Inhibition of Apoptosis and Proliferation in T Cells by Immunosuppressive Silymarine

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ABSTRACT

Silybum marianum, is known to have anti-inflammatory, hepatoprotective and anticarcinogenic effects. The aim of this study was to compare effects of Silymarin, Rapamycin and FK506 on proliferation and apoptosis of human T cells stimulated with Con A.

Peripheral blood mononuclear cells (PBMC) were stimulated with concavalin A (Con A) (5µg/mL) and then treated with different inhibitors (silymarin, rapamycin and FK506) in various concentrations (5 days). Cells were examined using carboxyfluorescein succinimidyl ester (CFSE) assay for proliferation. Then cell apoptosis was analyzed by FITC annexin V/PI staining and flow cytometry. The effects of drugs on the activation of poly ADP ribose polymerase (PARP) pathway in PBMCs stimulated with Con A and treated with IC50 dose of drugs for 5 days were evaluated using the PathScan cleaved PARP sandwich ELISA kit.

The results indicated that silymarin inhibited T cell proliferation. In addition, our results pointed out that 100 µM and 200 µM of silymarin significantly have more inhibitory effect on T cells proliferation than FK506 and rapamycin. None of these drugs at IC50 concentration had affected the level of cleaved PARP.

Overall, with superior efficacy and lesser toxicity in comparison with other immunosuppressive drugs, silymarin could be a suitable choice of therapy for certain diseases.

Keywords: Apoptosis; FK506; Immunosuppressive; Lymphocyte; Proliferation; Rapamycin; Silymarin

INTRODUCTION

A large number of researches within the last few years have shown that certain fruits, herbs, and plants exhibit chemo-preventive effects. Among them, silymarin, is a polyphenolic flavonoid isolated from the fruits and seeds of the milk thistle (also called artichoke) (Silybum Marianum). Silymarin is a complex of other components, mainly silybin (or silybinin), which is the most active component,
isosilybin, silydianin and silychristin. Currently, silymarin is widely used as a hepa-to-protectant and as a supportive therapy of liver disorders such as cirrhosis, hepatitis and fatty acid infiltration. Silymarin has been described to represent antioxidant, immunomodulatory, antiproliferative, antifibrotic, and antiviral activities, the underlying mechanisms remain to be fully defined and its clinical efficacy is currently uncertain. Several studies, have reported immunomodulatory actions of silymarin in vitro. Silymarin increases lymphocyte proliferation, interferon gamma (IFN-γ), interleukin 4 (IL-4) and IL-10 secretion by activated lymphocytes, in a dose-dependent manner. In addition, many studies have also reported immunosuppressive effects of silymarin in vitro and in vivo. It has been shown that silymarin treatment strongly impairs NF-κB activation in activated T CD4\(^+\) lymphocytes with consequent inhibition of IL-2 and IFN-γ production and cell proliferation. The effect of silymarin on immune cells such as T lymphocytes and the mechanisms responsible for it is largely unknown. Some papers have reported inhibitory effect of silymarin on the proliferation of T cells in vitro, which is associated with inhibition of IFN-γ and IL-2. Activation of T cells lead to initiate a series of intracellular signaling pathways that control different cellular activities such as expressing immune regulatory genes, whose function is essential for the initiation and coordination of the immune response. The aim of present study was to examine the effects of silymarin, rapamycin and FK506 on the proliferation and apoptosis of human activated T cells in vitro.

**MATERIALS AND METHODS**

**Study Design and Subjects**

Peripheral blood mononuclear cells (PBMC) from normal volunteer donors were obtained in heparinized tubes. All of the protocols had received the approval of the Local Research Ethics Committee (No. IR.MUI.REC.1393.3.573).

PBMCs were isolated using standard Ficoll-Paque Plus density-gradient centrifugation (Sigma- Aldrich, USA). Briefly, heparinized blood (10 mL) was mixed with an equal volume of phosphate-buffered saline (PBS). Samples were layered over Ficoll (5 mL) in a centrifuge tube. After centrifuging (400 x g for 20 min) at room temperature, the interface layer of PBMCs was carefully removed and washed twice with PBS (260 x g for 5 min). Cells were counted and cell viability was assayed by trypan blue dye exclusion (0.4% trypan blue in PBS). Cells with viability 98% were used for further experiments.

