Silymarin and hepatoprotection

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ABSTRACT

Objective: To determine the hepatoprotective effect of silymarin with Chang cell cultures. Specifically, to investigate the antioxidant properties of silymarin and its protective function in reducing pro-apoptotic markers.

Methods: Intracellular free radical levels were assessed with dichlorofluorescein (DCF) fluorescence after exposing cells to an oxidative stress of 400 μmol/L H_2O_2 for 20 min. Levels of cellular ATP and bax expression were examined to evaluate the protective effects of silymarin.

Results: Silymarin significantly reduced the DCF fluorescence signal. Cell viability, assessed by the MTT assay, showed that silymarin enhanced the cell growth. Drug treatment was also associated with enhanced ATP levels, and reduced Bax and protein mRNA levels.

Conclusion: Silymarin can function as a hepatoprotectant against free radical damage due to oxidative stress. The protective nature extends to reducing levels of pro-apoptotic Bax protein. Silymarin may be a useful adjuvant for the treatment of specific liver diseases.

KEY WORDS silymarin; liver disease; hepatoprotection; apoptosis

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水飞蓟素的肝脏保护作用研究

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[**摘要**] **目的：**研究水飞蓟素对人正常肝细胞 (Chang 细胞株) 的保护作用, 尤其是其抗氧化活性和对细胞抗凋亡的保护作用。**方法：**采用 400 μmol/L H_2O_2 处理 Chang 细胞 20 min 后, 通过二氯荧光黄 (dichlorofluorescein, DCF) 荧光强度检测细胞内自由基水平, 并检测了细胞活性及细胞内 Bax 表达水平和 ATP 水平。**结果：**水飞蓟素明显降低了 DCF 荧光信号, 显示细胞内自由基生成减少。MTT 实验结果显示水飞蓟素可增强细胞活性, 增加细胞内 ATP 水平, 而减少促凋亡蛋白 Bax 的转录和表达水平。**结论：**水飞蓟素可抑制 Bas 表达, 具有保护细胞抗氧化应激所致自由基损伤的作用。水飞蓟素可作为治疗特异性肝脏疾病的有效辅助药物。

[**关键词**] 水飞蓟素; 肝脏疾病; 肝脏保护作用; 凋亡

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Biography: Frank J. Burczynski, Ph.D,mainly engaged in the research of liver diseases and pharmaceutical treatment.

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Silymarin, a purified extract from Milk Thistle (Silybum marianun), is a poly-phenolic flavonoid that is comprised of four flavonolignans with silibinin being the main active ingredient. Silymarin has been suggested to possess many biological properties including stimulatory effects on key cells of the immune system[1], hepatoprotection[2-3], modulating the oxidant-antioxidant imbalance during drug induced oxidative stress[4]. Many of the functional attributes on liver disease are thought to be due to its antioxidant property[5-6]. In this study we investigated the hepatoprotective properties of silymarin using an oxidative stress cell culture model.

1 Materials and methods

1.1 Materials

All chemicals including silymarin and 2, 7-dichlorofluorescein diacetate ($DCFH_2$-DA) were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, were purchased from Gibco/BRL (Gaithersburg, MD, USA). MTT (cell proliferation) and ATPase kit were purchased from Sigma (St. Louis, MO, USA).

1.2 Methods

1.2.1 Cell culture and drug treatment

Chang hepatoma cells were maintained in 25 cm^2 culture flasks supplemented with DMEM containing 10% FBS (DF-10), penicillin and streptomycin (50 units/mL) at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2 for at least 24 h. Cells were plated onto culture plates (100 mm^2) and exposed to DF-10 media and drug treatment for 24 h for Western blot and RT-PCR studies. For DCF and MTT assays cells were subcultured onto 96-well black plates (Corning, NY, USA) and allowed to attach for 8 h prior to any drug treatment. Following plate confluence, cells were exposed to DF-10 media containing silymarin (1 000 μg/L) or vehicle (control cells) for 24 h.

1.2.2 Measurement of oxidative stress induced by H_2O_2

Chang cells were cultured in black plastic 96-well culture plates (Costar #3603, Corning Incorporated) at a cell density of 2.5×10^4 cells/well with DF-10 culture medium. After 24 h incubation in DF-10 and drug (or vehicle), cultures were washed twice with PBS and then incubated with 10 μmol/L $DCFH_2$-DA in 95% air/5% CO_2 for 30 min at 37 ℃ in the dark. Extracellular $DCFH_2$-DA was removed by washing cultures twice with warm PBS. Cellular oxidative stress was induced by incubating cells with 400 μmol/L H_2O_2 in PBS containing Ca^{2+} and Mg^{2+} for 20 min at 37 ℃ in the dark. Negative controls were performed using the same conditions but without H_2O_2. Cellular fluorescence intensity of emitted dichlorofluorescein (DCF) signal was immediately measured using a BMG Lab Technologies Inc. (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader (485 nm excitation wavelength/520 nm emission wavelength) equipped with excitation and emission probes directed to the bottom of the plate. Mean fluorescence intensity was calculated from triplicate cultures of control and drug treated cells. Results were expressed in arbitrary fluorescence units (AFU).

