Isorhamnetin exerts antifibrosis effects by attenuating PDGF-BB–induced HSC-T6 cells activation via suppressing PI3K-AKT Signaling Pathway, a new remedy for liver fibrosis

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Research Article

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Abstract

Background

Liver inflammation and fibrosis are the results of chronic liver injuries, which activated myofibroblasts in the liver to produce more proteins in the extracellular matrix. The origin of myofibroblasts is quiescent hepatic stellate cells. Isorhamnetin as a natural flavonoid widely distributed in fruits and vegetables has anti-inflammatory activity and avoids cell proliferation and migration.

Objectives

We studied the effect of Isorhamnetin as an antifibrotic following stimulation by PDGF- BB in the HSC-T6 cells, as well as related mechanisms in vitro.

Methods

First of all, we used PDGF- BB to activate the HSC-T6 cells. After that, we used Isorhamnetin to treat activated cells for 24h. Finally, we compared the mRNA expression amount of collagen1 (COLA1), Alpha Smooth Muscle Actin (α-SMA), and level of phosphorylated AKT protein with our control group.

Results

The obtained data revealed the expression of the COLA1, α-SMA genes, as well as the amount of phosphorylate-AKT protein in the cells treated with PDGF-BB, significantly rose in comparison with our control. In addition, 75 and 100 µM concentrations of Isorhamnetin markedly declined the COLA1, α-SMA expression, and the phosphorylated AKT protein in the HSC-T6 cells.

Conclusions

Isorhamnetin significantly decreased HSC-T6 activation by preventing the PI3K-AKT cell signaling and reducing the expression of COLA1, α-SMA within liver fibrosis in vitro, so Isorhamnetin can prohibit liver fibrosis progression. In terms of these data, Isorhamnetin use can be recommended as a helpful therapeutic factor to treat liver fibrosis.

1. Background

When chronic liver diseases last, due to different factors such as metabolic disorders, viral infection, drugs, or immune disease, lead to liver fibrosis (1). The result of sustaining chronic liver disease is progression to fibrosis, which eventually leads to cirrhosis and the development of hepatocellular
carcinoma. (2, 3). Liver fibrosis is a global problem today. Immunosuppressive factors or antiviral drugs may prohibit the progression of liver fibrosis at the first stages. However, efficient treatments are not available for many patients and proper treatment is still far from satisfactory (4, 5). Definite suppressive and therapeutic actions have not been found yet, so it is necessary to detect antifibrotic elements. Deposition of extracellular matrix (ECM) and mass production of Collagen type I (COLA1) are important signs of Liver fibrosis onset and progression (6, 7).

The major origin of ECM is hepatic stellate cells (HSCs). These cells are inactive during physiological situations and store retinoid and vitamin A (8, 9). In hepatic fibrosis, HSCs that have been activated, express much amount of fibrogenec genes such as Collagen1 α (COL 1α) and Alpha Smooth Muscle Actin (α-SMA). Increased producing COL 1α and α-SMA is known as markers which show HSCs have become activated. (10).

