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In Vitro Investigation of Silymarin Effects on HBV Infected Hepatocytes

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Abstract

Background and Objectives: At now, there are restricted therapeutic compounds for the real treatment of liver cancer, and HCC, to decrease the morbidity and mortality rate of HCC, development of chemo-preventive and therapeutic therapies is more important. This study aimed at evaluating the effects of Silymarin (Milk Thistle) on the hepatocytes infected with HBV *in vitro*.

Methods: HBV infected HepG2 cell lines were exposed to different concentrations of Silymarin (0.1, 1, 10 and 100 mg/L). The initial dose was repeated every 3 days. Control group was HBV infected cells that did not receive Silymarin. At first and after 12 days in both groups were measured the, HBsAg and HBeAg levels using ELISA and HBV DNA by Real-Time PCR. For evaluating the cytotoxicity effects of Silymarin, MTT assay was used. Data analyzed using SPSS software and non-parametric statistical test (Kruskal Wallis).

Results: In HepG2 cell lines, secretion of HBV DNA levels were not significantly inhibited by Silymarin ($p = 0.132$, 0.83 and 0.276, respectively). Also, Silymarin had no effect on the secretion of HBsAg and HBeAg. In all samples, at first HBsAg was positive and HBeAg was negative; and after 12 days, they did not change. Silymarin had hepatotoxic effect at a concentration of 100 mg/L.

Conclusion: Our study showed that the Silymarin decreased stopped the cell growth but these effects were not statistically significant. So, Silymarin had no significant effects on HBV activity and damaged hepatocellular in infected hepatocytes with HBV.

Keywords: *Silymarin; HBV; Hepatocellular*

Introduction

Chronic hepatitis B is one of the most common infectious diseases which accompanied a trend toward cirrhosis and hepatocellular carcinoma with significant morbidity and mortality [1,2]. Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world [3]. About 2 million people are infected with the virus worldwide and 350 million people (about 5%) of the world's population are chronic carriers of this virus [4].

Hepatitis B is the tenth leading cause of death in the world. The studies carried out in Iran have shown that up to 35% of Iranian people are exposed to the virus; three percentages of those have been chronic carriers of infection. And around 8 - 10000 people die annually due to the consequences of this infection [5]. The areas with high prevalence include Southeast Asia, China, and Africa, where approxi-

mately 10% of the population in these regions are chronic carriers for the hepatitis B virus but local levels of the virus in Australia, North America, and Western Europe are lower [5]. But the prevalence reaches to 5 - 20% in tropical countries, especially in patients with Down syndrome, polyarthritis' nodosa, lepromatous leprosy, leukemia, Hodgkin's disease, people with chronic kidney diseases and injecting drug users.

At now, there are restricted therapeutic compounds for the real treatment of liver cancer, and Hepatocellular Carcinoma (HCC), to decrease the morbidity and mortality rate of HCC, development of chemo-preventive and therapeutic therapies is more important [6], high cost, side effects and the high levels of standard treatment failures has led to exploring the alternative treatments [7].

Among these drugs, there is the herbal drug of Milk thistle that has been used since ancient times in the treatment of liver diseases. Silymarin is the active ingredient in milk thistle, which is mainly present in the fruit (seeds) of milk thistle plant [8]. The main components of the silymarin include flavonolignans namely silybin, silydianin, silycristin which the most of its hepatoprotective effect is referred to silybin that comprises 60 - 70% of the drugs [9]. It has become clear that this substance has hepato-protective, antioxidant, anti-inflammatory and anti-fibrotic effects and also has an immunoregulatory function [10,11]. Because silymarin is partly hydrophobic; its performance is possible through integrating into lipid membranes of both viruses and target cells [12]. However, several side effects of Silymarin such as headaches, gastroenteritis and dermatological symptoms were reported [13].

Detection of HBV infection is routinely through serological and virological markers. Hepatitis B surface antigen (HBsAg) is the hallmark of HBV infection and is the first serological marker to appear in acute hepatitis B, and persistence of HBsAg for more than 6 months suggests chronic HBV infection. Meanwhile, monitoring the serum HBV DNA level is valuable for assessing liver disease activity [14].

HepG2 cell line is hepatoma-derived perpetual cell line broadly used as a surrogate model for hepatocytes [15,16]. HepG2 cell does support complete HDV replication, where their co-transfection with plasmids encoding the HDV genome and HBV envelope proteins leads to the production of recombinant HDV virions with ability to infect susceptible cells [17,18]. Moreover, the transfection of hepatoma cells with replication-competent HBV DNA or circular HBV DNA causes the production of HBV particles [19,20].

For infection assays, two HepG2-derived cell lines (HepAD and HepG2.2.15) were steadily transduced with the HBV genome and are frequently used as a source of HBV infectious particles [16]. Thus, the HBV replicon system, particularly the hepG2 infectious culture system, is a powerful tool to characterize antiviral potential of botanicals, due to induce the production of low levels of cccDNA making this model suitable for the study of many steps in HBV replication, including cccDNA formation and its regulation [21,22].

