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Pilot Study: Effect of 3, 3′-Diindolylmethane Supplements on Urinary Hormone Metabolites in Postmenopausal Women With a History of Early-Stage Breast Cancer

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Abstract: *Dietary indoles, present in* Brassica *plants such as cabbage, broccoli, and Brussels sprouts, have been shown to provide potential protection against hormone-dependent cancers. 3,3′-Diindolylmethane (DIM) is under study as one of the main protective indole metabolites. Postmenopausal women aged 50–70 yr from Marin County, California, with a history of early-stage breast cancer, were screened for interest and eligibility in this pilot study on the effect of absorbable DIM (BioResponse-DIM®) supplements on urinary hormone metabolites. The treatment group received daily DIM (108 mg DIM/day) supplements for 30 days, and the control group received a placebo capsule daily for 30 days. Urinary metabolite analysis included 2-hydroxyestrone (2-OHE_1), 16-α hydroxyestrone (16α-OHE_1), DIM, estrone (E_1), estradiol (E_2), estriol (E_3), 6β-hydroxycortisol (6β-OHC), and cortisol in the first morning urine sample before intervention and 31 days after intervention. Nineteen women completed the study, for a total of 10 in the treatment group and 9 in the placebo group. DIM-treated subjects, relative to placebo, showed a significant increase in levels of 2-OHE_1 (*$P = 0.020$*), DIM (*$P = 0.045$*), and cortisol (*$P = 0.039$*), and a nonsignificant increase of 47% in the 2-OHE_1/16α-OHE_1 ratio from 1.46 to 2.14 (*$P = 0.059$*). In this pilot study, DIM increased the 2-hydroxylation of estrogen urinary metabolites.*

Introduction

A potential source of new classes of chemopreventive and chemotherapeutic agents for human cancers are natural compounds found in food-derived products.[1] Indole-3-carbinol (I3C) is a dietary indole found in *Brassica* cruciferous vegetables, such as cabbage, broccoli, and Brussels sprouts, and the major in vivo product of I3C is 3,3′diindolylmethane (DIM; 1,2). DIM is thought to be the main mediator of the chemopreventive and chemotherapeutic effects of I3C (2).

Multiple studies have shown inhibition and arrest of the growth of cultured human breast cancer cells when I3C or DIM is added to the culture medium (3–18). In addition, growth of cultured human endometrial and prostate cancer cells have shown inhibition of growth with the use of DIM (19–22). Animal studies with mice and rats using either cabbage, I3C, or DIM resulted in a decrease in both spontaneous and chemically induced mammary tumors, and no side effects were noted (23–27). A decrease in cervical dysplasia was noted in approximately 50% of the women given oral I3C, and no significant side effects were observed (28). Both human adults and children have benefited from oral I3C or DIM in the treatment of laryngeal papillomatosis, and I3C and DIM have been reported to be well tolerated in these human studies (29–31).

Many mechanisms have been suggested to explain the potential anticancer properties of cruciferous indole compounds (3–24,32). These range from C-2 hydroxylation of estrogen and changes in hormone levels, increased cell cycle arrest and apoptosis, control of antiproliferative signaling and transcription pathways, inhibition of invasion and migration of cancer cells, aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic mechanisms, and the antioxidant scavenging of free radicals (3–24,32).

Both I3C and DIM induce the metabolism of estrone to form the estrogen metabolite 2-hydroxyestrone (2-$0HE_1$; estrogen receptor antagonist) at the expense of 16α-OHE_1 (estrogen receptor agonist) (1). Cytochrome P450 CYP enzymes convert estrone to either 2-OHE_1 or 16α-OHE_1. The P450 CYP1A1 enzyme is responsible for favoring the metabolic pathway of 2-OHE_1, which is considered protective (32–35). In contrast, the production of 16α-OHE_1 can stimulate cell proliferation (36–37). Some observational studies have demonstrated a correlation between a low urinary 2-OHE_1/16α-OHE_1 ratio and breast cancer risk, whereas others have not shown the same correlation (38–43).

