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13. ABSTRACT (Maximum 200 Words) The purpose of this research project is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs. This information may be useful to decrease the toxicity and increase the effectiveness of chemotherapy. The scope of the research involves <i>in vivo</i> assessments of nutritional supplement-chemotherapeutic drug interactions and <i>in vitro</i> studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions. Neither low or high vitamin E supplements nor St. John's wort significantly changed the rodent LD50 for doxorubicin, docetaxel or cyclophosphamide. Low dose vitamin E supplementation was associated with higher nadir white cell counts after docetaxel and a smaller drop in white count after cyclophosphamide in rats. Neither vitamin E nor St. John's wort supplementation modified rodent hepatic mitochondrial DNA changes caused by docetaxel or doxorubicin. There was no effect of vitamin E or St. John's wort on doxorubicin pharmacokinetics. In women with breast cancer, the drop in neutrophil count after chemotherapy was less in women taking dietary supplements versus no supplements, and in those taking multivitamins or vitamin E. Our studies suggest that dietary supplements such as vitamin E may reduce the bone marrow toxicity in both rodents and humans associated with some cancer chemotherapeutic agents used to treat breast cancer.				
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INTRODUCTION

The general subject of this research project is the effects of dietary supplements on the pharmacokinetics and pharmacodynamics of chemotherapeutic drugs used to treat women with breast cancer. More specifically, the research focuses on the effects of St. John's wort as an example of a nutraceutical and Vitamin E as an example of a nutritional supplement on doxorubicin, docetaxel, and cyclophosphamide. The hypothesis to be tested is that nutritional supplements have important effects on the pharmacokinetics and pharmacodynamics of cancer chemotherapeutic agents. The purpose of the research is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs and then utilize this knowledge to alert patients and their physicians to these interactions. This information also may be useful to decrease the toxicity and increase the effectiveness of chemotherapeutic drugs. The scope of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions.

BODY

Task 1. Evaluate the effects of supplementation with St. John's wort and vitamin E on the pharmacokinetics of cyclophosphamide, docetaxel and doxorubicin. See appended manuscript (1) for details.

- a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.
 - b. Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.
 - c. Inject rats with chemotherapeutic agents and collect plasma samples.
 - d. Measure drug levels in plasma.
 - e. Analyze pharmacokinetic data.
 - f. Repeat pharmacokinetic studies with different doses.
- a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.**
- i. As reported previously, we have refined the technique to measure α -Tocopherol (vitamin E) concentrations in plasma using a reverse-phase high-performance liquid chromatography method with UV detection as previously reported by Julianto *et al.* (2). We used this method to measure vitamin E levels in rats maintained on diets with differing vitamin E contents.
 - ii. As reported previously, our original plan was to focus on analysis of hypericin as an indicator molecule for our pharmacological studies of St. John's wort. However, because some recent studies have identified hyperforin as a more important contributor to the pharmacological actions of this herbal product (3), we have employed an HPLC analytical method that accurately detects and determines hyperforin.
 - iii. As reported previously, doxorubicin concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using fluorescence detection as previously reported by Warren *et al.* (4) and as modified by us.
 - iv. The method for assaying plasma docetaxel concentrations was refined using a modification of the method described by Parise *et al.* (5). Docetaxel concentrations in plasma were measured

with a reverse-phase high-performance liquid chromatography with UV detection. Briefly, docetaxel was extracted from plasma by solid-phase extraction on 1mL Sep-Pak CN columns (Waters, Milford, MA). The columns were first conditioned with 2 1mL aliquots of methanol followed by 2 1mL aliquots of 0.01M ammonium acetate (pH 5.0). Samples were loaded onto individual columns and washed with 2 1mL aliquots of 0.01M ammonium acetate, 2 1mL aliquots of 20% methanol in 0.01M ammonium acetate (pH 5.0), and 1mL hexane. Docetaxel was eluted from the columns using 1mL of acetonitrile. The eluents were evaporated to dryness under nitrogen and the residues were reconstituted in mobile phase. The mobile phase consisted of 45% acetonitrile in water with a flow rate of 1.0mL/min through an Econosphere C18 5 μ column with a matching guard column (Alltech, Deerfield, IL). The detector was operated at a wavelength of 227nm and the samples were quantified using peak height.

