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Original Article

Silymarin inhibits cisplatin-mediated apoptosis via inhibition of hydrogen peroxide and hydroxyl radical generation

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Abstract

Cisplatin mediated nephrotoxicity has been continuously reported and recognized as a major obstacle for cisplatin-based chemotherapy. The present study aimed to demonstrate the potential use of silymarin, an extract from the seed of *Silybum marianum* L., as a combination therapy with cisplatin. Previous studies indicated that cisplatin-mediated toxicity was primarily caused by cellular oxidative stress. This study found that pretreatment with silymarin significantly attenuated oxidative stress induced by cisplatin in human renal epithelial cells (HK2-cells) and protected against cisplatin-mediated apoptosis. Moreover, the present study demonstrated that silymarin could attenuate hydrogen peroxide and hydroxyl radical generated by cisplatin while having minimal effect on superoxide anion level. In summary, these observation showed significant impact of silymarin in the inhibition of cisplatin-mediated renal cell death *in vitro* and could be beneficial for the development of this compound as a combination therapy in patients before receiving cisplatin.

Keywords: silymarin, cisplatin, renal epithelial cell, nephroprotective, ROS

1. Introduction

Cisplatin is a platinum-based chemotherapeutic drug belonged to a class of medicine known as alkylating agent (Harper *et al.*, 2010). Although cisplatin is recommended as an effective therapy for the treatment of various cancers including ovarian, bladder, head and neck, lung, breast and prostate cancers (Taguchi *et al.*, 2005; Azzoli *et al.*, 2007; Brito *et al.*, 2012), its severe toxic effect to the kidney limits the use of cisplatin in many patients (Arany and Safirstein, 2003; Sastry and Kellie, 2005; Xin *et al.*, 2007). In certain cases, nephrotoxicity induced by cisplatin occurred as an irreversible decrease in glomerular filtration and subsequently resulted in an enhanced accumulation of cisplatin in tubular cells leading to renal tubular toxicity and renal failure (Taguchi *et al.*, 2005; Ali and Al Moundhri, 2006). A number of *in vivo* and *in vitro* studies suggested that the mechanism of cisplatin-induced nephrotoxicity is closely associated with an ability of cisplatin to generate cellular reactive oxygen species (ROS) (Pongjit *et al.*, 2011; Sögüt *et al.*, 2004). The high accumulation of ROS namely superoxide anion, hydrogen peroxide, and hydroxyl radical in tubular cells during cisplatin treatment can cause oxidative damage to the cells, leading to both apoptosis and necrosis cell death (Fukutomi *et al.*, 2006; Baek *et al.*, 2003; Jiang *et al.*, 2007; Çetin *et al.*, 2006).

Among the various plant-originated antioxidant compounds, silymarin L, which is a standardized extract obtained from the seed of *Silybum marianum* (L.), has exhibited the most potential in effectively protecting the liver

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cells against oxidative stress-mediated damages (Saller *et al.*, 2001; Patel *et al.*, 2010; Velebny *et al.*, 2010). Not only does silymarin exhibited a strong antioxidant activity in hepatocyte, but also this agent enhances the cell regeneration (Sonnenbichler and Zetl, 1986; Magliulo *et al.*, 1973). Furthermore, the safety profile of silymarin was ensured by the approval of FDA for using in the treatment of liver disease. Although silymarin possesses several activities which might attenuate renal toxicity induced by cisplatin, the effect of this agent in renal cell death-mediated by cisplatin treatment and underlying mechanism is largely unclear.

The objective of this study was to determine the ability of silymarin to prevent cisplatin-induced cytotoxicity in human proximal tubular cells (HK2-cells) and to evaluate the underlying mechanism involving specific ROS scavenging activity of silymarin. The finding of present study may benefit the development of a novel strategy in using this considered safe compound in cisplatin-based chemotherapy.

2. Materials and Methods

2.1 Chemicals

Cisplatin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ), 2,7-Dichlorofluorescein diacetate (DCFH2-DA), Hoechst 33342, silymarin, and Propidium iodide (PI) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Ferrous sulphate (FeSO4.7H2O) was obtained from Ajax Finechem Pty Ltd (Australia).

