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Effect of Silymarin against CAF protocol Hepatotoxicity

Bahir Abdul Razzaq Mshemish*, Khalid Abdallaa Al-Khazragy, Munoor Abdul Ilah AL-Nakaash*** and Wiaam Abdul Fatah*****

**Pharmacotherapy dept., College of Pharmacy, Al-Must.Univ.*
***Medicine dept., College of Medicine, Baghdad Univ.*
****Oncology dept., College of Medicine, Baghdad Univ.*

الخلاصة:

يحتل سرطان الثدي لدى النساء اهمية كبيرة من حيث كونه الاكثر شيوعا وانتشارا بين باقي الاورام السرطانية في مجتمعنا. . يعتقد ان السبب الحقيقي وراء هذا المرض هو تغيرات في الحامض النووي منقوص الاوكسجين (دنا , DNA)نتيجة لافعال تأكسدية والتي تنتهي بانتاج الجذور الحرة. يعتبر خليط العلاج الكيمياوي CAF(Cyclophosphamide+ Adriamycin + 5- FU) من اهم الانظمة العلاجية المستعملة في سرطان الثدي لكن استخدامه مرتبط بتاثيرات سامة في أعضاء الجسم المختلفة (كالكبد) من خلال ألية الاجهاد التأكسدي وأنتاج الجذور الحرة. مادة السيليمارين(silymarin) هي الخلاصة ألجافة للنبتة*silybym marianum* والمكونة من مركبات تستخدم سريريا كمضادات تأكسد ومكملات غذائية لفعاليتها في حماية بعض اعضاء الجسم من الاضرار النسيجية التي تسببها بعض الادوية والسموم . صممت هذه الدراسة السريرية لغرض بحث دور الحماية المحتمل لمادة السيليمارين المعطاة فمويا كمضاد أكسدة للوقاية من تسمم الكبد الناشئ عن الاجهاد التأكسدي اثناء العلاج بألخليط الكيمياوي(CAF) لدى النساء اللواتي يعانين من سرطان الثدي .

أشتملت هذه الدراسة على 74 سيدة كن مصابات بسرطان الثدي وخضعن لنمط الدراسة باستخدام طريقة التوزيع العشوائي حيث تم توزيعهن على ثلاثة مجاميع:

المجموعة(أ): تضمنت 24 مريضة استلمن الخليط الكيمياوي (CAF) بواسطة الحقن الوريدي لثلاث جرع وبمعدل جرعة واحدة كل ثلاثة اسابيع.

المجموعة(ب): تضمنت 25 مريضة استلمن نفس الخليط الكيمياوي (CAF) في المجموعة (أ) مضافا له مادة السيليمارين على شكل كبسول اعد خصيصا لهذا الغرض وبجرعة 210 ملغم/يوميا.

المجموعة(ج): تضمنت 25 مريضة استلمن نفس الخليط الكيمياوي (CAF) في المجموعة (أ) مضافا له مادة السيليمارين على شكل كبسول اعد خصيصا لهذا الغرض وبجرعة 420 ملغم/يوميا.

أعتمدت طرق التقييم على قياس مستويات وظائف الكبد (AST,ALT,TSB) في مصل الدم. تم قياس هذه المؤشرات للمجاميع المرضية الثلاثة قبل أعطاء العلاج و بعد 21, 42 و 63 يوماً من أعطاءه.

تسبب خليط العلاج الكيمياوي (CAF) في حصول ارتفاع معنوي في مستويات كل من (AST,ALT) بينما اثر السيليمارين في حصول انخفاض معنوي لهذه المستويات وبدرجة تعتمد على الجرعة المستخدمة وفترة العلاج . بالنسبة لمستوى ال (TSB) فقد لوحظ انخفاضه بصورة معنوية بعد المعالجة بالخليط الكيمياوي (CAF) لكن مستواه لم يتغيربشكل ملحوظ بعد اضافة مادة السيليمارين لهذا الخليط . في ضوء النتائج التي افرزتها هذه الدراسة يمكننا الاستنتاج بان سرطان الثدي وخليط العلاج الكيمياوي (CAF) يؤديان الى تكوين الجذور الحرة وبالتالي تضعيف الية الدفاع بواسطة مضادات الاكسدة مسببة تاثيرات سمية على الكبد, ولذلك فأن استخدام السيليمارين كمادة مضادة للاكسدة في هذه الدراسة السريرية بامكانها ان تقلل (اعتمادا على مقدار الجرعة وفترة الاستخدام) من مضاعفات عملية فرط الاكسدة وما ينتج عنها من ضرر على الكبد.

