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Investigation the effect of Silymarin on Cyclooxygenase - 2(Cox-2) Gene Expression in HCV infected Cell Line

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ABSTRACT

Introduction
Hepatitis C infection causes an increase in oxidative stress in infected cells, followed by Cyclooxygenase-2 (COX-2) gene expression. *Silybum marianum*, is one of the most widely used medicinal plants that has been used to control and reduce hepatitis manifestations, which contains silymarin –the effective component- that could protect liver cells.

Methods
In this study, Huh7 cell line originated from liver were infected by HCV virus model and then treated by silymarin. In order to investigate the anti-oxidative effect, the expression of COX-2 gene that plays central role, was measured in serial concentration of silymarin by developed quantitative RT-Real time-PCR.

Results
Findings of the study showed that silymarin could control expression of COX-2 as well as oxidative stress. It was showed that, silymarin at the concentration of more than 200µM could reduce the expression of COX-2 and oxidative stress, and also it was concluded that silymarin above 300µM, could likewise have therapeutic effect on increased oxidative stress as well as COX-2 gene expression in HCV infected cells.

Conclusion It was concluded that inhibition of COX-2 by silymarin has been considered as a promising and effective strategy for prevention and treatment of HCV and liver injuries and cancer.

Keyword: Hepatitis C virus, Cox-2, Oxidative Stress, Silymarin.

Introduction

Hepatitis C virus (HCV) is one of the most important causes of chronic hepatitis all around the world. According to the World Health Organization (WHO), more than 170 million peoples are infected with HCV and may suffer from the liver injuries and disease. HCV infection causes progressive liver infection and is likely to eventually lead to liver fibrosis, cirrhosis and hepatocellular carcinoma [1, 2].

Based previous findings it has been showed that HCV infection leads to rise in oxygen free species (ROS) and an increase in oxidative stress, followed by an increase in COX-2 gene expression that plays the central role in this injuries. On the other hand, COX-2 cause the induction of prostaglandin E2 (PGE2) as well as vascular endothelial growth factor (VEGF) production. VEGF has an inhibitory effect on apoptosis and may play role in immortalization. Both PGE2 and VEGF also play an inductive role in angiogenesis that may leads to liver cancer.

COX was recognized as the major enzyme regulating prostaglandin production in the 1980s by Bailey and Needleman [3]. Until 1991, only one isozyme of this

enzyme or Constitutive enzyme was known. In 1991, Simmons et al., discovered COX mRNAs whose expression in mouse and chicken fibroblasts increased the responses to src and tumor-inducer phorbolesters, respectively [4]. Cox-2 enzyme increases the ability of cancer cells to invade surrounding cells and tissues [5]. When Cox-2 is overexpressed in cancer cell lines, prostaglandin production is increased and the cellular invasiveness would be increased [6].

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and ibuprofen bind to the hydrophobic channel of silicoxygenase isozymes and has inhibition effect [7]. Aspirin is the only inhibitor of COX through covalently acetylation binding [8].

According to several reports, the use of some medicinal plants could be effective in treating hepatitis. One of the most widely used medicinal plants to control of hepatitis is ancient Greece and Iran was *Silybum marianum*, which contains silymarin that could protect liver cells against toxins, and may increase the body's defense system against viruses [9, 10].

This plant is abundant in the pastures of South Australia, Europe and North America as well as central Iran. The seeds of this plant has been used to formulate medicine as a liver protection medicine. Silybin and other flavonoids in *Silybum marianum* seeds have also been reported to have antioxidant, lipid, and anti-fibrotic properties in patients [11]. The results of several studies indicate that Silymarin exerts a protective effect on the liver by preventing the entry of toxic substances into the cell by acting on the membrane of liver cells, affecting Liver cells accelerate protein synthesis, and by increasing the concentration of glutathione (detoxification agent) by preventing the production of LDL oxide [12].

Although to date, several studies have been conducted on the effects of herbal medications on HCV infection and disease, but the exact mechanisms of altered expression of lipid factors have not been investigated. Silymarin extract may have effective role in controlling hepatitis C infection due to controlling oxidative stress and COX-2 gene expression. In the current study, we focused on COX-2 gene expression that have central role and effect on HCV pathogenesis and controlling liver injuries.

Material and Methods

The study was approved by the Medical Ethics Committee of Tarbiat Modares University (Code No: IR. MODARES.REC.1396.4321).