1.2.3 Cell viability/cell proliferation: MTT assay

The MTT assay was used to assess cell growth and viability. Living cells reduce MTT to purple formazan by functional mitochondria. Assay conditions were conducted as outlined by the manufacturer. Briefly, Chang cells were cultured in 96-well plates at a concentration of 3×10^4 cells/well. Following exposure to silymarin (1000 μg/L) for 24 h, 10 μL of the MTT solution (10% of the culture volume) was added to each well. Cells were incubated with the MTT solution for an additional 3.5 h at which time the culture media was removed and the MTT solvent added to the wells (100 μL). Absorbance of the colored solution was recorded at 570 nm by using a spectra Max 190 (Molecular Devices) plate reader.

1.2.4 Measurement of cellular ATP levels

A bioluminescent assay was employed for the determination of total cellular ATP level. Procedural details were followed as outlined by the manufacturer.

1.2.5 Western blot

Cells were plated onto culture plates (100 mm^2) and exposed to DF-10 media and drug treatment for 24 h. Cells were then treated with 400 μmol/L H_2O_2 for 20 min and total cellular protein was determined using a protein assay kit (Life Science Research Division). Cellular proteins (20 μg) were subjected to SDS-PAGE with 15% SDS-polyacrylamide gel. Titration of primary antibody was 1∶1000 for rabbit anti-Bax and 1∶3000 for mouse anti-β-actin. The secondary antibody was anti-rabbit antibody for Bax and anti-mouse antibody for β-actin (1 ∶ 1000 dilution). Protein-antibody complexes were detected by enhanced chemiluminescence (ECL system, Amersham). The optical density (OD) values of each target protein band were determined by using National Institutes of Health (NIH) Imaging software. Protein loading variation was corrected by normalization of β-actin.

1.2.6 RT-PCR

Chang cells were plated onto culture plates (100 mm^2) and exposed to DF-10 media and drug treatment for 24

h followed by 20 min of 400 μmol/L H_2O_2 incubation. Total RNA was isolated from treated cells using TRIzol LS reagent as described in the manufacturer's manual. PCR was performed by using the Advantage PCR kit, polymerase mix, and oligonucleotides synthesized by Gibco-BRL (Burlington, ON, USA). Gene-specific PCR primers for bax were purchased from Sigma (St. Louis, MO). The oligonuleotide primers were 5′-GTTTCATCCAGGATCGAGCAG-3′(sense), 5′-CATCTTCTTCCAGATGGTGA-3′(antisense). Product length was 487 bp. The specific rat GAPDH amplimers were from Clontech (5507-3) with an expected GAPDH size of 986 bp. PCR amplification was carried out in 30 cycles of denaturation at 94 ℃ for 45 s, annealing at 53℃ (Bax) and 60℃ (GAPDH) for 45 s, elongation at 72℃ for 120 s with an additional 7-minute final extension at 72℃ using an Eppendorf MasterCycler (Eppendorf, Westbury, NY, USA). The PCR product was analyzed using a 1.2% agarose gel. Identity of PCR products was confirmed by sequencing at the DNA-sequencing facility of the Manitoba Institute of Cell Biology.

1.3 Statistical analysis

Data are presented as mean±SEM. The *n* value refers to the number of replicates performed for each study. Data were analyzed using *t*-test taking $P<0.05$ as the level for significance.

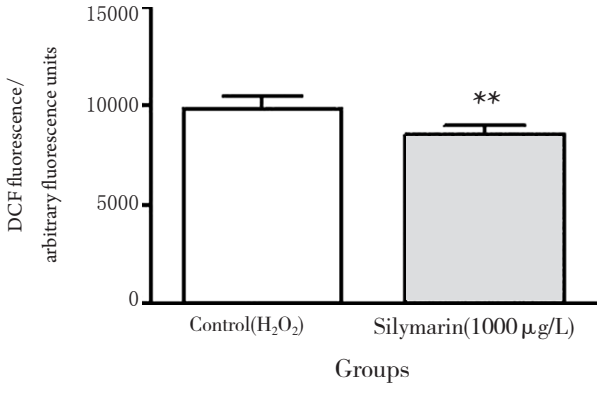

Figure 1 DCF fluorescence of Chang cells following oxidative stress induced by H_2O_2 (n=6). Cells were cultured in 96-well plates. $DCFH_2$-DA (10 mmol/L) was loaded onto cells for 30 min. Cells were subsequently exposed to 400 μmol/L H_2O_2 for 20 min. Cellular fluorescence in each well was measured and immediately recorded. Data are represented as mean ± SEM; ** $P<0.01$ vs control; one tail distribution.