**Determination of Minimum Toxic Dose**

To determine the proliferation capacity of PBMCs, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining was performed. The cells were centrifuged (at 200 x g for five min), washed with pre-warmed sterile PBS and centrifuged (at 200 x g), cell pellet was resuspended in PBS at a concentration of 5 x 10^6-1 x 10^7 cells/mL, then an equal volume of CFSE staining solution was added to the cell mixture. The cells were incubated at 37°C, 5% CO\(_2\) for 30 min. The cells were centrifuged (at 200 x g 5 min), the supernatant was removed and re-suspended in culture medium containing 10% FBS. The cells were incubated at 37°C, 5% CO\(_2\), for 30 min. Then cells were centrifuged at 200 x g 5 min. And at this stage, stained cells were stimulated by concanavalin A (Con A) (5 μg/mL) and then treated with silymarin, FK506 and rapamycin (at 0.001, 0.01, 0.1, 1, 10, 100, 200 μM) and the cells were cultured for 5 days in 37°C, 5% CO\(_2\) incubator. Negative control cells were treated with dimethyl sulfoxide (DMSO) and Roswell Park Memorial Institute (RPMI).

**IC\(_{50}\)**

The half maximal inhibitory concentration (IC\(_{50}\)) is a measure for the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half. In order to determine the dose of drugs that could cause proliferation inhibition in 50% of PBMCs, this method was used for each of the drugs separately. IC\(_{50}\) was calculated for each of drugs as effective dose using Graph pad Prism 6 (GraphPad Software, Inc, CA, USA).

**FITC Annexin V–propidium Iodide (PI) Staining for Apoptosis Assay**

Cytotoxicity of silymarin, rapamycin and FK506 was evaluated by FITC annexin V-PI (Sigma, USA) staining. PBMCs (10^6 cells /mL) were activated with Con A and incubated in 24-well culture plates...
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Sandwich ELISA Assay for Cleaved PARP (Asp214)

PBMCs (10^6 cells/mL) were cultured in 24-well culture plates in the presence of IC_{50} for each drug (silymarin, 3×10^{-5} µM; rapamycin, 10^{-6} µM or FK506, 10^{-5} µM) and stimulated with concanavalin A (5 µg/mL). After 5 days incubation with cells, media was removed and washed once with 5-10 mL ice-cold 1x PBS. Cells were harvested and lysed in 2 mL of 1x cell lysis buffer plus 1Mm PMSF. Lysates on ice microcentrifuge were centrifuged for 10 min (20000g) at 4°C and the supernatant was transferred to a new tube. The supernatant was the cell lysate and was stored at -80°C in single-use aliquots. CST’s PathScan Cleaved PARP (Asp214) Sandwich ELISA Kit (cell signaling Technology, USA) was used to evaluate the effect of different drugs on induction of apoptosis in cells. The kit was a solid phase sandwich ELISA that detected endogenous levels of cleaved PARP (Asp214) protein in cell lysates.

RESULTS

Effects of Silymarin, FK506 and Rapamycin on the Proliferation of PBMCs

To analyze the effect of silymarin treatment on activated PBMC, the proliferative response of PBMC was determined using a CFSE based flow cytometry assay. As shown in Figures 1 and 4 the proliferation of activated PBMC was significantly inhibited by 100 µM and 200 µM of silymarin in comparison with untreated control, FK506 (Figures 2 and 5) and rapamycin at the same concentration (Figures 3 and 6) in comparison with activated DMSO-treated controls as evaluated after 5 days.

IC_{50} Calculation

IC_{50} values represent the concentrations of drugs inhibiting proliferation of PBMC (after 5 days) by 50%. Our data showed that the doses of silymarin, FK506 and rapamycin effective on PBMC (after 5 days ) were 3×10^{-5} µM, 10^{-6} µM and 10^{-5} µM respectively. IC_{50} was calculated using Graph pad prism 6 (GraphPad Software, Inc, CA USA) (Figure7).

Figure 1. The effects of silymarin on the T cell proliferation in comparison with control (dimethyl sulfoxide). The proliferation of activated PBMC was significantly inhibited by 100 µM and 200 µM of silymarin.*The difference between mean of proliferation index and relevant control was significant (p< 0.05).
Figure 2. The effects of FK506 on the T cells proliferation in comparison with control (dimethyl sulfoxide) was statistically significant (\(p<0.001\)) \(n=3\). *The difference between mean of proliferation index and relevant control was significant (\(p<0.05\))

Figure 3. The effects of rapamycin on the T cells proliferation in comparison with control (dimethyl sulfoxide) was statistically significant (\(p<0.001\)) \(n=3\). *The difference between mean of proliferation index and relevant control was significant (\(p<0.05\))
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Figure 4. Suppressive effect of silymarin on the T cells. M1 represents suppressed cells and M2 proliferated cells.
Figure 5. Suppression effect of FK506 on the T cells (flow cytometric analysis). M1 represents suppressed cells and M2 proliferated cells.
Figure 6. Suppression effect of rapamycin on the T cells (flow cytometric analysis). M1 represents suppressed cells and M2 proliferated cells.
Figure 7. IC_{50} value for silymarin, FK506 and rapamycin in inhibition of the T cells by using Graphpad prism 6.