Cell culture

In this study, Huh7 cell line that is originated from human immortalized liver cells, was preferred and used. One of the most important applications of Huh7 cells is to identify and evaluate the effects of toxins and drugs against HBV and HCV [13, 14]. Live cell counts, Huh7 cell passage, and environment changes were performed sensibly and daily. Also the MTT assay was used to assess cell mortality and viability in different concentrations of Silymarin.

pFL-J6|JFH1 Plasmid amplification and extraction

pFL-J6|JFH1 plasmid has been received from addgene, USA. In order to perform transformation, the purified plasmid was introduced into the E.coli bacterial cell by heat shock as well as MgCl2 method. Since the plasmid had antibiotic resistance genes, the bacterial cell that had received plasmid could be grown in antibiotic medium. In order to ensure the entry of the plasmid into the bacterium and the plasmid evaluation, it was also extracted from the bacterium using the Qiagen miniprep kit. Plasmids extracted in agarose gel were evaluated. The Xba-1 restriction enzyme has been used for digestion and evaluation of the purified plasmid. Mung bean nuclease was also used to capture blunt ends of the digested plasmids.

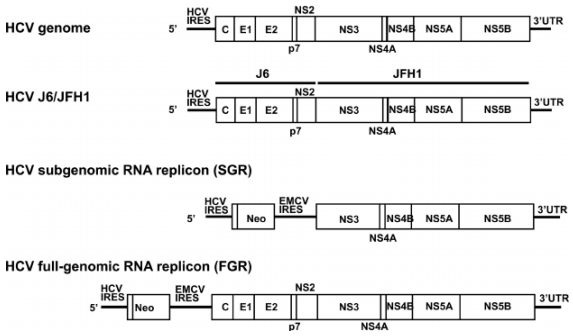

Figure 1: Schematic diagram of pFL-J6|JFH1 plasmid and its replicon.

Cells Transfection

Briefly the Huh-7 cells were cultivated and prepared 48 hours before transfection. To prepare the HCV infection model, pFL-J6|JFH1was purified and in-vitro transcription was performed by commercial available kit (MEGA script T7, Thermofisher, USA), and then the viral RNA was purified and transfected to the prepared cells by lipofectamine 2000 method.

Preparation of Silymarin concentration and treatment

The amount of one grams of silymarin powder (Sigma, Germany) was dissolved gradually in 5 ml of ethanol at room temperature, thus a solution with a concentration of 0.2 g/ml was prepared, the solution was kept away from light and in col place. To provide concentrations of 200, 400 and 800 µM, amount of 5, 10 and 20µl of the initial stock was added to the respective vials, it was also considered that the ethanol concentration in the culture medium should not exceed 0.1%, that may have some toxic effect on cell lines in higher concentrations.

RNA extraction and quantitative analysis

Huh-7 cells were harvested in different drug concentration, time laps and conditions. RNA extraction was performed using by commercial Bio-fact extraction kit (Bio Fact, South Korea) and the quantity and quality of the extracted RNA were evaluated using a nanodrop instrument (Thermofisher, USA). To remove the DNA contamination, the extracted RNA was also treated with DNaseI enzyme using Promega's RQ1 RNase-Free DNase kit.

Preparation of cDNA (Complementary DNA) and necessary tools

The expression of different genes at the RNA level were investigated by RT Real time PCR. For this purpose, total RNA pattern that has been extracted was first converted to cDNA by the reverse transcription enzyme and reaction. The cDNA was then amplified and detected by Real time PCR method.

First of all, conventional PCR for primers reaction, concentration and optimization was done, and fragment and quantitatively of the products was evaluate by agarose gel electrophoresis, then Real-time PCR reaction using SYBR Green method by Step One Applied Biosystems real-time device was developed.

In this study, primers were designed and selected for two genes targets, COX-2 and a reference gene HPRT (Hypoxanthine phosphoribosyl transferase) summarized in Table 1. Primer design was performed by selecting the reference sequences in the NCBI database and using Oligo analyzer and oligo7 software.

Statistical Analysis

In order to investigate the fold change of target genes expression, the relation $2^{-\Delta\Delta CT}$ method has been used, in which Ct is defined as a reaction cycle in which the fluorescence signal crosses the threshold and the difference Ct between the target gene and the reference gene is defined as ΔCT. ΔΔCT is the difference ΔCT between control and HCV-infected cell lines by Livak method [15]. Based on this procedure, all gene expression level and values would be normalized so the probable error rate would be omitted. In investigate the significance of the results, t-student statistical was selected for data analysis.

