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Original Article

Evaluation of the Silymarin Effect on IL-10 and IL-17 Production in Chronic HCV Infected Patients

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Abstract

Background and Aims: The use of herbal remedies, either in combination with conventional drugs or as an alternative, is accepted worldwide. Silymarin derived from Milk thistle has evidence-based therapeutic potency for wide spectrum of liver diseases. The current work aimed to study the immune-modulating activity of Silymarin in HCV-infected patients by measuring the effect of pure Silymarin solution on the production of IL-17 and IL-10.

Materials and Methods: Nine HCV-1a infected patients and three healthy controls were entered in this study. The mean age of patients and healthy controls were 45.53 (± 10.21) and 39.9 (±10.88), respectively. The PBMCs were isolated, cultured in 96-well plate and incubated with Silymarin solution (5µg/ml) for 24 hours. The cells and cell culture supernatant of three groups including patients treated with Silymarin, non-treated patients, and healthy controls were then subjected to Real Time PCR and ELISA to measure the levels of inflammatory and non-inflammatory cytokines including IL-17 and IL-10, respectively. Statistical analysis was conducted using SPSS software version 20.0.

Results: According to Real Time PCR and ELISA results, the level of IL-17 was significantly reduced in patients treated with Silymarin while the expression of IL-10 was remarkably increased in these patients.

Conclusion: The results of this study approved the immunomodulatory properties of Silymarin in HCV-infected patients. Hepatoprotective, antiviral, as well as immune-modulatory properties of the Silymarin make it a potential therapeutic option in patients with chronic hepatitis C.

Keywords: Silymarin; Hepatitis C Virus (HCV); Interleukin 10 (IL-10); Interleukin 17 (IL-17); Real Time PCR; ELISA

Introduction

The hepatitis C virus (HCV) is the major cause of chronic liver inflammations, which may progress to cirrhosis or liver cancers such as hepatocellular carcinoma (1). HCV, considered as one of the main global health concern, could be transmitted through transfusion of HCV-contaminated blood, blood products, and organ transplantation or verti-cally from mother to child (2, 3).

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Anti-HCV antibodies have not provided lifetime protection, and re-exposure to the HCV may result in a new asymptomatic infection. Since HCV RNA polymerase lacks proofreading activity, the HCV genome may undergo alterations leading to the development of genetically new and heterogeneous populations of the virus (4). Consequently, no anti-HCV vaccine has been successfully developed and approved by the FDA to prevent HCV infection to date (5).

Antiviral therapeutic approaches should either directly target the pathogen or boost the host immune system (6). Host humoral and adaptive immunities work harmoniously together to defend the body against viral infections,

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considering T cells and cytokines as the major effector cells and proteins, respectively.

Cytokines, the regulatory signaling proteins of the immune system predominately produced by T cells, can be categorized into two groups including pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines such as interleukin-17 (IL-17) are released during inflammations to reinforce immune responses against infections (7). Whereas, anti-inflammatory cytokines such as Interleukin-10 (IL-10) are immunosuppressors trying to reduce inflammation and possible tissue damages. Therefore, the two above-mentioned cytokine groups are inversely related and work in opposite directions, but under a dynamic balance.

Milk thistle (Silybum marianum), is a wild biannual flowering herb distributed in the Mediterranean regions, Middle East, North Africa, and North America (8). Milk thistle extract have been used for years to naturally treat liver diseases and modulate the immune system. Silymarin is the major constituent of the Milk thistle seeds and fruits, which has antioxidant and hepatoprotective potency (9).

This work was designed with the aim to study the immune-modulating activity of Milk thistle in HCV-infected patients by evaluation the effect of pure Silymarin solution on the production of IL-17 and IL-10.

Methods

Silymarin solution preparation: Silymarin powder was dissolved in PBS at 50000 ng/ml to obtain 10 mM stock solution. Different dilutions of Silymarin solution (50 µl/ml, 100 µl/ml, 200 µl/ml, 400 µl/ml) were prepared and subjected to the MTT assay. The MTT assay was performed to identify appropriate dose and incubation time of the drug .