Abstract:

Breast cancer became the commonest type of cancers among Iraqi women since the last two decades. The main underlying cause is thought to be DNA damage; much of which is oxidative in nature. CAF protocol (Cyclophosphamide + Adriamycin + 5-FU) associated with toxic effects in several body organs (like liver), mainly through production of free radicals and reactive oxygen species. Silymarin, the dried extract of a ripe seeds of the plant *silybium marianum*, was found to be a powerful antioxidant agent against toxin -induced tissue damage.

Aim of the study is to evaluate the possible time and dose-dependent protective effect of the orally administered silymarin as antioxidant agent against oxidative stress-related hepatotoxicity induced by CAF protocol in breast cancer wome.

Seventy four breast cancer women randomly distributed and allocated into three groups:

Group (A): 24 patients received CAF protocol by I.V infusion once every 21 days and for 63 days.

Group (B): 25 patients received 210mg/day of silymarin along with the same CAF protocol of group (A).

Group (C): 25 patients received 420mg/day of silymarin along with the same CAF protocol of group (A).

Indices of liver function (AST, ALT,TSB) were measured at baseline, after 21, 42, and 63 days of treatment.

The levels of AST and ALT, which were significantly elevated due to CAF protocol therapy, showed significant reduction when silymarin used with CAF protocol, in a time and dose-dependent manner. Mean while, TSB levels significantly reduced by CAF protocol but dose not show any significant change after treatment with silymarin.

Use of antioxidant agent (silymarin) in this study can ameliorate, in a time and dose-dependant manner, liver damage that induced by oxidative stress.
Keywords: Breast cancer, CAF protocol (cyclophosphamide+Adriamycin+5-fluorouracil), hepatotoxicity, silymarin.

Introduction:

Breast cancer is a malignant tumor that has developed from cells of the breast. It occurs almost in women, but men can get it, too [1]. According to cancer registry section (Iraqi Cancer Board) / Baghdad / MOH, breast carcinoma is the most common malignant tumor in Iraqi women and it comprise (31.3%) of all female malignant cases [2]. The most beneficial and commonly used staging system of breast cancer is the American Joint Committee on Cancer (AJCC) classification, which is based on the tumor size (T), the status of regional lymph nodes (N) and the presence of distant metastasis (M). Techniques that are commonly used to evaluate breast masses are: physical examination, mammography and fine needle aspiration cytology (FNAC) [3].

Local treatment (surgery or radiation) and systemic treatment (hormonal or chemotherapy) can be planned by number of ways. The most common sequence is: surgery, chemotherapy, radiation and then hormonal therapy. Combination of two or three chemotherapeutic drugs is used in breast cancer to avoid drug resistance and for better response. Several such combination regimens or protocols are available, such as CAF (Cyclophosphamide + Adriamycin + 5-FU), and CMF (Cyclophosphamide + Methotrexate + 5-FU) [4].

Adriamycin, or Doxorubicin, is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius var. caesius*; Cyclophosphamide is a synthetic antineoplastic drug chemically related to the nitrogen mustards; while 5-Fluorouracil (5-FU) is a pyrimidine analog (5-fluoro-2, 4(1*H*,3*H*)-pyrimidinedione). The use of these cytotoxic drugs against breast cancer is limited by number of adverse effects and toxicities, including cardiotoxicity (acute like tachyarrhythmia and ECG changes; and chronic like CHF and cardiomyopathy), nephrotoxicity, hepatotoxicity, neurotoxicity, myelosuppression and blood disorders. Much of these complications attributed to the induction of oxidative stress by CAF protocol (especially by doxorubicin) [5].

Silymarin is a mixture of flavonolignans isolated from the ripe seeds of the medicinal plant *Silybum marianum* (milk thistle), comprised mainly of silybinin, isosilybinin, silychristin, silydianin and taxifolin [6]. Multiple biological effects of flavonoids have been described, including anti-inflammatory, anti-allergic, anti-haemorrhagic, anti-mutagenic, anti-neoplastic and hepatoprotective activities. Most flavonoids, including silybinin, can protect cells and tissues against the harmful effects of reactive oxygen species (ROS). Their antioxidant activity results from scavenging of free radicals and other oxidizing intermediates, chelation of iron or copper ions and inhibition of oxidases [7]. Flavonoids from *Silybum marianum* have been widely used for treatment of liver disorders. No adverse reactions have been reported due to silymarin use in rats or human; either with short term or in long-lasting therapy [8].