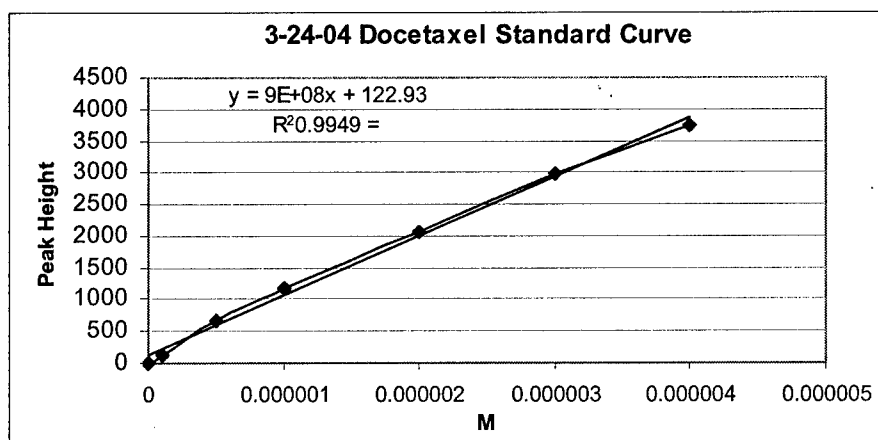


Figure 1. Docetaxel Standard Curve

b. Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.

- i. As reported previously, weanling female Fisher 344 rats, 4 or 5 rats per group, were fed either a cereal-based, standard rat diet that supports growth and maintenance (Harlan-Teklad Global, Product # TD 00217) that contains 16% protein, 3.5% fat and 102 I.U./kg Vitamin E (α -tocopherol), or the same diet supplemented with either a high level of vitamin E, 750 I.U./kg (Harlan-Teklad Product # T.D. 01375) or a low level of vitamin E, 50 I.U./kg (Harlan-Teklad Product # T.D. 01374). The plasma levels of vitamin E were measured and confirmed that dietary vitamin E content influenced plasma vitamin E levels. Thus, rats on the diet containing 102 I.U./kg vitamin E had plasma levels of 15.4 μ M, those on the diet containing 152 I.U./kg vitamin E had plasma levels of 20.0 μ M, and those on the diet containing 750 I.U./kg vitamin E had plasma levels of 26.2 μ M. These values are similar to levels reported in the literature for rodents maintained on diets enriched for vitamin E (6).
- ii. As previously reported, initial exploratory studies were performed with a standardized preparation of St. John's wort (HBC St. Johns wort) that was used in clinical trials and pharmacokinetic studies supported by the National Institutes of Health. A suspension was administered daily to rats by gavage. Unfortunately, all of the animals lost weight, and most died before the completion of a planned 14 day course. A review of the ingredients of this St. John's wort preparation indicated that the excipients included silicon dioxide. We believe that

this ingredient caused intestinal complications. However, several other St. John's wort preparations also caused toxicity when given by gavage. Further exploratory studies suggested that the stress of gavage in young rats contributed importantly to the observed mortality. Therefore we incorporated the St. John's wort into the diet rather than administer it by gavage. A custom diet was formulated that consists of Teklad Global 16% Protein Rodent Diet with 4 g of St. John's wort /kg of feed (4 mg/g). The St. John's wort preparation is Optical Nutrients product 14772 which consists of flowering tops and leaves and is standardized to 0.3% hypericin. Other ingredients include maltodextrin, rice powder, gelatin and magnesium stearate. A 150g rat ingests about 15 g of chow per day or 400mg/kg of body weight. This quantity of dietary St. John's wort has been calculated to approximate pharmacologically relevant doses of St. John's wort in humans (7). On this diet the rats grow at the same rate and ingest the same quantity of food as rats maintained on a control diet. The hematocrit and white blood cell counts measured after 2 weeks on the diet are not significantly different from rats maintained on the standard diet. After 2 weeks on the diet, the rats were found to have hyperforin levels of $2.50 \pm 0.69 \times 10^{-6}$ M. This drug concentration is comparable to levels we measured in rats that were given St. John's wort by gavage ($1.38 \pm 0.32 \times 10^{-6}$ M) and similar to levels of hyperforin reported in the literature by other laboratories (7). A lower dietary intake of 1 g of St. John's wort/kg of feed (1 mg/g) resulted in a hyperforin level of $2.04 \pm 0.69 \times 10^{-6}$ M.