Subjects: Nine patients with HCV-1a and three healthy controls were entered this study. Samples were collected from Dr Alavian Infectious Diseases Research Center and Imam Khomeini Hospital Infectious Diseases Research Center. The ethic committee of Tarbiat Modares University approved the study. The participants were asked to study, fill out and sign the written informed consent forms before participation.

The peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient from whole-blood specimens of each patient. Patients who had previously received HCV-related treatments or affected by other diseases in addition to HCV were excluded from the study .

Cell culture/ELISA: PBMCs were cultured in 96-well microplate containing RPMI-1640 media (Gibco, USA), 0.1% FBS (Gibco, USA), penicillin/streptomycin, and Silymarin solution, at a density of 100,000 cells/well.

According to the MTT assay results, the proper concentration of Silymarin solution was 5µg/ml. after incubation for 24 hours, cell culture supernatant was collected and subjected to ELISA (enzyme-linked immunosorbent assay). The levels of IL17 and IL10 was measured as pg/mL using ELISA kit (Beijing Solarbio Science & Technology Co., Ltd, China) .

Table 1. The sequences of primer sets used in this study

Target	Primer Sequences
IL10	Forward: 5' TACGGCGCTGTCATCGATTT 3'
	Reverse: 5' TCTCTTGGAGCTTATTAAAGGCA 3'
1L17	Forward: 5' ACCTCATTGGTGTCACTGCTA 3'
	Reverse: 5' CAGGTTGACCATCACAGTCC 3'

Real Time PCR: After 24 hours of treatment with Silymarin solution, cells were collected for Real Time PCR to evaluate the levels of IL-17 and IL-10 mRNA. Total RNA was extracted using High pure RNA isolation kit (BioFact™, Cat. No. RP101-050) according to the manufacture instruction and then converted to cDNA by BioFact™ RT Series kit. The quality of RNA and cDNA was assessed by NanoDrop 2000 (Thermo Scientific). Two pair of primers were designed using Laser gene primer design tool to amplify IL-17 and IL-10

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related-sequences. Prior to Real Time PCR, convectional PCR was performed to assess and optimize the primer sets performance. SYBR Green Real Time PCR was then conducted in the ABI StepOnePlus™ Real-Time PCR System using GAPDH as a reference gene. The sequences of primer sets used in this study are provided in Table 1 .

Statistics analysis: The data obtained from the experiments are presented as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) test was performed to compare three independent groups including patients treated with Silymarin solution, non-treated patients, and healthy controls. Paired t-test was used to compare data from each group before and after treatment. P values less than 0.05 were considered significant. Statistical analysis was conducted using SPSS software version 20.0 (Armonk, NY: IBM Corp). Graph Pad Prism 5 software (GraphPad Software Inc., La Jolla, San Jose, CA, USA) was used to plot the graphs.

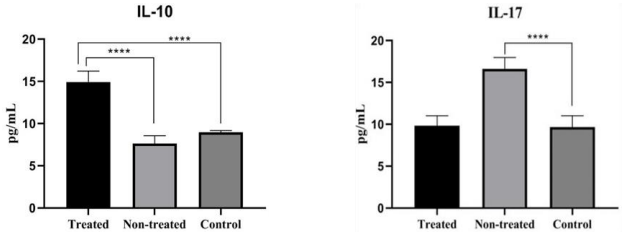

Fig. 1. The levels of IL10 and IL17, produced by PBMCs-stimulated with 5µg/ml Silymarin for 24 hours, measured by ELISA. The results were express as pg/mL .
****: p value ≤ 0.0001

Results

Subjects: Nine patients with HCV-1a (1 female and 8 males) and three healthy controls (1 female and 2 males) were entered this study. The mean age of patients and healthy controls were 45.53 (± 10.21) and 39.9 (±10.88), respectively.