In this study, we decided to study the effects of Silymarin (Milk Thistle) on the hepatocytes infected with HBV in culture medium (*in vitro*) to answer to this question; is Silymarin has an anti-hepatitis B effect or not? If it is confirmed, by adding this substance to the standard antiviral drugs can increase the success rate of the treatment, really.

Aim of the Study

This study aimed at evaluating the effects of Silymarin (Milk Thistle) on the hepatocytes infected with HBV *in vitro*.

Materials and Methods

Cell cultures and inoculum preparations

This study was performed on HepG2 (Institute Pasture, Iran). HepG2 cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 4.5g of glucose/L, 100 U of penicillin/mL, and 100, µg of streptomycin/mL. Cells were seeded at a concentration of 10^6 per dish in 4 mL of complete culture medium. Cultures were observed daily with a phase-

contrast microscope. The medium was changed every 1 or 2 days and stored at -20°C. The mixture of positive hepatitis B plasma (HBsAg and HBeAg) was prepared with high titers of the virus from patients with chronic hepatitis B. The serum titer of virus determined by quantitative-PCR and virus titer at 100,000 particles/mL in volumes of 400 mL were divided and kept at -70°C.

Infection of cell cultures with HBV and MTT assay

At 2 to 6 days after plating, HepG2 cells were infected with 1 mL of HBV particles from patient serum. After 3h of incubation at 37°C, the inoculum was removed. The cells were then extensively washed with phosphate-buffered saline, and complete culture medium supplemented with 10^4 M dexamethasone and $l0^5$ M insulin was added to the cells. The cultures were incubated for 1 day, washed three times, and incubated again with fresh culture medium. The media were changed after 1 day, and the cells were maintained thereafter in complete medium supplemented with 10^6 M dexamethasone and 10^6 M insulin. The medium was then changed every 2 days, harvested, and tested for HBV pregenomic RNA [23]. The viral titers of culture media determined by Real-Time PCR for, HBV pregenomic RNA and it then compared with primary viral titers at the beginning of culture. Increased the pregenomic RNA titers of culture media indicated the viability of the virus. Mitochondrial MTT assay test was used to investigate the effect of Silymarin on liver cell metabolism and its possible cytotoxic effects [23].

Analysis of HBsAg-HBeAg under treatment of Silymarin by ELISA and REAL-TIME PCR technique

Uninfected hepg2 cells exposed to the concentrations of 0.1, 1, 10 and 100 mg of Silymarin for 12 days (The initial dose of Silymarin was repeated every three days (and after 12 days, MTT test was conducted. After ensuring no toxic effect on HepG2 cells, they (HepG2 cells) infected with the hepatitis B virus which had been exposed with concentrations of 0.1, 1, 10 and 100 mg of Silymarin (The initial dose of Silymarin was repeated every three days). Control group were cells infected only with the B virus. After 12 days in both groups, levels of viral markers (HBsAg and HBeAg) in the liquid medium were measured using ELISA and the quantity of viral DNA was determined using REAL-TIME PCR technique.

Statistical analysis of data

For data analysis, SPSS software and non-parametric statistical test (Kruskal Wallis) was used.

Results

The effect of silymarin on AST

As shown in table 1, AST levels in the twelfth day compared to the beginning of the experiment in the samples of the control group have increased but in samples with receiving different concentrations of Silymarin decreased. The highest decrease was observed at a concentration of 1mg/L, but this difference was not statistically significant (P = 0.132). In the group treated with 100 mg/L of Silymarin, cell growth was stopped.

Groups	AST changes after 12 days	Mean changes(cv)	Percentage changes
Control	Increase	6 ± 3	25 ± 14
Silymarin (0.1 mg/l)	Decrease	22 ± 10	74 ± 12
Silymarin (1 mg/l)	Decrease	18 ± 2	81 ± 2
Silymarin (10 mg/l)	Decrease	13 ± 3	69 ± 6
Silymarin (100 mg/l)	Cell growth stopped		

Table 1: *The mean and percentage of AST changes in different groups.*

The effect of silymarin on ALT

ALT levels in the twelfth day compared to the beginning of the experiment have decreased in the control group and also in the group with receiving different concentrations of Silymarin. The highest percentage of decrease was detected at a concentration of 1 mg/L, and the minimum was seen in the control group but this difference was not statistically significant (P = 0.83). Also, in the group treated with 100 mg/L of Silymarin, cell growth was stopped (Table 2).

Groups	ALT changes after 12 days	Mean changes (cv)	Percentage changes
Control	Decrease	2 ± 5	10 ± 24
Silymarin (0.1 mg/l)	Decrease	21 ± 2	88 ± 1
Silymarin (1 mg/l)	Decrease	16 ± 0.5	100
Silymarin (10 mg/l)	Decrease	14 ± 1	93 ± 1
Silymarin (100 mg/l)	Cell growth stopped		

Table 2: *The mean and percentage of ALT changes in different groups.*

The effect of silymarin on HBV DNA

HBV DNA levels in the twelfth day compared to the beginning of the experiment have increased in the control group and also in the group with receiving different concentrations of Silymarin. The least increase was observed at a concentration of 10 mg/L, and the maximum of the increase was observed in the control group but this difference was not statistically significant (P = 0.276). Also, in a group treated with 100 mg/L of Silymarin, cell growth stopped (Figure 1).

