The Effects of Brussels Sprouts and Their Components, Indole-3-carbinol (I3C) and 1-cyano-2-hydroxy-3-butene (CHB) on Hepatic Detoxification Enzyme Activities

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Background. Brussels sprouts, one of many cruciferous vegetables, have been shown to have chemoprevention effects in both animal and human studies. The mechanism of chemoprevention activity is associated with the induction of drug-metabolizing enzymes, which modulate carcinogen metabolism. Whether indole-3-carbinol (I3C) and 1-cyano-2-hydroxy-3-butene (CHB), two major components in Brussels sprouts, contribute to the inductions of drug-metabolizing enzymes has not been determined yet. Therefore, the chemopreventive effects of Brussels sprouts and their components were separately investigated in 2 experiments by our group.

Methods. Freeze-dried Brussels sprouts (BS) powder at 20% diet (w/w) was fed to mice and rats (4/group) for 7 days. Pair-fed animals were used as the control group.

Results. Significant inductions were shown in both phase II enzymes, such as glutathione S-transferase (GST) and quinone reductase (QR), and phase I enzymes, such as cytochrome P450 1A (CYP IA), in the mouse and rat groups compared to their pair-fed groups. In the second study, the treatment diet included 6.25 mg I3C/g diet, 4 mg CHB/g diet, and 1% butylated hydroxyanisole (BHA) as a positive control.

Conclusions. No significant glutathione (GSH) induction was seen in any treatment group. I3C produced significant induction in GST, QR and CYP1A which were 2.4-, 2.4-, and 5.6-fold, respectively, over the control group. However, CHB did not show any significant inductive effects. Therefore, we concluded that I3C, not CHB, at least in part, contributes to the chemopreventive effect of inducing drug-metabolizing enzymes in Brussels sprouts. **(Mid Taiwan J Med 2002;7:28-37)**

Key words

brussels sprouts, detoxification enzymes, indole-3-carbinol (I3C), 1-cyano-2-hydroxy-3-butene (CHB)

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INTRODUCTION

Cruciferous vegetables, such as Brussels sprouts, broccoli, and cabbage, play a role in dietary prevention of cancers in humans [1].

Even eating as little as 10 g of cruciferous /day significantly decreases the risk of colorectal cancer [2]. Furthermore, experimental studies involving chemically-induced carcinogenesis have shown evidence of this protective effect, mainly through changes of detoxification enzyme activities [3,4].

I3C, a plant myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) hydrolysis product from glucobrassicin (GB), a major Glucosinolate (GS) found in cruciferous vegetables [5], has been isolated from cruciferous vegetables and identified as a potential bifunctional inducer of detoxification enzymes such as GST and cytochrome P450 (CYP) enzymes in rats [6]. Such induction requires the parental inducer, such as I3C, to bind to a cytosolic Ah-receptor and be translocated to the nucleus to bind a DNA regulatory site termed XRE. These processes then upregulate the transcription of detoxification enzymes [7].

The comparison of detoxification enzyme induction between dietary cruciferous vegetables and pure I3C has been reported in several published studies. Bradfield and Bjeldanes [8] showed that dietary exposure to I3C at doses as low as 50 ppm in diets for 10 days resulted in a 6-fold increase in activity of CYP 1A in intestine. A no-effect level for I3C on intestinal CYP1A induction was estimated to be between 16 and 25 ppm in diet. The researchers concluded that I3C can account for the CYP1A induction seen in rats fed cruciferous vegetables. In later studies, McDanell et al [9] fed rats the main GS in BS: sinigrin, progoitrin, GB, and glucotropaeolin, however, only GB caused significant CYP1A induction. Yet, they only measured phase I activity without measuring the phase II enzyme which is thought to have higher health benefit effects.

