\),\(^21\) (2) NASH group, received a high-fat liquid diet (HFD, 10 ml/kg)\(^22\) and 10% tween 80; (3) NASH + Chal group, received the HFD and *trans*-chalcone (20 mg/kg dissolved in 2 ml 10% tween 80).\(^19\) Rats received HFD by oral gavage at 6:00 p.m. once a day for six weeks. Moreover, *trans*-chalcone (Sigma-Aldrich, Germany, Product Number: 136123, 97%) or 10% tween 80 was administered by oral gavage at 8:00 a.m. once a day for two weeks (from fifth to sixth week). Treatment with *trans*-chalcone was performed after induction of simple
steatosis. Two rats were gavaged with HFD for four weeks to confirm the induction of simple steatosis by four weeks of HFD feeding. At the end of treatments, overnight fasted (8 hours) rats were sacrificed under deep anesthesia.\textsuperscript{19} Then, blood samples were collected, livers photographs were taken by a digital camera (Canon EOS 6D, Japan), and liver samples were removed. Liver samples were frozen for gene expression assay or fixed at 10% neutral buffered formalin for histological studies.

Biochemical measurements
Serum levels of liver injury markers, including alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT)\textsuperscript{23} were measured employing commercial kits (Pars Azmoon, Tehran, Iran). Fasting serum insulin concentration was determined through a rat specific insulin ELISA kit (Mercodia, Uppsala, Sweden). Moreover, fasting plasma glucose level was measured by a digital glucometer (Glucu Sure, Star, Taiwan). Then, insulin resistance was estimated by calculation of the homeostasis model assessment parameter of insulin resistance (HOMA-IR) through the following formula:\textsuperscript{24}

\[
\text{HOMA-IR} = \frac{\text{fasting serum insulin (µU/ml)} \times \text{fasting plasma glucose (mmol/l)}}{22.5}
\]

Real-time PCR
Real-time PCR was done to measure the expression levels of miR-122, 21, 34a, and 451 in the liver samples. In this regard, total RNA extraction and complementary DNA synthesis steps were done as previously described.\textsuperscript{21} SYBR Green PCR Master Mix (Fermentase, Germany) was used in real-time PCR reactions.\textsuperscript{21} Amplifications were performed in a rotor gene 6000 real-time PCR machine (Corbett, Life science, Sydney, Australia). Target genes were normalized with miR-191 (housekeeping gene). Finally, the 2\textsuperscript{−}Δ\textsuperscript{(ΔCt)} method was used to calculate the relative quantitative expression level of each target miRNA.\textsuperscript{21} Sequences of primers are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Target sequence a</th>
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<tbody>
<tr>
<td>rno-miR-122-5p</td>
<td>MIMAT0000827</td>
<td>UGGAGUGUGACAAUGGUUUG</td>
</tr>
<tr>
<td>rno-miR-21-5p</td>
<td>MIMAT0000790</td>
<td>UAGCUUAUCAGACUGUUGA</td>
</tr>
<tr>
<td>rno-miR-34a-5p</td>
<td>MIMAT0000815</td>
<td>UGGCAGUGUCUUAGCUGGUUGU</td>
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<td>rno-miR-451-5p</td>
<td>MIMAT0001633</td>
<td>AAACCGGUACAUCCACUGAGU</td>
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<tr>
<td>rno-miR-191-5p</td>
<td>MIMAT0000866</td>
<td>CAACGGAAUCCCCAAAAGCAGCU</td>
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\textsuperscript{a} Sequence was derived from miRBase (www.mirbase.org)

Histological assessment
Formalin-fixed liver samples were embedded in paraffin, stained with hematoxylin and eosin, and analyzed blindly by an experienced pathologist under a light microscope (Axioskop2; Carl Zeiss MicroImaging Inc., Germany). The degree of steatosis was scored as follows: (0) no steatosis, (1) microsteatosis, (2) microsteatosis plus mild macrosteatosis, (3) high macrosteatosis. The inflammation was also scored as follows: (0) none, (1) mild infiltration of lymphocytes into the portal triad, (2) high infiltration of lymphocytes into the portal triad, (3) high infiltration of lymphocytes all over the liver. Hepatocyte ballooning was scored as follows: (0) none, (1) low, (2) high.\textsuperscript{20}
Statistical analysis
Statistical analyses were done using SPSS 16 software (SPSS Inc., Chicago, IL, USA). Analyses of data obtained by real-time PCR or biochemical measurements were performed by one-way ANOVA with Tukey's post-hoc test. These data are shown as mean ± SEM. Besides, histological data were analyzed using Kruskal-Wallis and Mann Whitney test as post-hoc analysis. Histological data are expressed as median (min-max). Statistical significance was defined as a p-value <0.05. The graphs were plotted using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) software.