2.2 Cell culture

Human proximal tubular cells line (HK2-cells) was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37°C in 5% CO_2 humidified atmosphere with Dulbecco's modified eagle medium (DMEM), Life Technologies Inc. (Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), SUMMIT Biotechnology (Fort Collins, CO, USA), 1% L-glutamine, Life Technologies Inc. (Rockville, MD, USA) and 0.01% penicillin/streptomycin/amphotericin B, Sigma Chemical Inc. (St. Louis, MO, USA). HK-2 cells were grown and maintained in T 25 cm^2 tissue culture flasks at 37°C in 5% CO_2 humidified atmosphere. The cells were used for experiments between passages 25 and 40. For MTT, apoptosis and necrosis assay, the cells were seeded, 100,000 cells/well, in 96-well culture plates and allowed to grow to ~70%-80% confluence before the addition of test compounds.

2.3 Cell viability assay

The cytoprotective effect of silymarin on cisplatin-induced cytotoxicity in HK2-cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cells in 96-well plates were pretreated with silymarin (25-400 μM) for 30 minutes before the addition of cisplatin (IC50 = 100 μM) and continued to be cultured for 24 hours at 37°C. After 24 hours of treatment, the cells were incubated with 500 mg/mL of MTT for 4 hours at 37°C. The intensity of the MTT product was measured at 570 nm using a microplate reader. The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. The cultured cells incubated without the test compounds served as a control. Dose-response studies on cisplatin and silymarin were performed to determine the effect of cisplatin on HK2-cells viability and to see if silymarin could enhance HK2-cells proliferation. Cisplatin concentrations tested were 5, 25, 50 and 100 μM. Silymarin concentrations tested were 25, 50, 100, 200 and 400 μM. Each experiment was performed in triplicate and repeated for each condition tested.

2.4 ROS detection

The scavenging activities of silymarin against cisplatin-generated ROS in HK2-cells were investigated by a flow cytometric assay using dihydrodichloro-fluorescein diacetate (DCFH2-DA) and dihydroethidium (DHE) as the fluorescent probes. The cells in 96-well plates were incubated with the probes (15 μM) for 30 minutes at 37°C, after which the cells were washed, resuspended in phosphate-buffered saline and pretreated with silymarin (25-50 μM) for 30 minutes before the addition of cisplatin (100 μM). After 2 hours incubation time, the cells were immediately analysed for the fluorescene intensity by FACScan flow cytometer (Becton Dickinson, Rutheford, NJ, USA) at the excitation and emission wavelengths of 485 and 538 nm, respectively, for DCFH2-DA fluorescence measurements and at 488 and 610 nm for DHE measurements. The fluorescence response was evaluated by CellQuest software (Becton-Dickinson) analysis of the recorded histogram. To investigate further on the scavenging activities of silymarin toward hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\bullet -}$) and hydroxyl radical (OH), cells were treated with specific ROS generators before determining intracellular ROS level by flow cytometry. The ROS generators were DMNQ ($O2^{\bullet -}$ generator), hydrogen peroxide and the combination of hydrogen peroxide and ferrous sulfate ($^{\bullet}$OH generator).

2.5 Apoptosis and necrosis assay

The effect of silymarin on cisplatin-induced cell apoptosis and necrosis was determined by Hoechst 33342 and propidium iodide (PI) co-staining (Molecular Probes). The cells in 96-well plates were pretreated with silymarin (25-50 μM) before the addition of cisplatin (IC50 = 100 μM) and continued to be cultured for 24 hours at 37°C. After 24 hours incubation time, the cells were washed and incubated with 10 μg/mL Hoechst 33342 and 5 μg/mL PI for 30 minutes at 37°C before visualizing for apoptotic and necrotic cells under a fluorescence microscope (Olympus IX51 with DP70). The

apoptotic cells will exhibit a shrunken nuclei and an intense nuclear fluorescence in response to Hoechst staining. The necrotic cells will exhibit an intense PI nuclear fluorescence response, suggesting the loss of plasma membrane integrity. The cultured cells incubated without the test compounds served as a control. To demonstrate the induction effect of cisplatin on cell apoptosis and necrosis, HK2-cells were incubated with 100 µM cisplatin. Each experiment was performed in triplicate and repeated for each condition tested.

2.6 Statistical analysis

Data were expressed as the means ± SD from three or more independent experiments. Statistical analysis was performed by Student's t-test at a significance level of $p<0.1$.