HSCs can be activated by various factors and change to myofibroblast-like shape and express much COLA1 and α-SMA (11). Proliferative and Fibrogenic elements that raise the proliferation of HSCs and the generation of ECM are made by activated HSCs (12). Preventing of HSC activation and proliferation, inhibiting equal generation, and degradation of ECM, all are the most main agents for liver fibrosis treatment. Diverse inflammatory cytokines and growth factors can create the activation of HSCs. PDGF-BB (platelet-derived growth factor) is one of the most potential proliferative cytokines which can activate HSCs. After the liver injury, PDGF-BB expressions and related receptors (PDGF-R) are increased. (13) PI3K (The phosphatidylinositol3-kinase)-PKB/Akt (protein kinase B) is a signal transduction pathway that controls the survival and growth of cells in response to extracellular signals. PI3K produces 3'phosphoinositides to engage aim proteins (Akt which is located in the membrane of the plasma). Akt as a Ser-Thr kinase is an effector of PI3K which begin a kinase cascade that ends up controlling cellular activities (14, 15). Past studies have found the PI3K/Akt signaling pathway has the ability to impose proliferation of HSC, reduce HSC apoptosis, as well as regulate the liver fibrosis progression through its impact on the degradation of ECM (16, 17). Past studies show that phosphoinositide 3-kinase (PI3K) proliferative kinase signaling pathway could be activated in myofibroblast-like cells of rats and expression levels of COLA1 and α-SMA, an important marker of activated HSCs, are upregulated (17, 18). In contrast, various studies indicated PI3K activities inhibition represses proliferation of cells and COLA1 and α-SMA expressions in activated HSCs (19). Therefore, activation of PI3K plays a significant role in regulating HSC activation. Isorhamnetin, as a flavonoid, has antioxidant, anti-inflammatory, and antitumor activity. Isorhamnetin is gotten from the plant Hippophae rhamnoides L which owns hepatoprotective impacts by restricting autophagy of hepatocytes and increasing apoptosis (20). Lately, Yang et al stated Isorhamnetin weakens CCl4-induced hepatic fibrosis by reducing TGF-β Mediated SMAD Signaling (21). But, whether Isorhamnetin can reduce hepatic fibrosis in the PDGF-BB induced HSC-T6 Rat Hepatic Stellate Cell Line is unclear, and the involvement of through PI3K-AKT signaling pathway has not been investigated. The HSC-T6 rat hepatic stellate cell line is a valuable cell model for study of metabolism based on their similar retinoid phenotype to primary cells. There is currently little information on the mechanism of Isorhamnetin action in liver fibrosis, especially in the PI3K-AKT signaling pathway.
2. Objectives

In this study, we investigated Isorhamnetin effects as a factor on treating liver fibrosis through reducing COLA1 and α-SMA expressions in the HSC-T6 cells, and also we checked related signaling pathway. The purpose of our study was to check Isorhamnetin influence on PDGF-BB-induced liver fibrosis and also measure the expression of COLA1 and α-SMA in the HSC-T6 cell line, focusing on the involvement of the PI3K-AKT signaling pathway.

3. Materials And Methods

3.1. Chemicals and Reagents:

PDGF-BB specific for Cell culture (Sigma-Aldrich, Germany), MTT (3-(4, 5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide), FBS, DMEM, penicillin, and streptomycin antibiotics were gotten. (Sigma-Aldrich, USA). (FBS) was provided from Gibco (USA).

3.2. Cell Culture Conditions

The HSC-T6 cells were cultured in DMEM containing 10% FBS, 50 mg/ml streptomycin, 50 U/ml penicillin. To supply the best situation for the cells, we changed the culture medium every 24 h until the cells reach at least 80% confluence. Cells were incubated at 37°C in an atmosphere of 5% CO2.

For activating HSC-T6 cells, cells were kept in serum-starved in FBS-free DMEM condition for 24 h, later exposed to 10ng/ml PDGF-BB (22, 23).

3.3. Effect of various concentrations of Isorhamnetin on the viability of HSC-T6

First, the MTT assay technique was done to evaluate Cell viability. The HSC-T6 cells without PDGF-BB treatment were cultured in a 96-well (density of 2×10^6 cells/ml). After one night, several concentrations of Isorhamnetin (25, 50, 75, 100, 125, and 150 µM) were used to treat the cells for 24h. Next, the supernatant of the cells was removed. Then the cells were treated and incubated by MTT for 4 h. later, DMSO was used to solve the formazan crystals. Finally, an ELISA plate reader was used to read the absorbance at 570 nm.

3.4. Treatment of PDGF-BB activated HSC-T6 cell with different concentrations of Isorhamnetin

To examine the inhibitory impacts of Isorhamnetin on PDGF-BB-induced HSC-T6 cells activation, Isorhamnetin in various concentrations (25, 50, 75, 100, 125, and 150 µM) was used to treat the cells for 24 hours. Real-time PCR was done to evaluate the genes expression involved in hepatic fibrosis, and also western blot was used to check the phosphorylated AKT in cells activated with PDGF-BB and treated with
different concentrations of Isorhamnetin. Control had quite equal situation like the treated groups in the laboratory, but were treated with nothing.