Figure 8. Determination of silymarin cytotoxicity in IC_{50} (3×10^{-5} \mu M) on activated T cells after 5 days by FITC-annexin V/PI staining and flow cytometric analysis. Cells that are considered viable are both annexin V and PI negative (lower left), while cells in early apoptosis are annexin V positive and PI negative (lower right), and those in late apoptosis or already dead are both annexin V and PI positive (upper right).
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Figure 9. Determination of rapamycin cytotoxicity in IC_{50} (10^{-6} \mu M) on activated T cells (after 5 days) by FITC-Annexin V/PI staining and flow cytometric analysis. Cells that are considered viable are both annexin V and PI negative (lower left), while cells in early apoptosis are annexin V positive and PI negative (lower right), and cells those in late apoptosis or already dead are both annexin V and PI positive (upper right).

Effect of Silymarin, FK506 and Rapamycin on Cell Viability

We used FITC annexin V/PI to quantitatively determine the percentage of cells that are undergoing apoptosis. As shown in Table 1, cell viability in effective dose (IC_{50}) of silymarin (3\times10^{-5} \mu M) (Figure 8) was approximately similar to effective dose of rapamycin (10^{-6} \mu M) (Figure 9) and FK506 (10^{-8} \mu M) (Figure 10). Control for 3\times10^{-5} \mu M silymarin and 10^{-6} \mu M rapamycin and 10^{-8} \mu M FK506 were cells treated with DMSO in a final concentration equal to the test wells. Consequently, silymarin and FK506 and rapamycin were equivalent in cell viability (Table 1).

Effect of Silymarin, FK506 and Rapamycin on Activation of the PARP Pathway

To investigate whether the T cell apoptosis by silymarin, rapamycin and FK506 was due to the modulation of the PARP molecule, we used the PathScan cleaved PARP sandwich ELISA kit (Cell Signaling Technology, USA) to examine the effects of IC_{50} dose of silymarin, rapamycin and FK506 on the cleavage of PARP molecule in the T cells (stimulated by Con A). The results showed that none of these drugs at the IC_{50} concentration affected the level of cleaved PARP after 5 days of incubation.
Figure 10. Determination of FK506 cytotoxicity in IC_{50} (10^{-8} \mu M) on activated T cells (after 5 days) by FITC-annexin V/PI staining and flow cytometric analysis. Cells that are considered viable are both annexin V and PI negative (lower left), while cells in early apoptosis are annexin V positive and PI negative (lower right), and those in late apoptosis or already dead are both annexin V and PI positive (upper right).

Table 1. Data presents the effect of IC_{50} dose of silymarin, FK506 and rapamycin on the apoptosis of the T cells (stimulated with concavalin A) after 5 days incubation in vitro. Cells that are considered viable are both annexin V and PI negative, while cells in early apoptosis are annexin V positive and PI negative, and those in late apoptosis or already dead are both annexin V and PI positive.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Live cells % annexin V-/PI-</th>
<th>Early apoptosis % annexin V+/PI-</th>
<th>Late apoptosis % annexin V+/PI+</th>
<th>Necrosis %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silymarin 3×10^{-5} \mu M</td>
<td>94.0</td>
<td>3.6</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>FK506 10^{-8} \mu M</td>
<td>93.2</td>
<td>3.8</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Rapamycin 10^{-6} \mu M</td>
<td>96.5</td>
<td>1.3</td>
<td>1.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>
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DISCUSSION

Overall our result shows that silymarin as an immunosuppressive drug with fewer side effects could have an essential role to inhibit T lymphocyte proliferation. Consequently it could be considered as a therapeutic drug in the organ transplantation.

Transplantation medicine is one of the most challenging and complex areas of modern medicine. Some of the key areas for medical management are the problems of transplant rejection, during which the body has an immune response to the transplanted organ, possibly leading to transplant failure and a need to immediately remove organ from the recipient. The transplant rejection can be reduced through serotyping to determine the most appropriate donor-recipient match and use of immunosuppressant drugs such as rapamycin, cyclosporine and tacrolimus.14

In the present study, we compared the suppressive effects of silymarin with rapamycin and FK506 on the proliferation and apoptosis of T cells in vitro.