3. Results

3.1 Cisplatin induced cytotoxicity in HK2-cells

Cisplatin-mediated cytotoxicity in HK2-cells was characterized and is shown in Figure 1. HK-2 cells were treated with various concentrations of cisplatin (0-100 µM) for 24 hours. Cell viability was then evaluated using MTT assay. Results showed that cisplatin exposure for 24 hours caused a dose-dependent toxicity with approximately 60% and 40% of cells remained viably at the doses of 50 µM and 100 µM of cisplatin, respectively (Figure 1A). Since apoptosis and necrosis are two major pathways of cell death, mode of HK2-cells death in response to cisplatin treatment was clarified by Hoechst 33342 and PI co-staining assay. Hoechst and PI assay showed an increase in chromatin condensation and nuclear fluorescence response of cisplatin-treated cells compared with non-treated cells, suggesting an induction of apoptotic cells (Figure 1B) and necrotic cells (Figure 1C) by cisplatin.

3.2 Silymarin stimulated HK2-cells proliferation

The effect of silymarin on the vitality of HK2-cells is shown in Figure 2. Cells were exposed to 0-400 µM silymarin for 24 h in normal culturing condition. MTT results indicated that all concentrations of silymarin did not cause any toxicity to HK2-cells. In contrast, silymarin at the concentrations of 400 µM significantly enhanced the percentage of cell viability compared with the control (cells without silymarin pretreatment, $p<0.1$). There was no significant difference in the percentage of cell viability among cells treated with 0-200 µM silymarin. These results suggested that silymarin is not only non-toxic to the cells, but also enhance the vitality of HK2-cells at high concentrations.

3.3 Silymarin inhibition of cisplatin-induced cytotoxicity

The inhibitory effect of silymarin on cisplatin-induced toxicity in HK2-cells is demonstrated in Figure 3. HK2-cells were pretreated with silymarin (25-400 µM) for 30 minutes and exposed to cisplatin (100 µM). After 24 hours incubation, cell viability was determined, and the result showed that silymarin-pretreated cells exhibited a greater viability than cisplatin-treated cells without silymarin (Figure 3A, $p<0.1$). The percentage of cell viability increased from 48% to 103% as the concentrations of silymarin increased from 0 µM to 400 µM. In addition, Hoechst 33342 and PI co-staining assay suggested that silymarin was able to modulate cell apoptosis

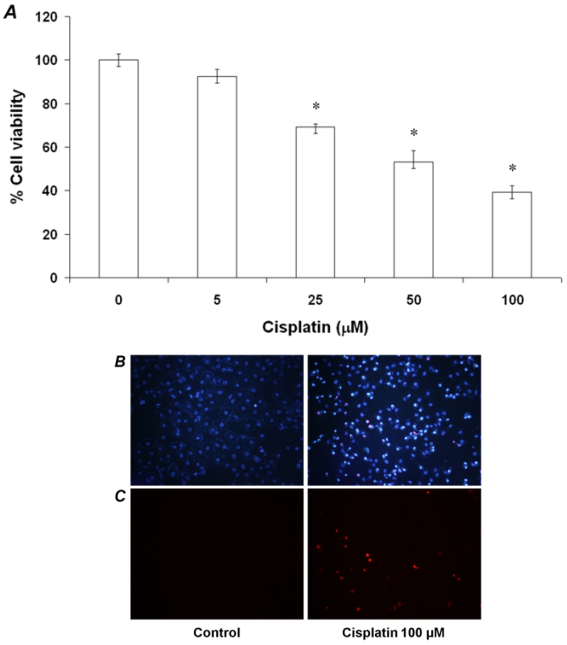

Figure 1. Cisplatin induced cytotoxicity in Human Kidney cell line (HK2-cells). Cells were treated with 0-100 mM cisplatin at 37°C for 24 hours. *A*, dose effect of cisplatin on cell viability was determined by MTT assay. *B*, effect of cisplatin on cell apoptosis was determined by Hoechst 33342 assay. *C*, effect of cisplatin on cell necrosis was determined by PI exclusion assay. *Columns*, mean (n=3); bars, SD; *, $P < 0.1$ versus non-treated control.