3.5. Measurement of genes expression via using Real-time PCR technique

After the treatments, first, we used an RNA extraction kit (QIAGEN Company, UK) to isolate the total RNA from the cells. After that, the cDNA synthesis process (QIAGEN Company, UK) was done by using oligo-dT primers and random hexamer. We did real-time PCR by ABI Applied Biosystems to have quantification. The used primers are written in Table 1. GAPDH was used as the internal control.

3.6. Western blotting

BCA Protein Assay Kit (Merck Millipore, GERMANY) was used to measure total protein concentrations. Samples were separated by using 10% gel electrophoresis. Then transferred to nitrocellulose membranes with electrophoresis which were incubated with antibodies (both primary and secondary antibody respectively). After that, a ChemiDoc system was used to detect signals. Finally, Image J software which check the proteins expression was utilized.

3.7. Statistical analysis

One-way analysis of variance ((abbreviated one-way ANOVA) was used to reveal the significance of the differences among treatment groups. To define the significance of differences between the groups, multiple post hoc tests (Tukey's) was done. Results are statistically notable at P values of less than 0.05 (p < 0.05).

4. Results

4.1. The Impacts Of Various Concentrations Of Isorhamnetin On The Hsc-t6 Cells Survival.

The effects of several concentrations of Isorhamnetin (25, 50, 75, 100, 125, and 150 µM) upon cells viability were checked by using the MTT assay. The percentage of viability at 125 µM of Isorhamnetin declined remarkably to the control (***p < 0.001, Fig. 1), thus concentrations less than IC50 were chosen to continue the experiment.

4.2. Impacts of various concentrations of Isorhamnetin upon fibrogenic genes expression in PDGF-BB treated HSC-T6 cell.

Considering the phenotype and appearance, converting quiescent HSC cells to fibrogenic Myofibroblasts is a main mark of these cells' activation. Activated cells have spindle-shaped and are more elongated and
convert to Myofibroblasts, but Inactive cells are virtually round (24). Images of Cells shape in the control group, treated with PDGF-BB and treated with Isorhamnetin, is shown. (Fig. 2A, B, and c).

Modulation of COLA1 gene expression was checked in the activated cells treated with 25, 50, 75, and 100 µM Isorhamnetin. Obtained data from RT-PCR indicate there was a significant decline in the mRNA expression for COLA1 and α-SMA in response to 75 and 100 µM Isorhamnetin in contrast to the cells treated only with PDGF-BB (control group) (2 fold-change vs. 3.3, and 1.7 fold-change vs 3.3## p < 0.01, ### p < 0.001, Fig. 2.D). Also, Real-time PCR data show that the mRNA expression for α-SMA considerably declined in response to 75 and 100 µM Isorhamnetin in comparison with the cells treated only with PDGF-BB (2 fold-change vs. 4, and 1.4 fold-change vs 4## p < 0.01, ### p < 0.001, Fig. 2.E)

The amount of mRNA expression of COLA1 and α-SMA genes reduced but not remarkably with 50µM and also did not change in response to 25µM, so according to these data, the effect of Isorhamnetin was dose-dependant manner.

**4.3. Impact of Isorhamnetin treatment upon the PDGF-BB - induced phosphorylated AKT**

Phosphorylated AKT was assessed to study the PI3K-AKT signaling pathway in the HSC-T6 cells with 75 and 100 µM Isorhamnetin and also to find out that Isorhamnetin could decline the PDGF-BB induced PI3K-AKT signaling pathway to prevent hepatic fibrogenesis. Western blot data showed that 75 and 100 µM Isorhamnetin treatment significantly suppressed the phosphorylated AKT level compared to treatment with the control group. (2.1 fold-change vs. 3.8, and 1.6 fold-change vs. 3.8) respectively (## p < 0.01, ### p < 0.001, Fig. 3). These results showed the modulation of the PI3K-AKT signaling pathway by Isorhamnetin.