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This is the first randomized controlled study examining the use of oral DIM supplementation in postmenopausal women with a history of early-stage breast cancer. This study monitored the effect of DIM on the C-2 hydroxylation of estrogen and on the 2-OHE_1/16α-OHE_1 ratio. In addition, this is the first study to measure urinary excretion levels of E_1, E_2, E_3, 2-OHE_1, 6β-OHC, cortisol, and DIM after supplementation with DIM. Changes in E_1, E_2, and E_3 are thought to influence breast cancer risk (40–44). Levels of 6β-OHC and cortisol were tested because the 6β-OHC/cortisol ratio is an indication of the activity of the CYP3A4 enzyme, which is important in many metabolic processes, including steroid and drug hydroxylations. The CYP3A4 enzyme also influences the total production of 16α-OHE_1 and 4-hydroxyestrone (4-OHE_1), which are mitogenic in breast cancer cells (45–46). Shanghai women with breast cancer have been found to have a higher 6β-OHC/cortisol ratio (45). In another study, Asian women without breast cancer were found to have a lower 6β-OHC/cortisol ratio than white women, and this could help explain the difference in rates of breast cancer between these populations (47).

Methods

Women from Marin County, California, have been reported to have the highest incidence of breast cancer in the United States and in the world (48). These women are a homogeneous group with regard to breast cancer risk factors and demographics and are predominantly white non-Hispanic (48). In addition, white women have a lower frequency of polymorphisms with regard to estradiol 2-hydroxylation metabolism than African American, Japanese, and Hawaiian populations (49). For these reasons, we chose to do research on this group of women.

Dose ranging studies done with I3C on healthy women at high risk for breast cancer showed that a dose of 300–400 mg/d was sufficient to induce an increased 2-OHE_1/16α-OHE_1 ratio without significant side effects (50). DIM was used in this study because it is a major product of I3C after digestion in the stomach and has been shown to be a primary circulating metabolite following oral use of I3C (2). Study of breast cancer survivors was the important step forward in this study, because no one had established that survivors of breast cancer were able to raise their 2-OHE_1/16α-OHE_1 ratio in the same way as healthy subjects who were at high risk for breast cancer. The level of DIM present in two capsules of absorbable BioResponse-DIM® (108 mg) is approximately equivalent to 400 mg of I3C. This is indicated by a similar post-treatment increase in the 2-OHE_1/16α-OHE_1 ratio in healthy women using BioResponse-DIM in the range of 100 mg (29). The dose used in this study was 108 mg/d of DIM.

The rationale for safety of the 108-mg/d dose comes from the history of exposure to DIM in the diet and from the history of supplemental use of DIM in the same formulation and dose as used in this study. Approximately 10 metric tons of BioResponse-DIM has been produced and sold for dietary supplement use at and above the 108-mg/d dose used for 1 mo in this study. Consumers of BioResponse-DIM have used the supplement continuously for periods of many years. No significant adverse effects have been reported to the manufacturer at this dose over 6 yr of use.

The study interval of 1 mo was chosen because prior studies have shown significant upward shifts in the 2-OHE_1/16α-OHE_1 ratio using I3C for 1 wk, 1 mo, and 3 mo (28,35,50). The 1-mo interval is within the 1-wk to 3-mo range shown to be reflective of effects from I3C. The shift in the ratio appeared to stay the same from 1 mo to 3 mo. Therefore, a longer interval than 1 mo was not judged necessary in this pilot study using DIM.

Cruciferous vegetables were not used because, based on studies of the 2-OHE_1/16α-OHE_1 ratio, consumption of these vegetables would have required greater than a pound a day to generate significant increases in the ratio. Addition of 500 g/day of broccoli resulted in a 30% increase in the ratio in a 2-wk study (33). Voluntarily adding as many cruciferous vegetables as possible resulted in a nonsignificant increase in the 2- OHE_1/16α-OHE_1 ratio when consuming about 200 g/day (34). Starting from the typical DIM precursor glucosinolate content of cruciferous vegetables, at least 2.2 lb (1 kg) of cruciferous vegetables would be required to deliver a dose of DIM close to the 108 mg/day used in the present study (51). Cruciferous vegetable intake at these levels are not practical or necessarily safe in view of the presence of the antithyroid compound, 5-vinyl-2-thiooxazolidone, also produced in these vegetables (52). This antithyroid compound is not present in the DIM supplement.