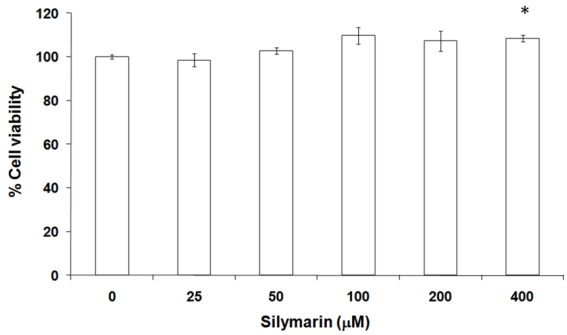

Figure 2. Silymarin stimulated Human Kidney cell line (HK2-cells) proliferation. Cells were treated with 0-400 mM silymarin at 37°C for 24 hours. Dose effect of silymarin on cell proliferation was determined by MTT assay. *Columns*, mean (n=3); bars, SD; *, $P<0.1$ versus non-treated control.

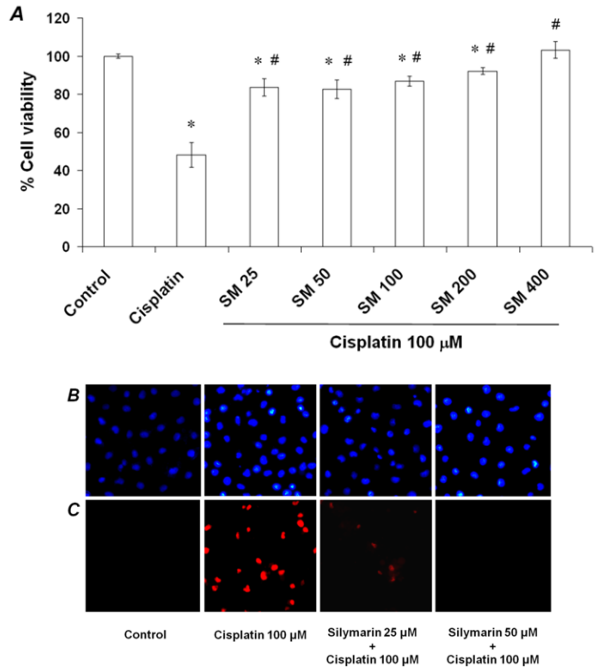

Figure 3. Silymarin inhibited cisplatin-induced cytotoxicity in HK2-cells. Cells were pretreated with 0-400 mM silymarin for 30 minutes followed by 100 mM cisplatin for 24 hours. *A*, effect of silymarin on the viability of cisplatin-treated cells was determined by MTT assay. *B,* effect of silymarin on cisplatin induced cell apoptosis was determined by Hoechst 33342 assay. *C*, effect of silymarin on cisplatin induced cell necrosis was determined by PI exclusion assay. *Columns*, mean (n=3); bars, SD; *, $P<0.1$ versus non-treated control; #, $P<0.1$ versus 50 μM cisplatin-treated control.

and cell necrosis induced by cisplatin, resulting in the improvement of cell viability. A decrease in chromatin condensation and PI nuclear fluorescence response of HK2-cells with 25 and 50 mM silymarin pretreatment compared to cisplatin-treated cells without silymarin, indicating the reduction of cell apoptosis and necrosis by silymarin (Figure 3B and 3C). At higher concentration of 50 mM silymarin, no necrotic cells were detected, suggesting the ability of silymarin to inhibit cell necrosis at high concentration.

3.4 Silymarin inhibition of cisplatin-induced ROS generation

Flow cytometric analysis of ROS using DCFH2-DA as a specific oxidative probe indicated that silymarin was able to inhibit cisplatin-induced ROS generation in HK2-cells. HK2-cells pretreated with silymarin (25 and 50 μM) before the addition of cisplatin (100 μM) showed a lower DCFH2-DA fluorescence response than cisplatin-treated cells without silymarin pretreatment (Figure 4, $p<0.1$). The effect of silymarin in inhibiting cisplatin-generated ROS in HK2-cells contributed to its antioxidant properties.

3.5 Silymarin primary moderation of hydrogen peroxide and hydroxyl radical

The ROS scavenging mechanism studies showed that silymarin had scavenging activity against hydrogen peroxide and hydroxyl radical induced oxidative stress in HK2-cells (Figure 4). The pretreatment with silymarin before the addition of hydrogen peroxide or the mixture of hydrogen peroxide and ferrous sulfate reduced the DCFH2-DA fluorescence response of HK2-cells compared with no silymarin pretreatment. In contrast, silymarin showed no scavenging activity toward superoxide anion. The DHE fluorescence response, a specific fluorescence dye for superoxide anion detection, after DMNQ exposure was slightly increased with silymarin pretreatment (Figure 4).