**5. Discussion**

If liver chronic injury continues due to different factors like metabolic diseases, viral, and toxins, the result is Hepatic fibrosis. If this condition progress, finally leads to cirrhosis, liver failure, and cancer (22). Extreme deposition of ECM proteins, like COLA1 and α-SMA, leads to liver fibrogenesis following the HSCs activation. These cells are not activated in the usual liver. During liver injury, they change, become activated, and convert to myofibroblast cells, which lead to liver fibrosis. (23). Akt phosphorylation is with HSC replication increase, mRNA levels, and COLA1 protein levels. Studies have shown that inhibition of PI3-kinase by inhibition of Akt by adenovirus with the mediated transmission of a dominant-negative form of Akt significantly declined HSC proliferation and production of COLA1 and α-SMA protein levels (24). After years of research and studies to treat liver fibrosis due to its prevalence in the world, no suitable drug has been introduced for the complete treatment of this disease, and on the other hand, the exact mechanism of liver fibrosis has not been fully determined. Therefore, studying possible mechanisms of this disease and introducing of factors that can improve liver fibrosis as a drug is very helpful. According to many studies, PDGF-BB is one of the main factors that can activate the genes involved in liver fibrosis development. Inhibition of those genes and reduction of their signaling pathway
can be a useful method to treat liver fibrosis (25). However, it has recently been observed that the use of flavonoid compounds can have beneficial effects on liver fibrosis (20), but its mechanisms of action have not been studied in vitro, so we investigated the effect of Isorhamnetin as a treatment for hepatic fibrosis on the expression of the important genes involved in the pathogenesis of liver fibrosis (COLA1 and α-SMA). Furthermore, we studied the influence of the PI3K-AKT signaling pathway in this disease. In this experiment, we had investigated whether isorhamnetin could reduce HSC-T6 cells activation in vitro. Our aim was to study Isorhamnetin effect on reducing the PDGF-BB – induced HSC-T6 cells activation and measuring the change in expression of COLA1 and α-SMA. Also studying whether the PI3K-AKT signaling pathway was involved in liver fibrosis. Isorhamnetin remarkably declined the expression of the important fibrogenic genes (COLA1 and α-SMA) in activated HSC-T6 cells. Isorhamnetin meaningfully repressed PDGF-BB-induced fibrosis and its inhibitor impact on HSC-T6 cells activation is maybe due to its inhibition effect on the PI3K-AKT signaling pathway. Yuqing and his colleagues in their study showed that stimulation of HSC by platelet-derived growth factor (PDGF) could lead to cell proliferation and the progression of fibrogenesis (26). Erwin et al. and their colleagues showed that during fibrosis, HSC cells undergo a complex activation process with increasing in proliferation, deposition of ECM, and study p70S6 K's role in HSC proliferation, controlling cell cycle, and COLA 1 and α-SMA expression. They showed that PDGF stimulated p70S6K phosphorylation, and rapamycin inhibited p70S6K phosphorylation but had no effect on Akt phosphorylation. Rapamycin treatment did not influence the mRNA level of COLA1 and α-SMA, but decreased the secretion of the COLA1 and α-SMA protein. Their results showed that p70S6K has an important role in HSC proliferation, controlling cell cycle and collagen expression (27). Ning Liu et al., in a study, investigated the protective effect of Isorhamnetin in preventing the progression of liver fibrosis in the presence of carbon tetrachloride (CCl4) in mice. Smad3 and p38 MAPK protein expression was assayed by Western blotting. Results showed that isorhamnetin inhibits HSC activation, deposition of ECM, and autophagy. These influences were linked with Transforming growth factor beta 1 (TGF-β1) as messenger signaling regulation and mitogen-activated protein kinase p38 (MAPK). Actually, Isorhamnetin has an effective protective for liver fibrosis by reducing ECM production and inducing autophagy by inhibiting TGF-β1/Smad signaling pathway .(28) Reducing HSCs activation through controlling the TGF-β1/Smads and PI3K/Akt signaling pathways is possible. So signaling pathways are good targets to treat diseases (29).