Subjects

The study proposal was approved by the Human Subjects Institutional Review Board at the University of California, Berkeley. Grant funding was received from the California Breast Cancer Research Program. An independent medical safety monitor was in place during the study. Informed consent was obtained from all subjects.

A database of 833 potential participants aged 50–70 yr living in Marin County with the diagnosis of early-stage breast cancer (Stages 0–2) from January 1, 1990, through May 1999 was obtained from the Northern California Cancer Center registry.

A letter explaining the study was sent to the database of subjects in April 2002 stating that interested potential participants should be postmenopausal women living in Marin County who were currently not on tamoxifen. A return postcard was included with the letter. Two hundred thirty-three of the returned postcards indicated that women were still on tamoxifen and therefore not eligible or that they were not interested in the study. Ninety-seven women indicated that they were interested in the study. A 1-hr physician telephone interview was made to further assess eligibility. Additional eligibility criteria were no recent history of smoking; no use of hormone replacement therapy (HRT), steroids, or medica-

tions such as cimetidine or thyroxine, or herbal supplements; or no use of soy or cruciferous vegetables that might influence their hormone excretion profile. Women were required to be postmenopausal, defined as 1 yr with no menstrual period and to have a weight not over 200 lb or less than 20% below ideal weight. They were also required to have no allergy to cruciferous vegetables and have no recent smoking history or a history of liver, kidney, thyroid, or adrenal disease. Twenty-three subjects were found eligible and scheduled to enter the study in September and October 2002. They were informed that letters would also be sent to their physicians explaining the study. Before starting the study, there was a 2-wk washout period to allow time for metabolic enzyme effects from over-the-counter medications, dietary supplements, and foods such as soy and cruciferous vegetables to be normalized. Subjects were asked to fill out a questionnaire at the beginning of the study regarding customary risk factors for breast cancer that also included body mass index (BMI), and waist/hip ratio. They were asked about their customary food frequency of soy, cruciferous vegetables, alcohol, tea, and exercise because these factors are most likely to influence the 2-OHE_1/16α-OHE_1 ratio. Another questionnaire at the end of the study asked if they noted any side effects from the dietary capsules and again asked for measurements of weight, BMI, and waist/hip ratio. Subjects were asked to return any unused capsules at the end of the study.

The 23 eligible subjects were randomized in a double-blind manner into two groups to receive either daily placebo capsules (made from defatted rice bran) in the control group or daily absorbable DIM (108 mg/d of DIM) for 30 day. Michael Zeligs, MD, from BioResponse, LLC, Boulder, CO, provided the BioResponse-DIM® capsules. Nineteen women completed the study according to protocol, for a total of 10 in the DIM group and 9 in the placebo group. A baseline first-morning urine sample was taken prior to receiving the capsules. DIM capsules or placebo capsules were then given for 30 days. On the 31st day, the first morning urine sample was collected again. All urine samples were stored at –70°C until completion of the study.

Urinary Metabolite Analysis

Samples were sent on dry ice to the laboratory of Daniel Sepkovic, PhD, at the David and Alice Jurist Institute for Research at Hackensack University Medical Center in Hackensack, New Jersey, for analysis of 2-OHE_1, 16α-OHE_1, E_1, E_2, E_3, 6β-hydroxycortisol, cortisol, DIM, and creatinine. Validity of the assays has been established in other studies (53–56). Baseline and 31-day post-treatment samples were analyzed together to avoid interbatch variation. All assays were subject to stringent quality control and good laboratory practice to ensure that the methods continue to perform in an accurate and precise manner. Quality control was based on the evaluation of precision. Quality control data were plotted using Levy-Jennings charts that allow determination of trends, shifts, random error, and control of outliers. The application of Westgard rules allows for the detection of random analytical errors vs. systematic analytical errors. Upon completion of laboratory analysis, the randomized code was broken to determine the identities of the placebo and DIM treatment subjects. Urinary E_1, E_2, and E_3 concentrations were determined by GC-MS (53). Briefly, estrogens in sample aliquots were deconjugated using β-glucuronidase and internally standardized with d_4-estradiol-17β. After extraction into chloroform and evaporation, samples were derivitized with BSTFA and analyzed by Isotope Dilution GC-MS. Analyses of 2-OHE_1 and 16α-OHE_1 were performed using a competitive solid-phase enzyme immunoassay (IMMUNA CARE Corporation, Bethlehem, PA; 54). Urine samples were internally standardized to account for extraction losses and were subjected to hydrolysis using a mixture of β-glucuronidase and arylsulphatase. Enzyme immunoassay was used to determine cortisol (Assay Designs Inc., Ann Arbor, MI) and 6β-OHC (Stabiligen, Viller Les Nancy Cedex, France). Results were presented as the 6β-OHC/cortisol ratio. DIM was determined in urine aliquots after β-glucuronidase hydrolysis and internal standardization with 4,4′-dichlorodiindolylmethane. Urine creatinine concentration was determined spectrophotometrically using a Beckman Creatinine Analyzer II. The individual metabolites in urine were expressed per mg creatinine.