4. Discussion

Cisplatin-mediated renal toxicity has been shown to be one important obstacle for efficient cisplatin-based chemotherapy. In the present study, cisplatin-induced dose-dependent toxicity in HK2-cells was observed and such cytotoxicity was attenuated by pretreatment of the cells with silymarin. Since many studies supported the possibility that cisplatin contributed cell damage through oxidative stress (Baek *et al.*, 2003; Matsushima *et al.*, 1998; Kruidering *et al.*, 1997; Zhang *et al.*, 2001), the protective effect of silymarin in the present study was likely due to antioxidant activity of the

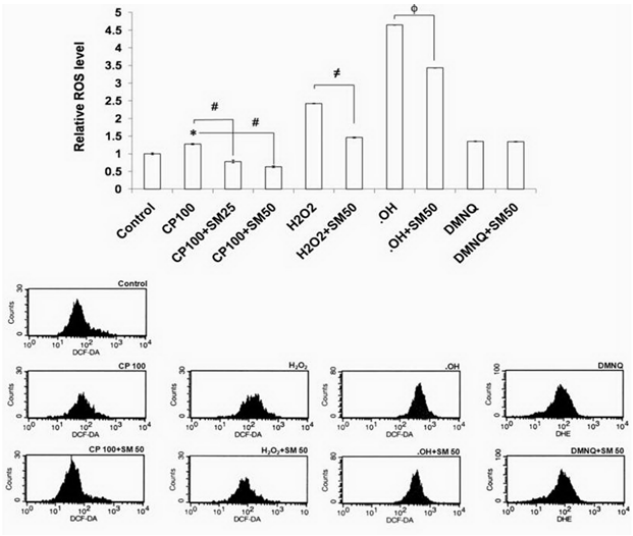

Figure 4. ROS scavenging activity of silymarin. HK2-cells were pretreated with 25 or 50 μM silymarin for 30 minutes. The cells were then treated with cisplatin (100 μM), hydrogen peroxide (400 mM), hydrogen peroxide/ferrous sulphate (1000 mM) or DMNQ (20 mM) for 30 minutes. ROS production was determined by flow cytometry using the fluorescent probe DCFH-DA. *Columns*, mean (n=3); bars, SD; *, $P<0.1$ versus non-treated control; #, $P<0.1$ versus cisplatin-treated control; ¹, $P<0.1$ versus H_2O_2-treated control; ϕ, $P<0.1$ versus $^{\bullet}OH$ -treated control.

compound. Although antioxidant capability of silymarin has been continuously revealed (Abdelmeguid *et al.*, 2010a; Mansour *et al.*, 2006; Abdelmeguid *et al.*, 2010b; Gholamreza *et al.*, 2005), the definite antioxidant mechanism, especially in renal epithelial cells, is not yet well elucidated. In an attempt to promote the development of silymarin to be used as additive drug benefiting cisplatin-based chemotherapy, the present study verified the specific mechanism of silymarin in protection of renal cell from cisplatin-mediated death. Indeed, an increased in cellular ROS can affect the integrity of mitochondrial membrane and other part of cells leading to the activation of apoptosis and/or necrosis (Sgonc and Gruber, 1998; Kanduc *et al.*, 2002). Previous studies indicated that cisplatin treatment contributed to an increase of specific ROS, namely superoxide anion, hydroxyl radical (Fukutomi *et al.*, 2006; Matsushima *et al.*, 1998) and hydrogen peroxide (Pongjit *et al.*, 2011). Accumulation data indicated that each species of ROS varying in chemical structure and reactivity could affect the cell differently. Hydroxyl radical has been shown to be the most reactive among the named ROS and was linked tightly to cell membrane damage resulting in necrotic cell death (Baek *et al.*, 2003; Shino *et al.*, 2003).