In a more recent and complete study [10], rats were fed 4 derivatives of GS in greatest abundance in BS: CHB, I3C, iberin, Phenylethylisothiolcyanate (PEITC) and a mixture of all four components in the diet. The results showed that CYP1A activity was induced only in the I3C and mixture groups. This indicated that the bifunctional induction observed in the mixture group was mainly attributed to I3C. Additionally, phase II enzyme QR and GST induction appeared in the I3C and CHB mixture; a synergistic effect between both compounds was evident in the induction effects in the mixture group. Furthermore, CHB, the derivative of epiprogoitrin in BS, was identified as a monofunctional inducer which is thought to be a potential chemoprevention agent. The monofunctional inducers are metabolized to a Michael acceptor that only causes induction of phase II enzymes [11]. Moreover, the proposed monofuctional induction of phase II enzymes by Michael acceptors is through a "sensor" protein in the cell which recognizes Michael acceptors and activates transcription of phase II genes [12]. Actually, using rat as an animal model, in addition to Staack's work, CHB at a dose of 50 mg/kg bw also induced GSH levels [13] and GST activities [14] in liver and pancreas. Davis et al [15] found that hepatic mRNA levels of gamma-glutamyl cysteine synthetase (GCS) were elevated following CHB treatment. In one study, mice were treated with a single intravenous dose of CHB (70 mg/kg bw) and found that the nitrile induced apoptosis in pancreas cells. However, the induction effects of detoxification enzymes were not examined in the study [16].

Very few studies have been done using a mouse model to compare the enzyme induction activities between vegetable and vegetable constituents. Whole vegetable (20% dry weight) feeding caused mouse liver GST activities to increase [17]. In the present study, we chose mouse hepatic detoxification enzyme activities as a bioassay to identify inducing agents present in cruciferous vegetables for the following reasons: 1) literature studies suggest that the murine liver may serve as a useful system for the identification of dietary inducing agents [17]; 2) the low food intake of mice minimizes the preparation of plant material in further

feeding studies; 3) for in vitro systems which our in vivo results could compare with, mouse celllines are used more often than rats; 4) mice are easier to take care of. Moreover, we directly compared the effects of enzyme inducers between whole vegetables and the vegetable constituents I3C and CHB in mice by measuring not only phase I and phase II enzyme activities, but also hepatic GSH levels which is another index of liver detoxification potential. Additoinally, in order to compare with other studies, and to more easily measure higher responses, the level of BS, 20% of the diet (w/w), was chosen.

MATERIALS AND METHODS

Chemicals

CHB was purified from seeds of Crambe abyssinica. The extraction procedure was carried out as described by Wallig [13]. All other chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri).

Experimental Design

Experiment 1: 8 female Fisher 344 rats and 8 female Swiss albino ICR mice (National Science Council Animal Center, Taipei, Taiwan, R.O.C.) were separated into treatment and pair-fed groups (4/group), respectively. Treatment groups were fed a 20% (w/w) diet of Brussels sprouts dry powder, which was completely chopped prior to freeze-drying. Experiment 2: 24 female Swiss albino ICR mice were divided into 6 groups of 4 mice each for 3 treatment diets: 1% butylated hydropxyanisole (BHA) as a positive control, 6.25 mg I3C/g, and 4 mg CHB/g diet, each with their own pair-fed groups.

Animal Housing and Treatments

Animals were housed in plastic cages. They were maintained in a controlled environment with 12-hr light/dark cycles under uniform temperature and humidity. The animals were acclimated on a AIN-76 semi-purified diet (Table 1) for 7 days and assigned dietary treatment and pair-fed groups at random. Experimental diets were based on the AIN-76 diet (Table 1) [18]. Throughout the 7 day experiment, water and food were provided from 6 PM to 10 PM (experiment 1), 8 PM to 8 AM (experiment 2) and changed daily to avoid oxidative changes. The body weight and food intake were recorded every day before feeding. At the conclusion of the experiment, the animals were killed by cervical dislocation.

Table 1. Composition of experimental diets

Component	Conc. (g/kg mixture)
Casein*	200
DL-Methionine†	3
Corn starch*	150
Sucrose*	500
Corn oil	50
Mineral mix‡	35
Vitamin mix‡	10
Choline bitartrate†	2
Cellulose *	50

*Casein, corn starch, sucrose and cellulose were obtained from ICN company. †DL-Methionine and choline bitartrate were from Sigma Chemicals. ‡AIN-76 Mineral mix and AIN-76 vitamin mix.

Tissue Preparation

All procedures were conducted at 4 ℃. Livers were perfused with 1.15% KCl. Half of the liver was used immediately for GSH analysis and the other half was homogenized in 4 vols 0.25 M-sucrose, 0.01M-K_2HPO_4 / KH_2PO_4 buffer pH7.4. The homogenate was centrifuged at 10,000 × g for 20 minutes followed by ultracentrifugation of the post-mitochon-drial fraction for 60 minutes at 105,000 × g. The supernatant (cytosol) was stored at −70℃ until being used for the determination of GST and QR activity. The microsomal pellet was resuspended in 1 mL freezing buffer and the mixture was stored at −70 ℃ until being used in the determination of ethoxyresorufin O-deethylase (EROD) activity, as a measure of CYP1A activity.