Statistics

Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS). Measurements of demographic and risk factors for breast cancer were analyzed by a two-sample *t* test for continuous variables and χ^2 or Fisher exact test for categorical variables. A two-tailed $P < 0.05$ was considered statistically significant (Table 1). Measurements of urinary metabolites were analyzed for change from baseline. The statistical method used was an analysis of covariance with the change score as the dependent variable, the DIM-treated subjects as the grouping variable of interest, and the baseline score as the covariate. A one-tailed *P* value of < 0.05 was considered statistically significant (Table 3).

Results

Participants ranged in age from 55 to 69 yr and averaged 63 yr. They were educated at the college level or above and lived in Marin County, California, for an average of 35 yr and were white, non-Hispanic. There were no significant differences in BMI between the placebo and the treatment group, which averaged 25.1. There also was no significant difference in waist/hip ratio, age at menarche, age at menopause, number of children, years on birth control pills (BCP), or years on HRT. Both groups used HRT for more than 5 yr and BCP for more than 5 yr. There was also no significant difference in stage of cancer or percentage of women who received chemotherapy or tamoxifen treatment (Table 1). The follow-up questionnaire showed no significant difference in BMI or waist/hip ratio after 30 days.

Table 1. Distribution of Selected Characteristics Among Study Subjects[a]

Continuous Variables	Placebo Mean (SEM)[b]	DIM Mean (SEM)[c]	P[d]
Age	63.1 (1.1)	62.7 (1.6)	0.83
Waist/hip ratio	0.81 (0.03)	0.77 (0.01)	0.21
Body mass index	25.1 (1.5)	25.1 (1.0)	1.00
Age at menopause	50.1 (1.5)	51.5 (0.7)	0.40
Age at menarche	13.1 (0.8)	12.9 (0.2)	0.79
No. of children	2.8 (0.3)	2.4 (0.5)	0.57
Years of BCP	6.3 (2.0)	5.3 (2.6)	0.78
Years of HRT	6.1 (1.9)	5.0 (1.9)	0.68
Years in Marin	35.3 (3.6)	34.6 (4.8)	0.90
Categorical Variables	**Placebo %[b]**	**DIM %[c]**	**P[e]**
Stage of cancer			1.00 (f)
Stage 0	44	40	
Stage 1–2	56	60	
Chemotherapy	22	20	1.00 (f)
Tamoxifen	55	50	0.81 (χ^2)
Education			
College or above	100	100	Same
Ethnicity			
White, non-Hispanic	100	100	Same

a: Abbreviations are as follows: DIM, diindolylmethane; BCP, birth control pills; HRT, hormone replacement therapy.
b: $n = 9$.
c: $n = 10$.
d: Two-tailed P value from two-sample t test.
e: Two-tailed P value from either Chi-Squared Test (χ^2) or Fischer Exact Test (f).