2 Results

2.1 Intracellular free radical levels

Cellular free radical levels were assessed using the DCF assay. Figure 1 shows DCF fluorescence from H_2O_2 (400 μg/L) induced oxidative stress treated with silymarin (1000 μg/L) in Chang cells. Silymarin statistically reduced the free radical levels (n=6, $P<0.01$).

2.2 Cell proliferation

Cellular viability and proliferation were assessed using the MTT assay. Silymarin produced a slight but statistically significant ($P<0.05$) increase in cell number from the control or no drug treatment levels [(13.1±1.5)%, n=12].

2.3 Cellular ATP levels

To understand the protective nature of silymarin, we investigated the cellular ATP levels following drug treatment. Silymarin treatment did not significantly affect cellular ATP levels when oxidative stress was absent. However, as shown in Figure 2 following H_2O_2 treatment (data not shown here), ATP levels were significantly increased after silymarin (1000 μg/L) treatment($P<0.001$).

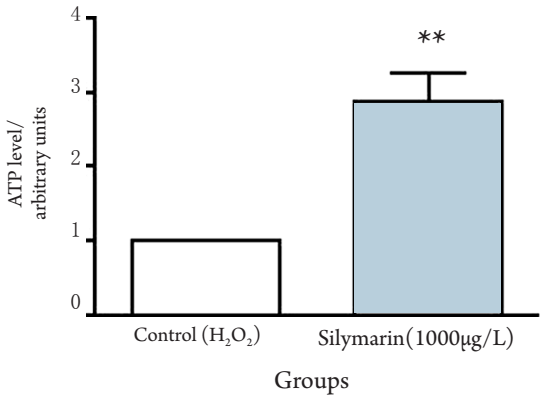

Figure 2 Cellular ATP levels in Chang cells as assessed by the bioluminescent assay(n=6). Data represent mean±SEM; **$P<0.001$ vs control; one tail distribution.

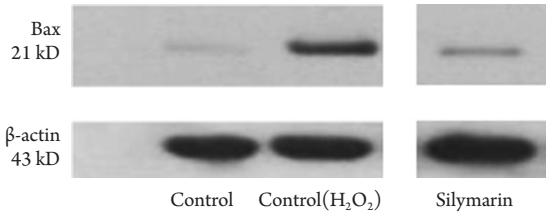

Figure 3 Expression of Bax protein in control and drug treatment Chang cells. Bax activity was used as the marker of apoptosis following H_2O_2 treatment. Blank contained serum-free media cells while control contained serum-containing media.

2.4 Bax protein level

To further understand the protective nature of silymarin we investigated whether drug treatment affected the pro-apoptotic protein Bax. Figure 3 shows a representative Western blot following drug treatment in Chang cells while Figure 4 summarizes the data. Silymarin treatment of Chang cells significantly reduced Bax to a greater extent than the control (H_2O_2) values (P<0.001).

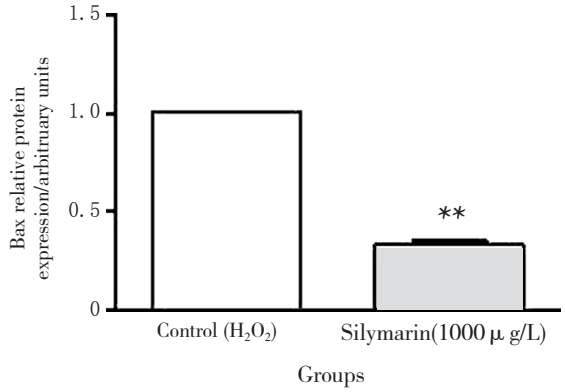

Figure 4 Relative Bax expression in Chang cells calculated by normalizing Bax optical density against actin (n=5). Data represent mean±SEM, **P<0.001 vs control; one tail distribution.

2.5 Bax mRNA level

To further elucidate the protective nature of drug treatment we investigated the effects of silymarin on Bax mRNA to understand if the protective function is related to enhanced degradation of the pro-apoptotic markers or transcription. Figure 5 shows a representative scan of RT-PCR bax mRNA while Figure 6 summarizes the data from the Chang cell line (n=3). Silymarin statistically reduced the bax mRNA from control levels (P<0.001).

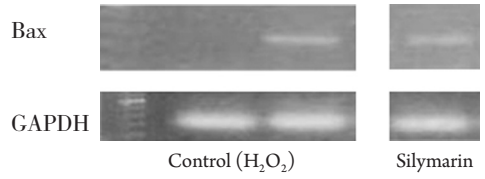

Figure 5 RT-PCR image of bax and loading control GAPDH in the control and drug treated cells.