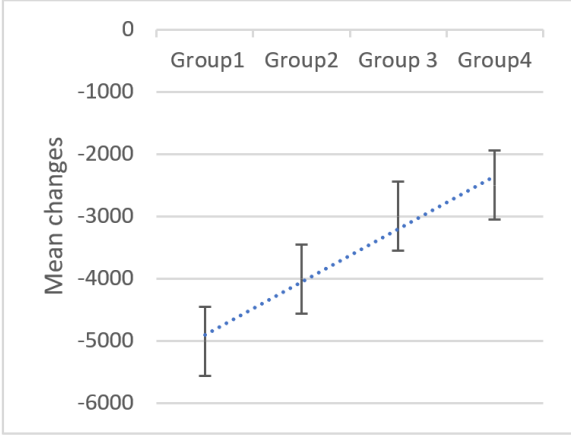

Figure 1: *Mean changes of HBV DNA level in different groups.*

The effect of silymarin on HBsAg and HBeAg

All of the groups at the beginning of the test were positive for HBsAg and negative for HBeAg and no change was observed after 12 days.

Discussion

The studies in both experimental and clinical fields have reported that regarding the pharmacological activities of silymarin, its use is supporting in patients with liver diseases [24,25].

Antioxidant, immunomodulatory, anti-fibrotic, antiproliferative, and antiviral are the known features of silymarin, though the real mechanism of its activity is not clear [26].

The most studies about the effect of Silymarin on viral hepatitis have been carried out particularly in animal models or as clinically [27]. The main difference between our results with other studies is that unlike the most studies, our study conducted *in vitro* as a non-clinical. In addition, it doesn't have limitations of previous studies such as; the low number of participants, the lack of appropriate control group, and the difference in dose/duration of treatment.

Our results showed that Silymarin significantly did not decrease liver enzymes compared with baseline and the control groups.

The decrease rate of ALT enzyme showed that the Silymarin prevented hepatocellular damage, but it is not clear whether this decreased rate has a clinical value?

In a long-term study conducted without control group, it was found that the patients positive for HCV which had continuous biochemical responses to the standard treatments(Interferon) had a low risk for cirrhosis and hepatocellular carcinoma compared to the group that didn't show biochemical responses, therefore, the biochemical weak response may be clinically useful [28].

In line to our study, another double-blind randomized placebo-controlled trial conducted in Nigeria to determine the effect of silymarin on liver biochemistry in treatment-naive patients with chronic viral hepatitis B infection after one month of treatment and compare it to placebo, evaluating the effect of silymarin on liver function test (bilirubin, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) was performed. Results showed that Silymarin could cause improve the serum alanine, aspartate aminotransferase, and HRQOL, while it could not change the serum bilirubin and alkaline phosphatase (ALT) levels [29].

In accordance with our findings, a study conducted as Randomized Controlled Trial(RCT) about the effect of Silymarin (Milk Thistle) on liver disease in patients with chronic hepatitis C, the higher doses of silymarin did not significantly decrease serum ALT levels more than placebo in patients with chronic HCV infection which unsuccessfully treated with interferon-based therapy, in total, Silymarin did not afford higher benefit than placebo for patients with treatment-resistant chronic HCV infection [30]. Of course, this should be noted the mentioned study conducted as a clinical trial on HCV patients, but our study conducted on the HBV virus *in vitro*. However, because both studies dealt with liver cells, partly, could compare their results. Although, the previous studies were performed as clinically and many factors may be prevented of drug effects, but in our studies, because evaluated the only effect of the drug without other factors, with more possibility can be stated that Silymarin doesn't have an effect on HBV replication.

As well as, our study revealed that Silymarin didn't have an effect on HBsAg and HBeAg which was inconsistent with results of a study carried out by Mehrotra., *et al* [11]. In our study, the highest effect on the levels of liver enzymes (ALT and AST) was reported in concentration 1 mg/L and also on the HBV DNA in the concentration 10 mg/L, but this difference was not statistically significant. So, it does not seem to be a dose-dependent effect of Silymarin.

Yi-Fang Wu., *et al*. conducted a study about chemo-preventive effect of Silymarin on liver pathology in HBV X protein transgenic mice, their data presented that Silymarin has therapeutic effects on the early stages of liver damage, retreating fatty changes and improving liver histopathology in a dose-dependent mode, but Silymarin was unable to prevent cancer development [6], which their results were

in contrast with our findings. In the current study, Silymarin couldn't cause a decrease in HBV DNA level that this result was in line with above-mentioned studies.

With all interpretations and agreed and opposed studies, the possible benefits of silymarin in the clinical healing of liver illnesses remained a controversial topic. In regards to the differences in results of our research with other studies, the importance of the issue and the lack of enough clinical trial studies; more studies with higher volumes, a longer period of time and measuring the more parameters are needed.

Conclusion

Our study showed that the Silymarin decreased the ALT and AST levels also stopped the cell growth, but these effects were not statistically significant. So, Silymarin had no significant effects on HBV activity and damaged hepatocytes in infected with HBV.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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