The aim of the present study was to evaluate the possible time and dose-dependent effects of the orally-administered silymarin as a protective agent against oxidative stress–related hepatotoxicity which could be induced by CAF protocol in women with breast cancer.

Patients and Methods:

Drugs used in this study involve Doxorubicin, Cyclophosphamide, 5-Fluorouracil (from Ebewe Pharma, Austria) and standardized powder of Silymarin (Luna comp.,Egypt). Ready made kits (Bio Merieux/France) were used to determine serum aspartate and alanine transaminase activity (AST and ALT) and total serum bilirubin (TSB) levels by using UV Spectrophotometer (Jenway 6300, U.K).

This randomized clinical study was carried out on 80 female patients with different stages of breast cancer, all pass through one type of operative mastectomy and this is the first time they receive chemotherapy in their life's. These patients were with age range of 41-60 years (mean: 49± 1.5) and body weight range of 65-96 kg (mean: 76± 2.5). Certain exclusion criteria were followed to avoid interference of any other factors and include: those with history of previous chemotherapy, hepatic disorders, pregnant and breast feeding women, and those for whom any of CAF protocol components is contraindicated.

Only 74 female patients completed this study, others were excluded due to poor compliance with the follow up program. These patients were diagnosed and treated in Baghdad Teaching Hospital/ Department of Surgery/ Unit of Oncology under follow up of specialist doctors during the period from March 2009 to September 2009. Our patients were randomly allocated in three groups as follow:

Group (A): Include 24 patients who received CAF protocol (Cyclophosphamide 600 mg/m² + Adriamycin 60 mg/m²+ 5- FU 600 mg/m²) by intravenous infusion once every 21 days and for 63 days.

Group (B): Include 25 patients who received 210 mg/day of silymarin (given as single dose in a capsule dosage form especially prepared for this purpose) along with same CAF protocol of group (A).

Group (C): Include 25 patients who received 420 mg/day of silymarin (given as 210 mg/12hour in a capsule dosage form especially prepared for this purpose) along with same CAF protocol of group (A).

After overnight fasting, venous blood (5 ml) was obtained from the forearm of each patient by veine puncture at baseline, after 21, 42 days of treatment and at the end of 63 days for all patient groups. Each blood sample was placed in EDTA-free tube to be centrifuged for 10 minutes at 3000 rpm. Serum was then divided into several eppendrof tubes and kept frozen until time for assay of hepatic transaminases activity (AST and ALT) and total serum bilirubin (TSB) levels. Colorimetric determination of AST and ALT activity was described by Reitman and Frankel method [9]. Colorimetric method for TSB was based on that described by Jendrassik and Grof [10].

Statistical analysis: results were expressed as mean ± standard error of means (SEM). Student's paired t-test and ANOVA test were used to examine the degree of significance and *P* values < 0.05 were considered significant.

Results:

Significant elevation ($P<0.05$) in serum AST levels was observed as a result of treatment with CAF protocol (18%,33%) after 42 and 63 days respectively, compared with that of baseline (table 1) . Regarding treatment with CAF protocol and 210 mg/day of silymarin, there was significant reduction ($P<0.05$) in serum AST levels (15%) just at the end of the third treatment cycle compared with baseline. Those patients who received CAF protocol and 420 mg /day of silymarin produced significant reduction ($P<0.05$) in serum AST levels (11%,21%) after 42 and 63 days of treatment respectively compared with that of baseline. There was significant difference ($P<0.05$) in serum AST levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol (table-1).

Group	AST (U/l)				
	Number of patients	Baseline	21 days post treatment	42 days post treatment	63 days post treatment
CAF protocol	24	14.78±1.23 [a]	15.32±1.47 [a]	17.48±1.53 [b]	19.64±1.47 [c]
CAF+Sily. (210mg/day)	25	14.32±2.31 [a]	14.20±2.71 [a†]	13.72±2.11 [a†]	12.15±1.84 [b†]
CAF+Sily. (420mg/day)	25	15.11±1.99 [a]	14.35±1.74 [a†]	13.50±1.72 [b†]	12.01±1.56 [c†]

Table -1: Effects of treatment with 210 and 420 mg/day of silymarin on serum AST levels in breast cancer patients treated with CAF protocol.