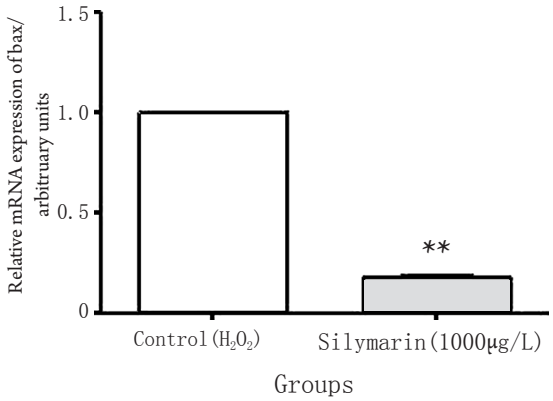

Figure 6 RT-PCR results of bax mRNA expression in Chang cells calculated by normalizing bax mRNA optical density against GAPDH (n=3). Data represent mean±SEM; ** P<0.001 vs control; one tail distribution.

3 Discussion

The objective of the present study was in determining the mechanism for the hepatoprotective effect of silymarin. Silymarin has been used for many years in the treatment of liver diseases[7] including alcoholic induced liver disease[3,8] and CCl_4-induced oxidative damage[2]. The mechanism for this effect is thought to be its ability to trap free radicals, thereby, producing a membrane stabilizing effect[9]. Treatment with low dose silymarin is not thought to provide any beneficial effect while higher doses have been shown to provide a therapeutic effect in reversing the fibrosis produced from prolonged CCl_4-treatment in animals[9]. Moreover, because of its hepatoprotective properties silymarin, as a plant extract, is used as a standard in models of liver disease to which other plant extracts are measured[10].

Silymarin is known to reduce the rise in intracellular Ca^{2+} levels induced by ter-butyl hydroperoxide in rat hepatocytes, suggesting that the hepatoprotective effect of silymarin is not only due to the inhibition of lipid peroxidation but also modulation of intracellular calcium levels[11]. The reduction in reactive oxygen species (ROS) levels in Chang cells was not surprising given the many reports suggesting silymarin's hepatoprotective effect is through its antioxidant activity[2, 4]. Thus, the reduction of free radical damage to phospholipids within the cell membrane would further limit any increase in nonspecific calcium permeability induced by membrane oxidation. Silymarin may also have an indirect effect on cells. Since the ATP levels of cells in the presence of silymarin were much greater than those not exposed to the drug, silymarin may mediate preservation of mitochondrial function such that the cell is able to continually excrete calcium ions during phases of intracellular calcium overload. The ability

to maintain calcium flux may be due to either silymarin's effect as an antioxidant by reducing intracellular free radical levels and/or some direct effect on mitochondria through modulation of mitochondrial calcium ion channels.

Silibinin, the active constituent of silymarin, has been shown to protect cardiac myocyte injury through the mitochondrial pathway[12]. Silibinin also has been shown to inhibit the release of the pro-apoptotic cytochrome c from mitochondria and increase the expression of the anti-apoptotic Bcl-2 family protein. Thus, silibinin inhibits Bax translocation from the cytoplasm to the mitochondria. Similar results have been reported in UV-induced cell apoptosis in human malignant melanomas[13]. Whether this effect is primarily a direct result of its antioxidant action or secondarily to the inhibition of the release of the pro-apoptotic factors is not clear. In our study silymarin reduced these levels, which were likely a result from reduced bax mRNA as shown by RT-PCR. Thus, silymarin's effects are upstream from the translocation of bax from the cytoplasm to the mitochondria and may occur at the gene level.

Silymarin was effective in enhancing cell proliferation. The modest increase in proliferation rate is in contrast to that reported by Bhatia et al[14].That group reported that silymarin at doses of 100 μmol/L inhibited cell growth of prostate, breast and cervical human carcinoma cells grown in culture but had little effect on cell viability. In the present study the addition of 1000 μg/L silymarin to cells enhanced cell growth. Whether it is an actual increase in proliferation or enhanced cell viability is not clear. Since silymarin increased ATP levels in cells subjected to oxidative stress, enhanced viability is likely. Similar results were reported by Ligeret et al[15]. Using a cold preservation-warm reperfusion isolated liver model, they reported that silibinin increased ATP levels (39%) and decreased oxidative stress to values observed in control non-ischemic livers. A direct antioxidant effect on the mitochondrial membrane may also contribute to cell viability. In conclusion, the present study shows that silymarin substantially reduced ROS levels, enhanced ATP levels as well as cell proliferation when hepatocytes were subjected to oxidative stress by the addition of hydrogen peroxide. The improved cell function was accompanied by reduced bax mRNA resulting in reduced bax levels. Silymarin can function as a hepatoprotectant against free radical damage due to oxidative stress and may be a useful adjuvant for treatment of specific liver diseases.

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