Glutathione S-transferase Assay

Total GST activity was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate with 1.0 mM glutathione. The reaction was started by adding CDNB. We

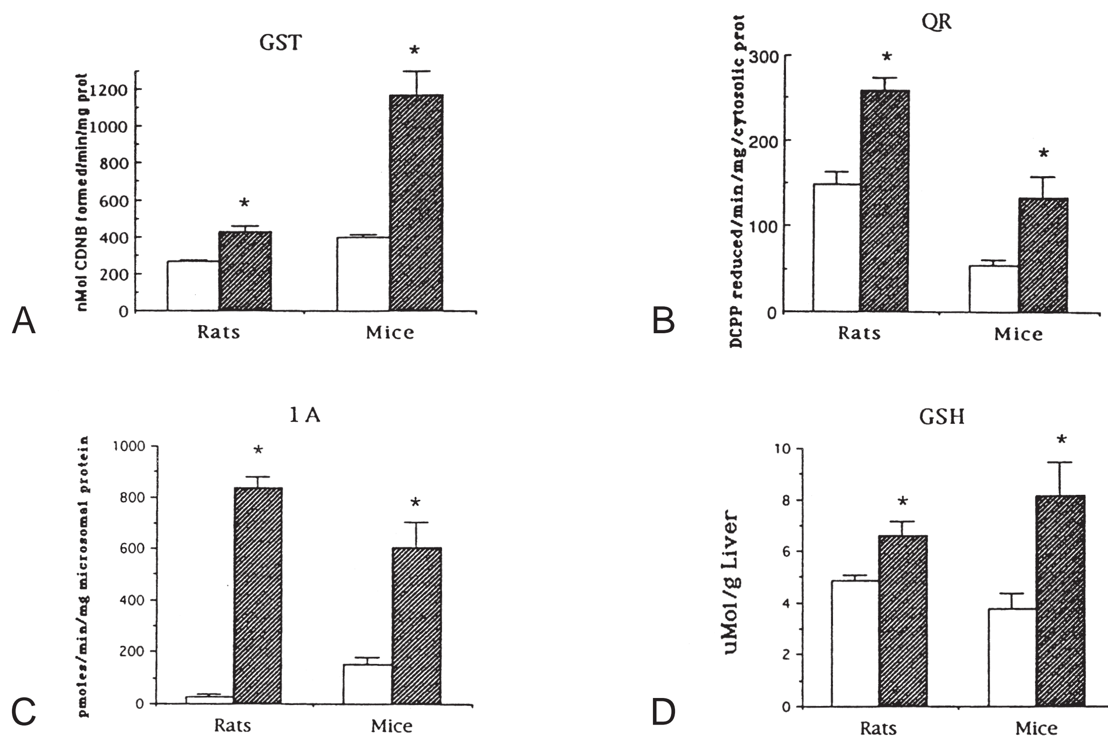

Fig. 1 The comparison of examined enzyme activities between control and experimental groups of rats or mice. A: Glutathione S-transferase activity. B: Quinone reductase activity. C: Cytochrome P450 1A activity. D: Glutathione contents in livers of rats and mice fed Brussels sprouts diets (gray bars). Values marked with an asterisk differ significantly (ANOVA + Fisher PLSD test) from the corresponding control (open bars) value (* $p < 0.05$).

then measured the linear increase in absorbance at 340 nm over 90 seconds, following the spectrophotometric method of Habig et al [19]. Cytosolic protein was determined by the method of Lowry et al [20] using bovine serum albumin as a standard.

Quinone Reductase Assay

QR activity was measured by using 2,6-dichlorophenolindophenol (DCPP, 12 mM) as substrate. The enzyme reaction was started by adding DCPP. We measured the linear increase in absorbance at 600 nm over 90 seconds, following the spectrophotometric method of Ernster [21] as modified by Benson et al [22]. Cytosolic protein was determined by the method of Lowry et al [20] using bovine serum albumin as standard.