Results were expressed as mean± SEM
Results with non identical superscripts (a, b, c) within the same group were considered significantly different at $P<0.05$
†= Significant at $P<0.05$ as compared with CAF protocol values
Kit normal values: up to 20 U/l

Significant elevation ($P<0.05$) in serum ALT levels was observed as a result of treatment with CAF protocol (19%) just after the end of the third treatment cycle compared with that of baseline (table-2). Concerning treatment with CAF protocol and 210 mg/day of silymarin, there was significant reduction ($P<0.05$) in serum ALT levels (19%) just at the end of the third treatment cycle compared with baseline. Those patients who received CAF protocol and 420 mg /day of silymarin produced significant reduction ($P<0.05$) in serum ALT levels (12%, 30%) after 42 and 63 days of treatment respectively, compared with that of baseline. There was significant difference ($P<0.05$) in serum ALT levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol. Meanwhile, the reduction in this parameter values was significant ($P<0.05$) after 63 days of treatment with CAF protocol and 420 mg/day of silymarin (9%) compared with those on CAF protocol and 210 mg/day of silymarin (table-2).

Group	ALT (U/l)				
	Number of patients	Baseline	21 days post treatment	42 days post treatment	63 days post treatment
CAF protocol	24	12.96±2.31[a]	13.11±2.24 [a]	13.25±1.97 [a]	15.37±1.88 [b]
CAF+Sily. (210mg/day)	25	12.44±1.87 [a]	12.23±1.94 [a†]	11.90±2.25 [a†]	10.05±2.33 [b†]
CAF+Sily. (420mg/day)	25	13.01±1.79 [a]	12.44±1.82 [a†]	11.49±1.74 [b†]	9.11±1.35 [c†*]

Table-2: Effects of treatment with 210 and 420 mg/day of silymarin on serum ALT levels in breast cancer patients treated with CAF protocol

Results were expressed as mean± SEM

Results with non identical superscripts (a, b, c) within the same group were considered significantly different at $P<0.05$

†= Significant at $P<0.05$ as compared with CAF protocol values

*= Significant at $P<0.05$ as compared with CAF protocol and silymarin (210mg/day) values

Kit normal values: up to 20 U/l

Significant reduction ($P<0.05$) in TSB levels was observed as a result of treatment with CAF protocol (12%,22%) after 42 and 63 days respectively, compared with that of baseline (table 3). Combination of CAF protocol with 210 or 420 mg/day of silymarin produced no significant reduction ($P>0.05$) in TSB levels after the end of each treatment cycle compared with baseline. There was significant difference ($P<0.05$) in the reduction of TSB levels for patients treated with CAF protocol and 210 mg/day (10%,23%) or 420 mg/day (8%,22%) of silymarin after 42 and 63 days of treatment respectively, compared with those who received just CAF protocol (table-3).

Group	TSB (µmol/l)				
	Number of patients	Baseline	21 days post treatment	42 days post treatment	63 days post treatment
CAF protocol	24	10.32±0.98 [a]	10.06±0.86 [a]	9.11±0.57 [b]	8.07±0.66 [c]
CAF+Sily. (210mg/day)	25	10.24±1.09 [a]	10.12±1.14 [a]	10.03±1.07 [a†]	9.91±1.21 [a†]
CAF+Sily. (420mg/day)	25	9.99±1.87 [a]	9.93±2.01 [a]	9.87±1.65 [a†]	9.82±2.11 [a†]

Table-3: Effects of treatment with 210 and 420 mg/day of silymarin on TSB levels in breast cancer patients treated with CAF protocol.

Results were expressed as mean± SEM

Results with non identical superscripts (a, b, c) within the same group were considered significantly different at $P<0.05$
†= Significant at $P<0.05$ as compared with CAF protocol values
Kit normal values: 5-17 µmol/l

Discussion:

In spite of the fact that doxorubicin is extensively metabolized in the liver, its hepatotoxicity is uncommon since liver antioxidant capacity, including that provided by glutathaione (GSH) production, may protect against free radical injury. Doxorubicin contains a quinone nucleus which undergoes redox cycling and is reduced to a semiqunone free radical which in turn reacts with molecular oxygen to produce superoxide ($O2\cdot^-$) and regenerate the quinine [11]. The superoxide anion can then enter into a series of reactions which ultimately cause cell injury. Doxorubicin was found to be a periportal toxin in perfused liver and toxicity was preceded by increase in oxygen uptake due to redox cycling. Therefore, redox cycling as well as doxorubicin toxicity was limited to regions with high oxygen tension [12].