Dietary history of intake of cruciferous vegetables, tea, alcohol, and soy was similar in both groups. All of these factors can influence 2-hydroxylation and the 2-OHE_1/16α-OHE_1 ratio (57). Consumption of red meat and dairy products was similar in both groups. Moderate exercise history was also similar in both groups. Overall, the amount of usual cruciferous vegetable consumption was low and corresponds to the amount consumed in the general population (34). History of consumption of cruciferous vegetables in this study ranged from never to every week but not every day. Although subjects were asked not to eat soy or cruciferous vegetables for 2 wk before the study and during the study, there was DIM detected in the urine of some subjects at baseline. It may be that because subjects thought that cruciferous vegetables were beneficial, some subjects started eating more of these vegetables prior to the start of the study. All results are adjusted for baseline. History of soy intake was similar to history of cruciferous vegetable intake, ranging from never to weekly but not daily. The two groups were well matched as to the proportion of alcohol consumed. Daily alcohol consumption was reported in 33% of the controls and in 30% of the DIM subjects. History of tea consumption was similar at 1–3 times per day in 40–44% of both groups (Table 2).

Four of the original 23 subjects were excluded from the final analysis. One subject reported eating large amounts of cruciferous vegetables during the study and for this reason was excluded. In a similar manner, one subject continued to eat soy during the study and was excluded. One subject stopped taking supplements because of travel. One DIM-treated subject developed a rash and arthralgias and for this reason stopped the supplements. The rash resolved with discontinuation of the supplements and taking antihistamines. The arthralgias improved with time. Two DIM-treated subjects noted an increase in hot flashes, but this did not stop them from taking the capsules. One DIM-treated subject noted some nausea when taking the capsules without food. All other subjects took both the placebo and DIM capsules without significant side effects.

Table 2. Usual Dietary and Exercise History of Study Subjects

Variable	Placebo %[a]	DIM %[b]
Cruciferous vegetables		
Once a wk or less	100	100
Soy		
Once a wk or less	100	90
Alcohol		
Daily	33	30
Never or weekly or monthly	66	70
Tea		
1–3 times per day	44	40
Once a wk or less	55	60
Red Meat		
Three times a wk or less	100	100
Dairy		
Once a day or less	100	100
Moderate Exercise		
Once a wk or less	77	90
1–2 times/day	22	10

a: $n = 9$.
b: $n = 10$. Abbreviation is as follows: DIM, diindolylmethane.

There was a significant rise in the level of 2-OHE_1 (P = 0.020), relative to placebo. This elevation was responsible for a 47% increase in the 2-OHE_1/16α-OHE_1 ratio (P = 0.059). There was no significant difference in the change between the two groups for the levels of 16α-OHE_1 (P = 0.257), E_1 (P = 0.447), E_2 (P = 0.109), or in E_3 (P = 0.137). Relative to placebo, the DIM level in the urine of DIM-treated subjects rose significantly (P = 0.045) indicating overall compliance with taking the DIM capsules. All DIM-treated subjects, except for one, had an increase in urinary DIM. This subject returned one unused capsule. There was also a significant rise in the level of cortisol (P = 0.039). There was a nonsignificant decrease in the 6β-OHC/cortisol ratio (P = 0.160) compared with the placebo (Table 3).

Discussion

The baseline morning urinary 2-OHE_1/16α-OHE_1 ratio in the controls was 1.79, and the baseline for the DIM-treated group was 1.46 (Table 3). In both groups, the baseline value was below the desirable ratio, which is considered to be greater than 2.0 (56). The 2-OHE_1/16α-OHE_1 ratio in our study subjects was also much lower than the ratio found in healthy postmenopausal women, where the average baseline ratio was found to be 2.27 (34).

Baseline levels of DIM prior to the start of the study were above zero. This may be because women made aware of the benefits of cruciferous vegetables were eating cruciferous vegetables prior to the start of the study. Adjustment for baseline was made in the statistical analysis.

Table 3. Unadjusted Mean Values and Standard Error of the Mean (SEM) of Urinary Metabolites in ng/mg Creatinine in Placebo and Diindolylmethane (DIM)-Treated Subjects Before and 31 Days After the Study and Net Difference