Results

trans-Chalcone and biochemical parameters
As shown in Table 2, NASH induction significantly (p<0.001) increased the HOMA-IR score as well as serum levels of ALP, AST, and ALT. Conversely, trans-chalcone significantly (p<0.001) inhibited HFD-induced changes in HOMA-IR score and serum levels of liver injury markers (ALP, AST, and ALT) in rats of NASH + Chal group compared with the NASH group (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NASH</th>
<th>NASH + Chal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IR</td>
<td>6.36 ± 0.183</td>
<td>16.77 ± 1.595***</td>
<td>5.653 ± 0.138###</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>459.667 ± 13.776</td>
<td>933.5 ± 7.756***</td>
<td>581 ± 3.183###</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>102.6667 ± 4.255</td>
<td>161.75 ± 1.264***</td>
<td>92.8 ± 0.771##</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>41.66667 ± 1.453</td>
<td>80 ± 0.598**</td>
<td>45 ± 0.471##</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *p<0.05 compared with the control group, **p<0.01 compared with the control group, ***p<0.001 compared with the control group, ###p<0.001 compared with the NASH group. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NASH, non-alcoholic steatohepatitis.

trans-Chalcone and hepatic miRNAs
Hepatic expression levels of miR-122, 21, 34a, and 451 are shown in Figure 1. Induction of NASH significantly (p<0.001) decreased miR-122, 21, and 451 expression levels. Besides, expression levels of miR-34a were significantly (p<0.001) higher in the NASH group than the control group. On the contrary, trans-chalcone significantly (p<0.001) inhibited NASH-related changes in hepatic expression levels of miR-122, 21, 34a, and 451 (Figure 1 a-d).

trans-Chalcone and NASH-related histological changes
Development of simple steatosis after four weeks and NASH after six weeks of HFD feeding were confirmed by macroscopic and microscopic studies. In this regard, the livers of rats with NASH and simple steatosis became yellow. However, trans-chalcone protected the livers of rats in the NASH + Chal group against this color change (Figure 2a). In the histological studies, livers of rats with simple steatosis showed lipid droplets accumulations. Also, livers of rats in the NASH group showed accumulations of lipid droplets, severe infiltration of lymphocytes, and hepatocellular ballooning. On the
contrary, trans-chalcone reversed NASH-related histological changes in the NASH + Chal group compared with the NASH group (Table 3 and Figure 2b).

Table 3. Histological results of study groups (control: received 10% tween 80 for two weeks, NASH: received the high-fat liquid diet for six weeks and 10% tween 80 for two weeks, NASH + Chal: received the high-fat liquid diet for six weeks and trans-chalcone for two weeks).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NASH</th>
<th>NASH + Chal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>0 (0-1)</td>
<td>2 (2-3) *</td>
<td>1 (1-2) #</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0 (0-1)</td>
<td>2 (1-3) *</td>
<td>1.5 (1-2)</td>
</tr>
<tr>
<td>Ballooning</td>
<td>0 (0-0)</td>
<td>2 (0-2) *</td>
<td>1 (0-2)</td>
</tr>
</tbody>
</table>

Values are expressed as median (min-max). * p<0.05 compared with the control group, # p<0.05 compared with the NASH group. Chal, trans-chalcone; NASH, non-alcoholic steatohepatitis.

Discussion

In this study, two weeks of treatment with trans-chalcone could inhibit the transition from simple steatosis to NASH in rats fed the HFD for six weeks. This chalcone reduced serum levels of liver injury markers (ALP, AST, and ALT) and also improved histological abnormalities in the livers of HFD-fed rats, including lipid accumulation, infiltration of lymphocytes, and hepatocellular ballooning. Consistent with these findings, a recent study indicated that co-administration of trans-chalcone with HFD for six weeks could protect HFD-fed animals against NASH induction.20

Insulin resistance is strongly linked to severe forms of NAFLD.25 In the current study, trans-chalcone inhibited insulin resistance in HFD-fed rats. Consistent with this finding, the protective effects of this chalcone against hyperglycemia, hyperinsulinemia, and insulin resistance have been suggested by previous studies.19,26 Hence, it seems that trans-chalcone has a protective role against NAFLD progression by its inhibitory effect on insulin resistance.

The progression of NAFLD/ NASH is linked to the altered hepatic levels of specific miRNAs. Dysregulation of these miRNAs can affect inflammation, insulin signaling, response to tissue injury, and lipid metabolism.27 In the current study, trans-chalcone had a hepatoprotective role against NAFLD progression by inhibiting changes in the hepatic expression levels of miR-122, 21, 34a, and 451. It has been known that miR-122, a liver-specific miRNA, accounts for 70% of the total miRNA pool in the liver.28 Loss of this miRNA in knockout mice results in lipid accumulation, inflammation, fibrosis, and finally, the development of hepatocellular carcinoma.29 In the present study, the expression of hepatic miR-122 was lower in the NASH group. However, trans-chalcone could inhibit this HFD-induced change. Consistent with this study, Cheung et al.27 indicated that NASH patients had decreased miR-122 levels. Moreover, in a study by Yang et al.,30 reduction of miR-122 led to insulin resistance, and licorice flavonoid could reverse this change.