6. Conclusion

In conclusion, Isorhamnetin inhibitory effect against hepatic fibrosis through reduction of HSC-T6 cells activation may come from its debilitation of the PI3K-Akt signaling pathway. Understanding the mechanisms of hepatic fibrosis has progressed vastly, but yet, no proper remedy for liver fibrosis has not been found, and still, finding helpful antifibrotic remedies is a problem in medicine. Our study shows Isorhamnetin can inhibit the PI3K-Akt signaling pathway and profibrogenic genes expression (COLA1 and α-SMA) in PDGF-BB – inducing HSC-T6 activation. Overall, our findings suggest that Isorhamnetin can be introduced as a possible anti-fibrotic factor and a remedy to reduce and treat chronic liver diseases.
Declarations

Ethics approval to participate

Ethical clearance was not needed and not sought from the Review Board of Ahwaz Jundishapur University of Medical Sciences, because the study was done on cell lines in vitro, and did not use human samples.

Conflict of Interest

The authors have no conflicting financial interest.

Publication consent

No applicable.

Potential Conflict of Interest

The authors have no conflicting financial interest.

Authors' contributions

ESH and MR designed the study. EM, HBN and ESH performed all assays. MCH and MR analyzed the data. ESH wrote the first draft. HBN and EM revised the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The data in this study are present from the corresponding author if requested.

References


Tables

Table 1. Primer sequence for RT-PCR
<table>
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<th>Gene</th>
<th>Primers</th>
<th>Sequence</th>
<th>Size of PCR Product (bp)</th>
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<tr>
<td>COLA1</td>
<td>R. 5'-ACCATCATTCCACGAGCA-3'</td>
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<tr>
<td>α-SMA</td>
<td>F. 5'-TGGTGTCACCCACAATGTCC-3'</td>
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<tr>
<td>α-SMA</td>
<td>R. 5'-ATCTCACGCTCAGCAGTAGT-3'</td>
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<tr>
<td>GAPDH</td>
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<td>181</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>R. 5'-CTTCCCGTTCTCAGCCTGA-3'</td>
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</tbody>
</table>

RT-PCR: Reverse transcription-polymerase chain reaction; COLA1: Collagen type I; α-SMA: Alpha-smooth muscle actin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; F: Forward; R: Reverse

**Figures**
Figure 1

Isorhamnetin effects in diverse concentrations on the HSCs-T6 survival. The MTT assay technics data reveal the viability of the cells after 24 h. the results are presented with mean ± SEM. Analysis was done by Tukey test, one-way ANOVA, and GraphPad Prism 8 program. (**p <0.01, ***p <0.001).
Figure 2

Effects of different concentrations of Isorhamnetin on the COLA1 and, α-SMA genes expression in Isorhamnetin-treated cells (A). α-SMA expression in Isorhamnetin-treated cells (B). Provided data are with the mean ± SEM and 3 replicates. They are shown as fold changes in expressions compared to the control. GAPDH was the reference gene. (** p <0.01, **** p <0.0001 vs treated control, ## p <0.01, ### p <0.001 vs treated).
Figure 3

(A) analysis of PI3K-AKT Signaling Pathway by Western blot. The cells were treated with 25, 50, 75, and 100μM Isorhamnetin. (B) The relative PI3K-AKT level was expressed as the ratio PI3K-AKT / GAPDH. Analyzing the bands was with ImageJ software. The results were done with ANOVA and Tukey’s test. Provided data are the Mean ± SEM of three replicates; ****p < 0.0001, vs vehicle-treated control, ## p < 0.01, ### p < 0.001 vs PDGF-BB alone.