Cytochrome P450 1A Assay

Cytochrome P450-dependent ethoxyresorufin o-deethylase (EROD) activity was determined by using ethoxyresorufin as substrate, following a modification of the methods of Pohl and Fonts [23]. The enzyme reaction was started by adding 0.2 mL microsomal suspension with a NADPH-generating system for 4 minutes at 37 ℃, and stopped by the addition of 2 mL methanol. The formation of resorufin was determined by comparing relative fluorescence of samples to that of known amounts of resorufin standard, using excitation at 550 nm and emission at 585 nm. Microsomal protein was measured by the method of Lowry et al [20] using bovine serum albumin as standard.

Glutathione Assay

Glutathione level was measured by a modification of the method of Asaoka and Takahashiissue [24]. Tissue samples were homogenized in cold 4% sulfosalicylic acid followed by adding sodium phosphate buffer (1.0 M, pH=7.0). After being centrifuged at $10{,}000 \times g$ for 20 minutes, an aliquot of the supernatant was added to phosphate buffer (1.0 M, pH=7.0) containing GST and o-

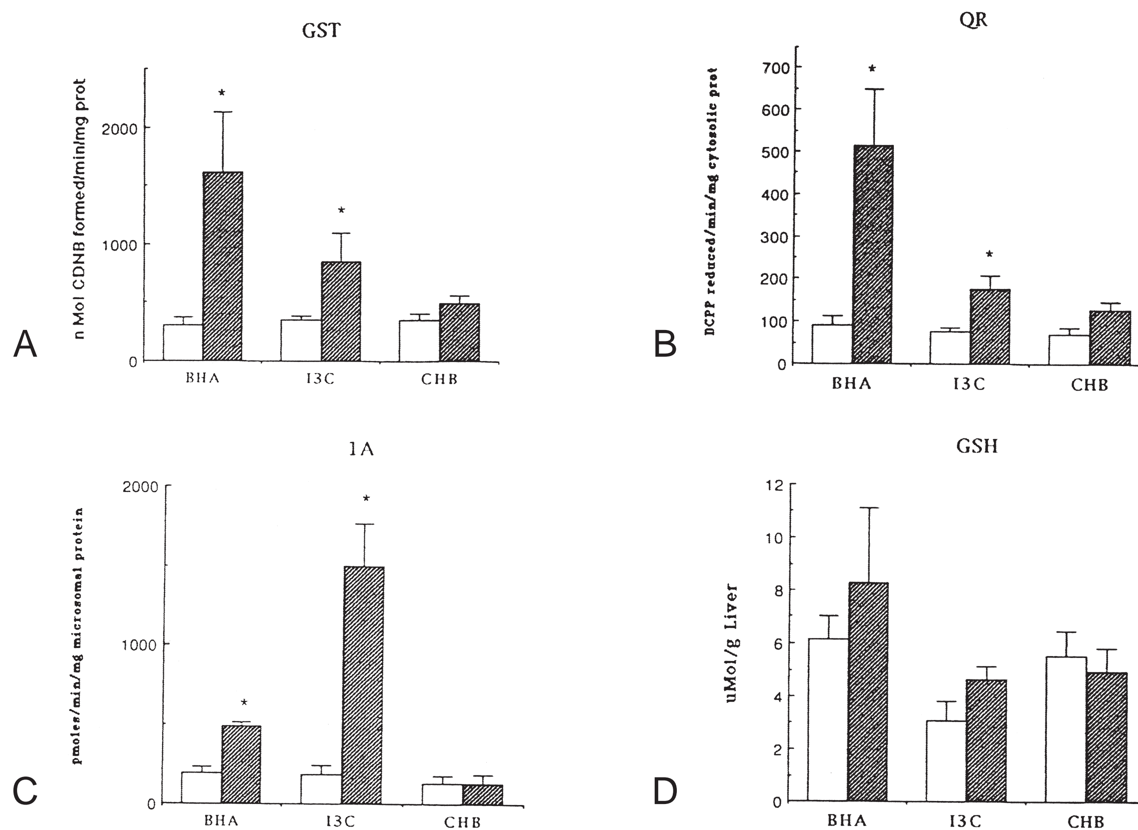

Fig. 2 The comparison of examined enzyme activities between control and experimental groups. A: Glutathione S-transferase activity. B: Quinone reductase activity. C: Cytochrome P450 1A activity. D: Glutathione contents in livers of mice fed BHA, I3C and CHB diets (gray bars). Values marked with an asterisk differ significantly (ANOVA + Fisher PLSD test) from the corresponding control (open bars) value (*$p < 0.05$).

dinitrobenzene. Then, the sample tubes were incubated for 30 minutes at 37 ℃. The reaction was stopped by the addition of a mixture of n-(o-naphthyl) ethylenediamine and sulfanilamide at 1:1 proportion, and absorbance was read at 540 nm. A standard curve was generated using freshly prepared GSH in 1.15% KCl.