Results:

Evaluation of plasmid amplification of pFL-J6|JFH1 and its cleavage

The pFL-J6|JFH1 vector was transferred to E. coli for replication and after extraction and purification of the plasmid, its quality was demonstrated by gel electrophoresis (Figure 2) and then the plasmid was digested and linearized using Xba-1 enzyme.

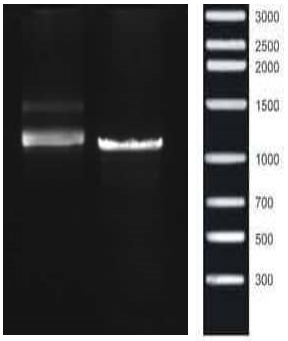

Figure 2: Undigested pFL-J6|JFH1 plasmid (Line 1 from left) and digested plasmid (Line 2 from left) and 1kb DNA ladder in gel electrophoresis. The original weight was 12372 bp

In-vitro viral RNA transcription

In order to confirm the quality of viral RNA transcription from linear plasmid that had been digested, gel electrophoresis was done and its quality was confirmed (Figure 3).

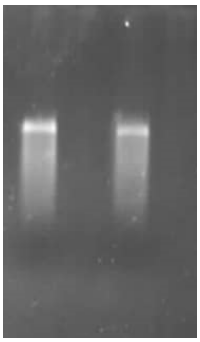

Figure 3: Transcribed HCV RNA from in-vitro transcription system on gel electrophoresis. The samples were diluted to one-twentieth in both lines.

Confirmation of the amplified Huh7 cells

Huh7 cells were cultivated in high glucose DMEM media containing 5% bovine serum and prepared for transfection with the purified HCV RNA and gene expression investigation. The health, viability and quality of the cultivated cells were confirmed by inverted light microscopic examination (Figure 4).

Silymarin possible toxicity evaluation

Before evaluating the antiviral effect of silymarin, the toxicity of silymarin should be evaluated by MTT assay, which is reduced by mitochondrial succinic dehydrogenases in living cells and turns into purple formazan crystals that are not soluble in water. The amount of formazan uptake in the visible range correlates with the number of living cells. Figure 5 shows the cytotoxic analysis of silymarin at different concentrations.

As it was shown in the figure, the viability of Huh7 cells was not affected up to 400µM of silymarin. However, when the concentration of silymarin increased to 500 to 800µM, the toxicity of silymarin on the living cells was observed. The results were also confirmed by microscopic investigation, so, in microscopic observation, trypan blue dye method was used, it was also found that a concentration of 400µM silymarin has none toxic effect on Huh7 cells.

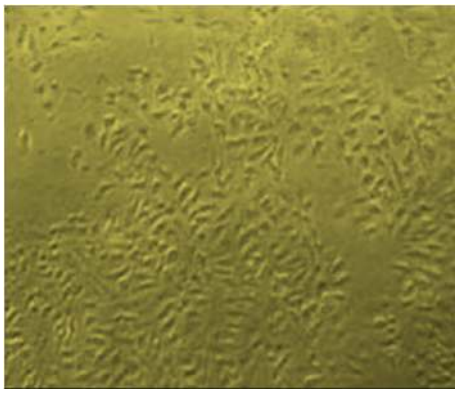

Figure 4: Huh7 cultivated in high glucose DMEM with 5% bovine serum.

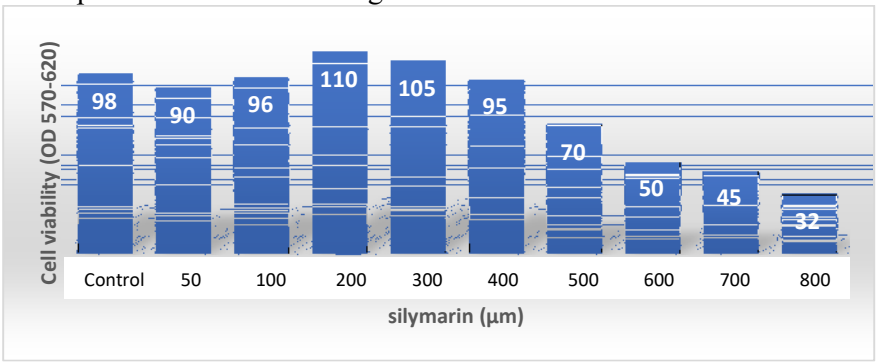

Figure 5: Toxicity effect of silymarin by MTT method in Huh7 cells. $2x10^4$ cells were cultivated in 96 plates, after 24 hours, the cells were treated with different concentrations of silymarin, and after 24 hours, MTT assay has been performed. Finally, by DMSO solution the formazan crystals were dissolved and then optical density was read at the 570nm to 620nm.