In another previous study, HFD increased the hepatic expression levels of miR-122. In contrast, lychee pulp phenolics ameliorated HFD-induced change in miR-122 expression and alleviated steatosis in the liver of HFD-fed animals.31 Similarly, proanthocyanidins could reverse the up-regulation of miR-122 in dyslipidemic obese rats.32 Therefore, it seems that miR-122 has different roles in HFD-induced dyslipidemia/ simple steatosis and NASH conditions. In the present study, HFD-
induced NASH was linked to the down-regulation of hepatic miR-122 levels. However, trans-chalcone could reverse this HFD-induced change. In this study, NASH induction was associated with decreased expression levels of miR-21. There are controversial results about the link between HFD feeding and hepatic miR-21 levels. Blasco-Baque et al. indicated that miR-21 expression was negatively correlated with the hepatic triacylglycerol content during metabolic adaptation of the liver to HFD. Furthermore, Ahn et al. suggested that lycopene could inhibit liver steatosis through the up-regulation of hepatic miR-21. However, a previous study indicated that this miRNA is overexpressed in NASH condition. It seems that the hepatic expression level of miR-21 is dependent on both animal model and diet, and trans-chalcone could reverse its pathologic decrease in NASH.

The next part of the present study suggested the ability of trans-chalcone to reverse HFD-induced up-regulation of hepatic miR-34a in rats with NASH. Similarly, the inhibitory effects of trans-chalcone on hepatic expression levels of this miRNA in normal and HFD-fed rats were observed in recent studies. On the other hand, miR-34a is overexpressed in NASH, steatosis, and insulin resistance. A previous study by Choi et al. suggested that overexpression of this miRNA in the liver tissue caused obesity-mimetic consequences. Conversely, antagonism of miR-34a could inhibit liver inflammation and glucose intolerance in obese animals. Furthermore, dysregulation of miRNA-451 is involved in NASH development. In the current study, NASH development was linked to the down-regulation of this miRNA, and trans-chalcone could reverse this alteration. In this regard, the reductions of hepatic expression levels of miR-451 were observed in NASH patients and a mouse model of HFD-induced NASH. It seems that miR-451 negatively regulates inflammation and gluconeogenesis in the liver. Zhuo et al. suggested that overexpression of hepatic miR-451 improved glucose intolerance in HFD-fed animals. Moreover, this miRNA negatively regulated the production of the inflammatory cytokines. It seems that the anti-NASH effect of trans-chalcone is partly mediated by inhibition of the HFD-induced changes in hepatic levels of miR-34a and 451.

Conclusions
Protective role of trans-chalcone against the progression form simple steatosis to NASH is linked to the ability of this chalcone to inhibit dysregulation of miR-122, 21, 34a, and 451 in the liver of HFD-fed animals.

Ethical Issues
All animal experiments were approved by the Ethics Committee of Tabriz University of Medical Sciences (Approval ID: IR.TBZMED.VCR.REC.1397.278).

Conflict of Interest
All authors stated no conflicts of interest.

Acknowledgment
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References
1. Shojaei Zarghani S, Abbaszadeh S, Alizadeh M, Rameshrad M, Garjani A, Soraya H. The Effect of Metformin Combined with Calcium-Vitamin D3 Against Diet-Induced


32. Baselga-Escudero L, Arola-Arnal A, Pascual-Serrano A, Ribas-Latre A, Casanova E, Salvado MJ, et al. Chronic administration of proanthocyanidins or docosahexaenoic acid reverses the increase of miR-33a and miR-122 in


Figure 1. Hepatic levels of miR-122 (a), 21 (b), 34a (c), and 451 (d) in the study groups (control: received 10% tween 80 for two weeks, NASH: received the high-fat liquid diet for six weeks and 10% tween 80 for two weeks, NASH + Chal: received the high-fat liquid diet for six weeks and trans-chalcone for two weeks). Data are expressed as mean ± SEM. ***p<0.001 compared with the Control group, ###p<0.001 compared with the NASH group. Chal, trans-chalcone; NASH, non-alcoholic steatohepatitis.

Figure 2. Liver morphology (a) and hematoxylin and eosin-stained liver specimens (b) in study groups (control: received 10% tween 80 for two weeks, NASH: received the high-fat liquid diet for six weeks and 10% tween 80 for two weeks, NASH + Chal: received the high-fat liquid diet for six weeks and trans-chalcone for two weeks). Long arrows show inflammatory cells infiltrations and short arrows show lipid droplets. Steatosis and severe lymphocyte infiltration were observed in the NASH group. However, these histological features of NASH were reduced in NASH + Chal group. Moreover, accumulations of lipid droplets were observed in the livers of rats with simple steatosis. Scale bar: 20 μm. Chal, trans-chalcone; CV, central vein; NASH, non-alcoholic steatohepatitis.