Statistics

The data were compared between groups of animals and treatments of samples, using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference test was used to determine differences. $p < 0.05$ was taken as significant.

RESULTS

The Comparison of Induction Effects by BS of Rats and Mice

There was no significant difference in body weight gain between treatment and pair-fed groups in rats or mice. Based on consumption, the dose of Brussels sprouts was 11.3 g/Kg body weight for rats and 35.2 g/Kg body weight for mice. In rats, GSH level, GST, QR, CYP1A activities increased 1.4-, 1.6-, 1.7-, and 29-fold, respectively (Fig. 1). In mice, GSH level, GST, QR, CYP1A activities increased 2.2-, 2.9-, 2.4-, and 4.0-fold, respectively (Fig. 1).

The Comparison of Induction Effects by I3C and CHB in Mice

All the animals looked healthy at the end of the experimental period. No significant differences were noted in body weight gain in any group compared to their pair-fed groups. Based on food intake, the daily doses of I3C and CHB were 793 mg/kg bw and 143 mg/kg bw, respectively. No significant GSH induction

was seen in any treatment group. I3C produced significant induction in GST, QR and CYP1A (Fig. 2) which were 2.4-, 2.4-, and 5.6-fold over the control group, respectively. However, CHB did not show any significant inductive effects.

DISCUSSION

On a per Kg basis, mice ate over 3 times more Brussels sprouts than rats. Compared with the studies of Jeffery's laboratory [10,25,26] rats fed 56 mg I3C/Kg showed significant enzyme induction. The I3C dose administered here to mice was over ten times greater, yet induction was of a similar magnitude. The difference between rats and mice probably results from the different metabolic rates, since adult metabolism for rats and mice are 130 Cal/Kg/day and 600 Cal/Kg/day, respectively [27].

The GSH levels significantly increased in rats in experiment 1 (Fig. 1). There are two studies that can explain this phenomena. 1) GCS, the rate limiting step of GSH synthesis, has been identified to be transcriptionally up regulated by monofunctional inducers [15]. 2) Four ARE sequences were found in the 5' regulatory region of the gene sequence of the GCS heavy subunit [28]. Hence, the elevations of GSH levels were expected in rats due to the presence of monofunctional inducers in BS. However, in the mouse system, whether there are significant numbers of ARE in the regulatory region of the GCS gene or whether other compounds in the whole vegetable also could induce or enhance GSH synthesis remain unknown. Therefore, the mechanism of GSH elevation in mouse liver is not clear. In addition, in experiment 2, for the monofunctional inducer CHB, the means of GSH levels of CHB and control groups were similar. We conjecture that either there is no or less ARE on the upstream of GCS gene or that inhibitors do exist in the promoter region in mice.

I3C, a bifunctional inducer, caused phase II enzyme induction through the regulation of XRE, not ARE. Consequently, significant GSH induction was not expected either, since no XRE has been found in association with GCS. Additionally, since the mice did not favor the diets, in order to increase their food intake, we extended the feeding period from 4 to 12 hours (8PM-8AM) in experiment 2. This may have caused the larger variation seen in GSH results. (Fig. 1).

Significant increases in hepatic glutathione S-transferase (GST), quinone reductase (QR) and cytochrome P450 IA (CYP IA) activity were produced by Brussels sprouts in rats (Fig. 1), results which are similar to other studies [10,29]. Also, significant activities of GST, QR and CYPIA were induced in mice (Fig. 1), and the results are similar to Bradfield and Bjeldanes's work [17]. Then, it was confirmed that in addition to the rat model, the mouse model also showed the expected response. Hence, mice were chosen to be the animal model in our future studies.