Antiviral effect of silymarin on cyclooxygenase 2 gene by RT-PCR

To determine the anti-oxidative effect of silymarin on control and HCV infected Huh-7 cells line, different concentrations of silymarin was introduced to Huh-7 cultivated cells. After 24 hours, total RNA was extracted by Bio-fact kit. cDNA was produced using oligo dT primer, Random-Hexamer as well as reverse primers. The Cox-2 cDNA was amplified by Real time PCR by specific primer pairs. The HPRT gene was also amplified and used as reference gene control. It was showed that silymarin could reduce and inhibits the expression of the Cox-2 gene in different concentration (Figure 6) with regard to HPRT as reference gene.

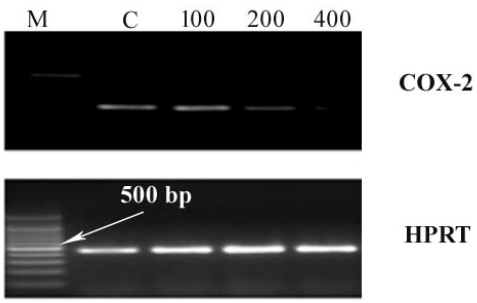

Figure 6: Anti-oxidative effect of silymarin, Cox-2 and HPRT genes in Huh-7 cell line. The DNA ladder was 100kb.

Evaluation of Cox-2 gene expression in HCV-infected cells by Real time-PCR

Gene expression fold change was investigated at two different concentrations. The relative gene expression of control and HCV infected cells model showed that the Cox-2 gene in HCV-infected Huh-7 cells was significantly reduced. In the case of HCV-infected cells, the expression of Cox-2 before treatment with silymarin, it was increased more than 7-fold, so it was concluded that infection of hepatocytes with hepatitis C leads to increased expression of the Cox-2 and oxidative stress (Figure 7). In order to evaluate the relative quantitation, the HPRT gene has been used as a reference control for ΔCT calculations.

At a concentration of 200µM silymarin, a decrease in expression was observed in the Cox-2 gene expression. The change in expression in HCV-infected Huh7 cells treated with 200µM silymarin was still significantly and was higher than control sample. The expression level in HCV-infected Huh7 cells treated with 300µM silymarin was also significant. However, cells treated with 400µM silymarin, a greater decrease in expression was observed. Therefore, the use of silymarin at a concentration of more than 200µM reduces the expression of HCV Cox-2 gene, and when the dose of silymarin increased to 300µM, the effect of silymarin is significantly increased and COX-2 gene expression was observed and decreased to almost similar to normal values (Figure 7). The CT curves and melting temperature of the samples and the Cox-2 gene was shown in Figure 8.

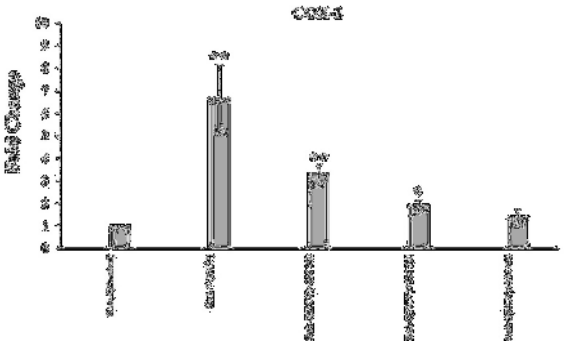

Figure 7: Alteration of Cox-2 gene expression in Huh-7 with different concentrations of silymarin by semi-quantitative RT-Real time-PCR. Calculations were performed based on the ΔΔCT method and the t-student statistical method was used to calculate the significant levels (p-value<0.01).