Metabolite	Before	After	After–Before
Placebo (n = 9)			
2-OHE_1	7.96 (1.62)	8.05 (1.92)	0.09 (1.01)
16α-OHE_1	4.84 (0.53)	4.59 (0.57)	–0.24 (0.61)
Ratio (2-$0HE_1$/16α-OHE_1)	1.79 (0.31)	1.82 (0.37)	0.04 (0.17)
E_1	0.44 (0.18)	0.53 (0.11)	0.09 (0.13)
E_2	0.20 (0.18)	0.20 (0.05)	0.00 (0.04)
E_3	0.00 (0)	0.09 (0.08)	0.09 (0.08)
6β-0HC	371.5 (38.1)	337.9 (52.8)	–33.6 (52.5)
Cortisol	38.45 (14.67)	29.84 (8.24)	–8.61 (7.07)
Ratio (6β-0HC/Cortisol)	14.6 (2.6)	14.1 (2.6)	–0.5 (3.0)
DIM	26.63 (15.74)	22.87 (8.79)	–3.76 (15.18)
DIM-Treated (n = 10)			
2-OHE_1	7.50 (1.73)	10.15 (1.89)	2.65 (0.55)
16α-OHE_1	4.97 (0.52)	5.17 (0.65)	0.20 (0.43)
Ratio (2-$0HE_1$/16α-OHE_1)	1.46 (0.26)	2.14 (0.39)	0.68 (0.32)
E_1	3.17 (1.64)	3.81 (2.96)	0.64 (2.40)
E_2	0.17 (0.02)	0.12 (0.03)	–0.05 (0.03)
E_3	0.04 (0.03)	0.00 (0)	–0.04 (0.03)
6β-0HC	427.4 (85.8)	363.5 (232.8)	–64.0 (114.0)
Cortisol	21.86 (2.35)	46.50 (13.45)	24.64 (12.07)
Ratio (6β-0HC/Cortisol)	20.7 (4.2)	10.7 (2.3)	–10.0 (4.9)
DIM	5.48 (4.30)	101.3 (36.10)	95.78 (37.87)

Metabolite	Net[a]	P[b]
2-OHE_1	2.57	.020
16α-OHE_1	0.48	.257
Ratio (2-$0HE_1$/16α-OHE_1)	0.64	.059
E_1	0.38	.447
E_2	–0.06	.109
E_3	–0.09	.137
6β-0HC	22.4	.409
Cortisol	27.3	.039
Ratio (6β-0HC/Cortisol)	–3.78	.160
DIM	76.6	.045

a: Net is the change for the DIM-treated group minus the change for the placebo group adjusted for baseline.

b: P value is one-tailed from an analysis of covariance with the change score as the dependent variable, the DIM-treated subjects as the grouping variable of interest, and the baseline score as the covariate

With DIM treatment, relative to placebo, the most significant finding was an increase in 2-OHE_1 ($P = 0.02$). There was a 47% nonsignificant increase ($P = 0.059$) in the 2-OHE_1/16α-OHE_1 ratio. In addition, we found a significant increase in cortisol and a nonsignificant decrease in the 6β-OHC/cortisol ratio ($P = 0.160$), relative to placebo. Consistent with this finding, DIM has been shown in animal models to have less effect on CYP3A4 enzyme activity than I3C (26). The fact that the first morning cortisol level was elevated may indicate that the adrenal gland is stimulated by DIM. Twenty-four-hr cortisol analysis would help to further clarify this possibility.

The study subjects underwent strict selection criteria and could not be on tamoxifen, hormones, or medications that could affect the estrogen hydroxylation profile for 2 wk before the study and during the study. Thus, our final pool of subjects for this pilot study was small. Future studies could examine the effect of DIM on women who are taking tamoxifen because there is known to be synergy of I3C and DIM with tamoxifen (5). This would allow the enrollment of a greater number of subjects and could lead to information that may allow a decrease in the recommended dose of tamoxifen.

Multiple other potential anticarcinogenic mechanisms of DIM, seen in breast cancer cell cultures and in animal studies, include free-radical scavenging, antioxidant effect, cytostatic and apoptotic effects, and changes in the genetic profile (3–24). These effects are more difficult to measure in a short-term study in humans and often require examination of human breast cancer tissue. Longer-term and larger studies are needed to determine if indole compounds such as DIM can prevent or treat breast cancer in humans. The goal is to provide a safe and useful oral compound that can decrease the incidence of breast cancer.

This is the first population-based, randomized, controlled, double-blind pilot study to examine the effect of DIM supplements on urinary hormone metabolites in postmenopausal women with a history of early-stage breast cancer. It may serve as a model for future studies.

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