Based on significant CYPIA induction results of mice fed Brussels sprouts, mice exhibited bifunctional induction in response to crucifrous vegetables. In experiment 2, I3C, a potent bifunctional inducer and a hydrolysis product of indole glucosinolate, caused significant induction in CYPIA, GST and QR activities. These data confirmed Shertzer's finding that administered I3C by gavage caused phase I and phase II inductions in mice [30]. Since some phase II enzyme inductions of rats fed BS were also identified [31], the GST and QR inductions of mice fed BS may also be contributed to other isothiocyanates. However, the CYP1A induction could be from some bifunctional inducer. The induction of CYPIA subfamily by both cruciferous vegetables and isolated indoles measured as AHH or EROD have been identified by other investigators [8,9,32]. A recent paper [10] supports that I3C can account for much of the CYP1A induction observed when experimental animals are fed a diet containing cruciferous vegetables. These conclusions support our findings that the bifunctional induction observed in tissues from mice receiving Brussels sprouts was essentially attributable to I3C. We also found

another explanation for these effects. Indole GS, the precursor of I3C, is the dominant GS in Brussels sprouts [33]. We checked all the main GS of BS individually from different sources [34] and only indole GS is known to cause significant bifunctional induction.

In the present study, the dietary I3C dose was 6250 ppm. The GB level in BS diet was suggested to be between 40-220 ppm [35]. Despite the potency of GB autolysis products, it is apparent that the doses used did not provide levels of I3C which occur naturally in the diet, which could be due the to differences in bioavailibility of isolated I3C and vegetal I3C. Some synergistic effects could be resposible. For instance, I3C and crambene caused a synergistic effect on the induction of phase II enzymes [10,26]. (However, CHB didn't show the effect in the present study). In addition to the GS derivatives, some other non-GS compounds in the plant might also enhance the enzyme induction effect.

CHB has been well identified as a potent monofunctional inducer in rats [10,25,26]. In a previous experiment, we found no consistent induction in mice at doses of 50, 100 and 200 mg/Kg by gavage (data not shown). One published study [16] indicated that CHB induces apoptosis of pancreatic cells at a dose of 70 mg/Kg in mice. However, it was an intravenous dose, and so far, little is know about the absorption of CHB from the GI tract. Therefore, either mice poorly absorb CHB in the GI tract or the dose of CHB was not high enough to stimulate the detoxification enzyme system.

It has been proposed that dietary cruciferous vegetables protect against cancer through the induction of detoxification enzymes [36-38]. However, I3C, a strong inducer of CYP1A which could activate carcinogens, is present in large amounts in cruciferous vegetables, which contain significant amounts of indole GS, precursor of I3C. So far, a significant number of published papers indicate that I3C enhances tumor development. This could affect the health benefits of eating cruciferous vegetables. Interestingly, dose-dependent CYPIA induction by I3C has been identified in both rat [29] and mouse models [39]. This suggests that CYP1A induction could be modulated by controlling the I3C intake. Moreover, the indole GS content is known to vary over a large range in different types of vegetables [40]. Lower content of indole GS could be produced through genetic manipulation to produce the maximun health benefits of BS.

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抱子甘藍和其成份物I3C和CHB對肝臟解毒酵素活性調節之探討

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背景 屬於十字花科的抱子甘藍，無論是在動物或人體實驗都顯示有預防癌症效果而此機轉是由於抱子甘藍能夠刺激致癌物代謝的解毒酵素活性，然而是否抱子甘藍的主要成份I3C及CHB主導了解毒酵素活性之產生，則尚待驗證。

方法 實驗分成兩部份來進行。抱子甘藍之冷凍乾燥粉末以飼料重量20%含率餵以大白鼠及小白鼠7天，並以控制相同攝取量爲對照組，在大白鼠及小白鼠實驗中，和對照組比較。

結果 無論解毒酵素phase I，如CYP1A，及phase II，如GST及QR，活性皆顯著上升，在進一步之實驗中，以 1% BHA爲正控制組，6.25 mg I3C/g及4 mg CHB/g爲實驗組。結果顯示，肝臟中GSH含量在任何一組皆無明顯活性上升。I3C則刺激GST，QR及CYP1A活性上升，分別爲對照組之2.4，2.4及5.6倍，但是CHB並無任何顯著效果。

結論 我們認爲抱子甘藍防癌效果，可能(至少部分)是來自於成份I3C，非CHB之對解毒酵素活性的刺激所造成。（中台灣醫誌 2002;7:28-37）

關鍵詞

抱子甘藍，肝臟解毒酵素，I3C，CHB

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