The results of this study revealed that doxorubicin treatment (within CAF protocol) significantly increased the activities of serum AST (at the end of last two treatment cycles) and ALT (at the end of last treatment cycle) compared with baseline values by increasing lipid peroxidation, where the permeability of plasma membranes severely affected and may lead to leakage of the enzymes and increase their activities in the serum (table 1,2). Results of this study was consistent with that recorded by Tomoki *et al.,*who found that doxorubicin elevates plasma AST and ALT activities in mice [13]. Other study established that there was close correlation between the administration of doxorubicin and the appearance of hepatic dysfunction [14].

Regarding this study, TSB levels were consumed and significantly reduced with CAF protocol therapy after the end of the last two treatment cycles compared with their baseline values (table 3). The reduction in TSB levels may attributed to its potent antioxidant properties which prevent the oxidative damage triggered by a wide range of oxidant related stimuli at a low physiological plasma concentrations by scavenging peroxyl radicals via donating a hydrogen atom attached to the C-10 bridge of the tetrapyrrole molecule to form a carbon-centered radical (Bil•) [15].
The presumptive mechanism of cyclophosphamide hepatotoxicity with Veno Occlusive Disease (VOD) is through hepatocyte metabolism of cyclophosphamide to acrolein, which is toxic to the sinusoidal endothelial cells adjacent to the hepatocytes. Acrolein causes profound GSH depletion, leading to death of the sinusoidal endothelial cells and elevation of AST and ALT activities [16]. A large

retrospective study of patients with breast cancer treated with cyclophosphamide, doxorubicin, and 5-fluorouracil reported liver test abnormalities in around 85% of patients without known liver metastases, and this was consistent with the results in (table-1,2). These abnormal liver tests may require discontinuation of the treatment regimen [17].

Silymarin antioxidant effect was observed in rats with acute intoxication by ethanol, phenylhydrazine, and acetaminophen, which are powerful inducer of lipid peroxidation that produce marked depletion of GSH levels in the liver. This indicated that silymarin was able to protect animals against oxidative stress produced in the liver by these substances [18]. The antioxidant effect of silymarin not only related to its action as a scavenger for the free radicals that induce lipid peroxidation, but also because it influences enzymatic systems associated with GSH (GSH-Peroxidase, GSH-Reductase) in liver cells [19].

It has been found that silybinin could inhibit several specifically induced P450 enzymes in mice, whereas other researchers have noted the lack of stimulatory effect on the P450 detoxification system. This effect may explain some of the hepatoprotective effects of silymarin, especially against amanita poisoning and acetaminophen, where they become lethal for hepatocytes only after being activated by CYP system [20].

Certain study indicated that, silymarin can protect against the alterations induced by CCL_4 on the hepatocyte plasma membrane through its antioxidant activities by modifying the plasma membrane phosphotidyl-ethanolamine, so silymarin has plasma membrane stabilizing and permeability regulating properties which prevents hepatotoxic agents from entering hepatocytes and block receptor binding of various toxins [21].

Silymarin stimulates regeneration of hepatocytes by increasing protein synthesis in the injured liver. In an *in vivo* and *in vitro* experiments performed in the liver of rats from which part of the organ had been removed, silybinin produced a significant increase in the synthesis of ribosomes, DNA and proteins [22].

The combination of silymarin and CAF protocol results in significant reduction in serum AST and ALT activities in dose-dependent manner at the end of different treatment cycles compared with their baseline values (table 1,2), which could provide a cytoprotective effect by stabilizing hepatocyte plasma membrane and prevent delivery of AST and ALT to the extracelluler fluid. Results of this study were consistent with that reported by Yusuf *et al.*, who found that the antioxidant effect of vitamin E significantly reduce doxorubicin-induced hepatotoxicity in rats [23]. Fariss *et al.* indicated that, by pretreatment with silymarin, serum AST and ALT activities stay approach to their normal limits after CCl_4-administration to rats [24].

In an experimental model of choleostasis induced by 17α-ethynylestradiol (EE) in rats, silymarin exerted protective effects by normalizing the EE-induced decrease in bile salt pool size and $HCO3^-$, and by counteracting the cholestatic effect of its glucuronidated metabolite [25]. The non significant reduction in TSB levels along this study for those patients who received different doses of silymarin with CAF protocol, compared with their baseline levels, suggest the reduction in the persistent consumption of endogenous bilirubin antioxidant activity by silymarin (table -3).

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