ELISA Results: According to ELISA results, IL17 level was significantly increased in the non-treated patients group compared to healthy control group while no significant differences were observed between patients treated with Silymarin and healthy control group. Furthermore, the level of IL10 was signify-cantly higher in patients treated with Silymarin compared to both healthy controls and non-treated patients (Fig. 1)

Real Time PCR Results: The level of IL17 was significantly increased in non-treated patients compared to healthy controls while the level of this cytokine was reduced in patients treated with Silymarin. Also, the IL10 level was significantly elevated in patients treated with Silymarin compared to both healthy controls and non-treated patients (Fig. 2).

Discussion

Chronic hepatitis C by affecting 3% of the world population, is a global concern imposing a huge burden on individuals, health care providers, and governments. The rate of HCV-related deaths is 0.3 million annually. Since no vaccines have been developed against HCV to date, finding effective ways to stop virus transmission or developing potent therapeutic approaches to reduce HCV-associated complications or deaths is necessary .

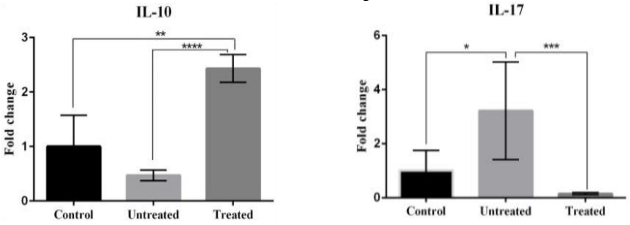

Fig. 2. The levels of IL10 and IL17, produced by PBMCs-stimulated with 5µg/ml Silymarin for 24 hours, measured by Real Time PCR.
:*p value ≤ 0.05
:**p value ≤ 0.01
:***p value ≤ 0.001
****: p value ≤ 0.0001

The use of herbal remedies, either in combination with conventional drugs or as an alternative, is accepted worldwide. They have drawn a great deal of attention due to their safety, low cost, and availability. A great number of preclinical and clinical researches have been performed with the focus on herbal drugs to identify their therapeutic potential, validate their safety and efficacy, or discover the cellular and molecular mechanisms of action. Milk thistle (also known as Silybum marianum), is a flowering herb belonging to

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the Asteraceae family, whose seed and fruit extracts has been traditionally used for wide spectrum of liver diseases. Milk thistle can also provide benefits for people with diabetes, heart diseases, kidney disorders, etc. (10-12).

In 2007 Polyak and his colleagues investigated the molecular mechanisms underlying the antiviral and anti-inflammatory activities of Silymarin extract in patients infected with HCV. They suggested that Silymarin by inhibition of nuclear factor kappa-B (NF-kB) could mitigate oxidative stress and inflamemation induced by HCV and other viruses (13).

Note that the NF-kB is a trans-cription factor involved in the regulation of various signaling pathways associated with cell proliferation, inflammation, and cancer (14-16).

Furthermore, based on the evidence, they proposed that the induction of the JAK-STAT pathway could be a result of the antiviral activity of Silymarin (13).

Dupuis and et al. (2018) used silibinin, the active component of Silymarin, as the natural ligand of estrogen receptor β (ERβ) to investigate the therapeutic potency of silibinin on T cell-mediated immunity in patients with rheumatoid arthritis (RA). They observed that in both RA-affected patient and healthy control groups, silibinin binding to ERβ could suppress T cell proliferation, induce T cell apoptosis, and also inhibit the expression of IL-17 and TNF-α (17).

In this study the Silymarin-treated PBMCs had decreased level of IL17 and increased level of IL10. It has been previously reported that depending on the concentration and timing, Silymarin could show dual effects on the immune system acting as either an immunosuppressor or immune-stimulator (18). While, by the concentration used in this study (as low as 5µg/ml). the Silymarin could modulate the immune responses.

The current study was limited by the lack of equipment including High-performance liquid chromatography (HPLC) to determine the exact composition of the Silymarin.

Conclusion

The results of this study approved the immunomodulatory properties of the Silymarin in HCV-infected patients. Hepatoprotective, antiviral, as well as immunomodulatory pro-perties of the Silymarin could be beneficial for patients with chronic hepatitis C. Therefore, it seems that the Silymarin and its components worth serious attention as a natural option to treat patients with liver problems.

Acknowledgment

None.

Conflict of interest

The authors declare that they have no competing interests.

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