Our results fit with the above finding that silymarin has potent antioxidant acivity against hydroxyl radical (Figure 4), and is able to reduce cisplatin-mediated necrosis (Figure 3). Furthermore, the ability of silymarin in attenuated cellular hydrogen peroxide presented in Figure 4 of this study may in part render the cell to cisplatin-induced apoptosis since hydrogen peroxide mediated cell apoptosis has been observed in several studies (Lee *et al.*, 2006; Ponnusamy *et al.*, 2009; Ueda and Shah, 2000). The ratio of necrosis to apoptosis in 100 μM cisplatin treated cells was 1:2 showing that apoptosis was predominant. It was believed that the intracellular ROS (oxidative stress) generated by cisplatin is a key initiator for such cell deaths. It is widely accepted that ROS generated by cisplatin are important inducer of apoptosis as well as necrosis cell death in many cells (El-Garhy *et al.*, 2014; Gatti *et al.*, 2014; Gonzalez *et al.*, 2001; Zhou *et al.*, 2013). Also, our results fit with the above mentioned since the ROS suppressing effect of silymarin was correlated with its protective effect. It has been intensively reported that oxidative stress cause either by other inducers or cisplatin (Florea and Busselberg, 2011; Luanpitpong *et al.*, 2012; Moungjaroen *et al.*, 2006) mediates cell death through mitochondrial (intrinsic) apoptosis pathway. Therefore, in the present study cisplatin mediated cell apoptosis via mitochondrial pathway. Although verifying the mitochondrial activity should reflect the activation of intrinsic apoptosis pathway, in our opinion MTT cell viability assay measuring the activity of mitochondrila reductase may imply the conception mentioned above, because ROS-cisplatin induced cell death through mitochondrial pathway is well known. The ROS scavenging activity of silymarin could possibly depend on the arrangement of functional group around the flavan backbone. The presence of free 3-OH is important for the scavenging effect of silymarin toward OH. A 3-OH can donate hydrogen to OH, yielding a relatively stable silymarin radical and water, thus reducing the detrimental effect of OH (Heim *et al.*, 2002; Varga *et al.*, 2006). A 5-OH and 7-OH may also contribute to the scavenging effect of silymarin toward OH but their impact on the scavenging activity is of questionable significance (Heim *et al.*, 2002).

For a superoxide scavenging activity, a 3′-OH in ring B, a 3-OH in ring C, and the double bond at C_2 and C_3 position are essential (Varga *et al.*, 2006; Svobodova *et al.*, 2006; Cos *et al.*, 1998). Among all the components of silymarin, only silychristin possesses a 3′-OH. However, the contribution of silychristin on the $O_2^{\bullet -}$ scavenging activity of silymarin is considered insignificant due to its low content (Kren and Walterova, 2005; Šimánek *et al.*, 2000). Moreover, silymarin has no double bond between C_2 and C_3 in its structure, making it unsuitable for $O_2^{\bullet -}$ scavenging.

Another mechanism that might be responsible for ROS scavenging effect of silymarin is its ability to stimulate glutathione synthesis (Alidoost *et al.*, 2006) and prevent the depletion of glutathione. For H_2O_2, silymarin may reduce its oxidizing capacity through glutathione related pathway. Glutathione functions as a cofactor for glutathione peroxidase enzyme in cellular oxidation-reduction systems. Reduced glutathione (GSH) can donate electron to the reactive hydrogen peroxide, yielding oxidized glutathione (GSSG) and water, thereby reducing the harmful effect of the reactive peroxide. Moreover, glutathione is involved in the detoxification of cisplatin. It could bind with cisplatin to form a less toxic and more water soluble compound, bis-(glutathionato)-platinum, thus also reducing cisplatin toxicity (Zhang *et al.*, 2001; Kosmider *et al.*, 2004).

Dose dependence studies showed that none of the concentrations of silymarin caused toxicity to HK2-cells. Furthermore, silymarin at concentration of 400 μM showed an increase in the percentage of cell viability compared with the untreated cells, suggesting that silymarin could stimulate HK2-cells proliferation. Silymarin is an antioxidant that capable of modulating ROS and stimulating RNA, DNA and protein biosynthesis (Sonnenbichler *et al.*, 1999), which are the important steps for cell growth and proliferation, while cisplatin can cause damage to RNA, DNA and protein through a ROS dependent mechanism (Jordan and Carmo-Fonseca, 2000; Jamieson and Lippard, 1999; Tay *et al.*, 1988). Therefore, the effect of silymarin on inhibiting cisplatin-generated ROS and reducing its toxicity in HK2-cells may contribute to its abilities to scavenge hydrogen peroxide and hydroxyl radical and to stimulate HK2-cells proliferation at high concentration.

In summary, treatment with silymarin could protect HK2-cells from the toxic effect of cisplatin. The cytoprotective effect of silymarin is associated with its antioxidant properties, as silymarin was shown to scavenge $^{\bullet}OH$ and H_2O_2, the harmful ROS induced by cisplatin. These results provide a further rationale for examining the beneficial use of silymarin in patients receiving cisplatin and other platinum-based chemotherapy treatment.

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