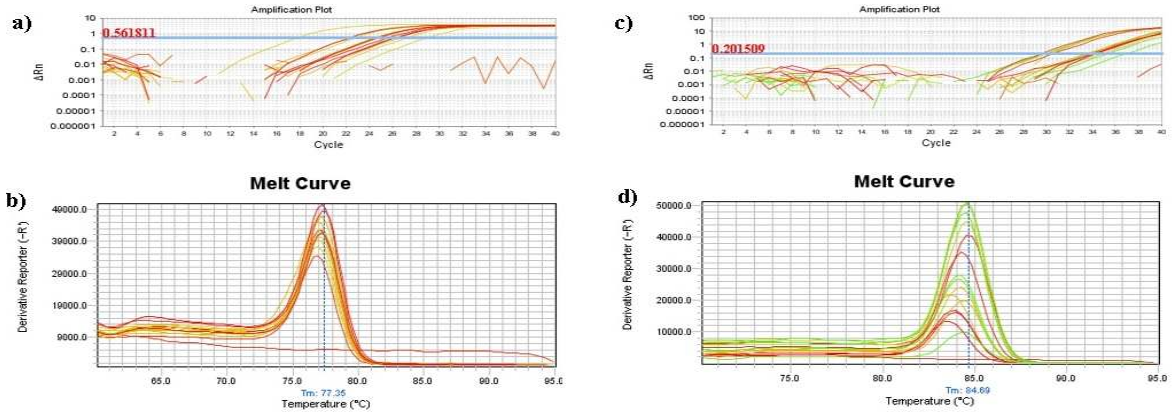

Figure 8: HPRT reference gene amplification in PCR cycles (a) and melting curve (b); Cox-2 gene amplification curve in PCR cycles (c) and its melting curve (d).

Table 1: List of the developed primers in this study for detection Cox-2 and HPRT gene expression.

Name	Forward Primer	Reverse Primer
Cox-2	5-CCTGAGCATCTACGGTTTG-3	5-TAGTCTCTCCTATCAGTATTAG-3
HPRT	5-GCTTGCTGGTGAAAAGGACCTC-3	5-GGAAATCGAGAGCTTCAGACTCGT-3

Discussion

The aim of the current study was to evaluate the medicinal and anti-oxidative effects of silymarin in the treatment of infected hepatitis C cell line. Numerous studies have examined the effect of silymarin or its constituent component silybin on patients with chronic liver disease, but there are little evidences about its effect in HCV infected patients. In a randomized study, it has been reported that patients who have received 12 months of polyethylene-interferon and ribavirin with a supplement containing silybin-vitamin E phospholipid compared with patients who received only polyethylene-interferon and ribavirin had better prognosis [16, 17].

In this study, the inhibitory effect of silymarin in different concentration with regard to toxic concentration used on Huh7 cells was investigated and it was observed that silymarin at the dose of 500μM and above has a toxic effect on Huh7 cells. As a result, in this study, further studies focused on the lower doses and below 500μM to alter the expression of the Cox-2 gene. Silymarin has been reported to have anti-oxidative properties by altering specific messaging pathways, transcription factors, and gene expression [18].

Previous research has shown that hepatitis C infection leads to an increase in reactive oxygen species (ROS) followed by an increase in oxidative stress leading to increased expression of the Cox-2 gene, which in turn increases prostaglandin E2 (PGE2) and Vascular endothelial growth factor (VEGF) production. VEGF has an inhibitory effect on programmed cell death, and both PGE2 and VEGF together play an inductive role in angiogenesis which leads to liver cancer [3]. Our study also showed and concluded that that HCV has effective role in oxidative stress and increased at least 7fold Cox-2 RNA expression.

Recent studies have shown that inhibition of the expression of Cox-2 gene, plays an important role in preventing liver injuries [7, 19-21].

Thus, silymarin by reducing the inhibition of Cox-2 can reduce the production of PGE2 and VEGF and ultimately reduce angiogenesis and prevent liver disease and cancer. The Cox genes has been reported to be consistently expressed in most tissues, while the Cox-2 gene is rapidly induced and expressed as part of inflammatory reactions and cycle in response to viral infections. Therefore, inhibition of Cox-2 has been considered as a promising and effective strategy for the treatment and prevention of liver injuries and cancer [3, 11].

Conclusion

In this study, the anti-oxidative effect and Cox-2 expression reduction of silymarin was observed by Cox-2 gene activity in Huh-7 cells infected with HCV model with regard to control cell lines. Increasing the concentration of silymarin, would also decrease the expression of Cox-2 expression geneat the level of normal and hence would control oxidative stress. On the other hand, reduction of Cox-2 that may be promoted by HCV, would also control liver injuries as well as angiogenesis and also may rise programmed cell death. The main finding of this study is the anti-oxidative effect of the active ingredient of the medicinal plant *Silybum marianum* on the HCV infection through regulating the Cox-2 gene has many beneficial effect and silymarin can be considered as an active supplement for controlling oxidative stress induced by HCV pathogenesis.

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Conflict of interest: There is no conflict of interest to declare.

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