Silymarin and silybinin are well known for their anti-inflammatory, hepatoprotective and anticarcinogenic effects.5,5 Several studies have also reported immunosuppressive actions of silymarin in vitro and in vivo.

Silymarin treatment strongly suppresses NF-κB activation in activated T CD4+ lymphocytes.15 In the mouse model of Con A-induced T cell-dependent hepatitis, silymarin was proven to suppress T cell-dependent liver injury, inhibiting intrahepatic expression of tumor necrosis factor (TNF-α), IFN-γ, IL-4, IL-2.16 It has been also reported that silybinin and silymarin inhibit in vitro T cell proliferation induced by stimulation via anti-CD3 monoclonal antibody or mitogens such as phytohemagglutinin (PHA), Con A and pokeweed.17,18 Several genes coding for transcription factors are involved as regulators of T cell activation, proliferation and apoptosis.19

The aim of this study was to compare the effect of various doses of silymarin on T cells proliferation with rapamycin and FK506 using the CFSE assay.

The result showed that silymarin had the ability to inhibit T cell proliferation. Moreover, our results indicated that silymarin at the concentrations of 100 µM and 200 µM had more inhibitory effect on T cells compared with the FK506 and rapamycin. This finding is consistent with recent reports regarding the effect of silymarin on T cells function and supports the notion that silymarin and its components have immunosuppressive effects on T cells.

A previous study on mice revealed that silymarin at 50 µM concentration, significantly inhibited CD4+ T cells proliferation, IL-2 and IFN-γ production.15

Another study has shown that the expression of IL-2 and IL-4 were reduced in thymocytes of the BABL/c mice, treated intra-peritoneally with silymarin.20 Moreover, in a mouse model of T cell-dependent hepatitis, the active compound of silymarin proved to be an immune-response modifier in vivo, by inhibition of intrahepatic expression of IL-4, IL-2, and inducible nitric oxide synthase (iNOS).16

The results of this study suggest that silymarin can inhibit T cell activation and proliferation in the human as well.

In the present study, we showed that the IC50 doses of silymarin (at the concentration of 3x10^5 µM), rapamycin (at the concentration of 10^5 µM) and FK506 (at the concentration of 10^-5 µM) inhibited T cells proliferation. In addition, the cell viability of treated T cells by silymarin, FK506 and rapamycin were equal. This study also showed that silymarin at the concentration of 3x10^5 M had a little cytotoxic effect on the T cells viability after 5 days of incubation. It has been shown that silymarin treatment at 100 µM cause G1 arrest in activated T lymphocytes after 72-hour incubation without significant cell death.21

Another study has shown that silymarin at highest concentration (100 µM) after 72-hour incubation significantly inhibited T cell proliferation compared with controls, and this inhibitory effect was not due to induction of cytotoxicity. This effect was detected less at 50 µM and was not detected at lower concentration (10 µM).22

To investigate whether silymarin, FK506 and rapamycin had cytotoxic effects on T cells, we examined the effects of IC50 dose of silymarin, rapamycin and FK506 on the cleavage of PARP molecule on T cells (stimulated by Con A) using the PathScan cleaved PARP sandwich ELISA kit. Our data showed that none of these drugs at IC50 dose affected the level of cleaved PARP after 5 days of incubation.

A family of cysteine proteases, the caspases, is known to play a central role in various models of cell death.23-25 The activation of effector caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates including PARP. These downstream cleavage events mark many of the
morphological features of apoptotic cell death. Given the important role of caspases as effector molecules in various forms of cell death including drug-induced apoptosis, the ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies. PARP is a key signaling nuclear protein involved in DNA repair and apoptosis. As a downstream substrate, PARP is cleaved by activation of caspas-3. Therefore, cleavage of PARP is also widely used as an indicator of apoptosis. Studies showed that silymarin did not have cytotoxic effects on T cells proliferation. However, the results of this study showed that silymarin had a little cytotoxic effect on T cells proliferation following 5 days of incubation. The discrepancy between this study and previous study may be due to a longer period of incubation.

This study shows that silymarin has the ability to inhibit T cell proliferation in vitro and exerts immunosuppressive effects. Silymarin could be a good candidate for immunosuppressive therapy for certain medical conditions with superior efficacy and lesser toxicity in comparison with other immunosuppressive drugs.

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