- c. **Inject rats with chemotherapeutic agents and collect plasma samples.** As reported previously, all samples were obtained from the saphenous vein of the animals. Both lower hind legs on each animal were shaved. A thin layer of silicone grease was applied, and the leg was held with sufficient pressure to cause the vein to become clearly visible. Using a Microlance blood lancet a small puncture wound was made in the saphenous vein. The blood was collected in a StatSpin heparinized collection tube, mixed, and centrifuged at 12,000 rpm for 10 minutes. The plasma was removed, placed in microcentrifuge vials and stored at -80°C until testing. Blood samples were obtained prior to injection of the drug and at 10, 20 and 30 min, and 1, 3, 5, 7, 24 and 48 hrs.
- d. **Measure drug levels in plasma.**
 - i. Pharmacokinetic parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats are presented in Table 1. In preliminary experiments rats were injected intravenously with doxorubicin at a dose of 5 mg/kg, but plasma concentrations at later time points were at or below the limit of quantitation. Therefore the dose was increased to 7.5 mg/kg. Blood samples were obtained prior to injection of the drug and at 10, 20 and 30 min, and 1, 3, 5, 7, 24 and 48 hrs. For all conditions there was a biexponential elimination pattern, with rapid distribution and slow elimination typically described for doxorubicin in rodents with doses comparable to the 7.5 mg/kg dose used (data not shown). Estimated pharmacokinetic parameters were comparable for rats fed control, low supplement vitamin E and high supplement vitamin E diets. For example, in animals receiving the control diet the elimination half-life was in the 2 hour range and volume of distribution and clearance were 4.5 liters and clearance was 32 ml/min/kg respectively; these parameters were closely similar to those estimated recently for doxorubicin in rats in a study of the effects of a P-glycoprotein inhibitor on the pharmacokinetics of doxorubicin (8). Estimated "peak" plasma concentrations of doxorubicin 10 minutes after intravenous administration were in the 500- 600 nanomolar range for rats in each study group (data not shown). Estimated plasma concentrations of doxorubicin 24 hours after intravenous administration were also comparable in rats on the high and low vitamin E diets. For example the mean doxorubicin concentrations at 24 hours were 35 and 39 nanomolar respectively for the low and high vitamin E diets. (data not shown). Pharmacokinetic

parameters for St. John's wort treated rats was similar to parameters in rats fed a standard diet and those supplemented with vitamin E (Table 1). None of the treatment means were significantly different from the control means.

Table 1. Pharmacokinetics parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats analyzed by Win-Nonlin.

<i>Diet</i>	<i>AUC</i> ¹	<i>Cl (ml/min)</i>	<i>T_{1/2} (min)</i>	<i>V_{ss} (liters)</i>	<i>N</i> ²
Control	6.1 ± 2.5 ³	32.5 ± 18.9	96.5 ± 28.0	4.5 ± 1.6	4
High E	7.4 ± 1.1	22.0 ± 4.5	132.3 ± 30.1	4.6 ± 1.0	5
Low E	8.5 ± 4.1	22.5 ± 9.6	86.2 ± 44.7	3.5 ± 0.7	4
St. John's wort	9.8 ± 3.9	21.7 ± 7.5	108.1 ± 31.5	3.7 ± 2.7	6

¹AUC x 10 = micromoles/liter x minutes

²N = number of animals

³mean ± SD

ii. Pharmacokinetics of docetaxel in control, vitamin E or St. John's wort-treated rats.

Animals were injected with 10mg/kg of docetaxel. Blood samples from rats on the control diet, the vitamin E supplemented diets, and the St. John's wort supplemented diet were collected and frozen. We collected two sets of samples for pharmacokinetic analyses. In the first set, we used reconstituted powdered docetaxel. Unfortunately we got unsatisfactory values, with unmeasurable early time points despite IV administration of the drug. We considered that either 1) we needed to draw earlier time points, 2) we were having precipitation of the reconstituted drug or 3) our detection system was not sensitive enough. Therefore a second set of samples was collected using the commercially available drug, with earlier time points. We also asked Dr. Merrill Egorin at the University of Pittsburgh to run a set of samples, since we are using UV detection and he is using mass spectroscopy. His more sensitive detection system could measure docetaxel in the early time points. Again our laboratory could not detect docetaxel in the blood samples, and although Dr. Egorin's laboratory could detect docetaxel in some samples, the results were not interpretable. Therefore we could not obtain pharmacokinetics of docetaxel in rats.

iii. Pharmacokinetics studies of cyclophosphamide were not done because of a shortage of time and funding. Much more time and expense were expended on the docetaxel studies described above than was originally anticipated.

Task 2. Measure the effects of supplementation with St. John's wort and vitamin E on the toxicity of cyclophosphamide, docetaxel and doxorubicin. See appended manuscript (1) for details.

- Maintain 3 groups of rats on diet alone, or diet plus St John's wort or vitamin E.
- Administer chemotherapeutic drugs in LD50 doses.
- Observe for toxicity and collect blood samples.
- Analyze blood samples for evidence of hematologic, renal, hepatic and cardiac toxicity.
- Analyze toxicity data.
- Repeat toxicological studies with different doses.

- i. **Doxorubicin.** After 8 weeks on the cereal-based diet, or that diet supplemented with a low or high dose of Vitamin E, the rats were injected with increasing amounts of doxorubicin (5.0, 7.5, 9.8, 12.8 and 17.9 mg/kg). The LD50 of doxorubicin for rats on the standard diet and that for rats ingesting the supplemented diets were not significantly different, and there was no dose-response relationship for Vitamin E (Table 2). Measurements of Hct and WBC counts on Days 4, 9 and 14 following injection of doxorubicin were obtained. Since the nadir for the Hct and WBC count was on Day 4, the nadir Hct and WBC and the drop from the initial to the nadir level of the Hct (Hctfall) and WBC (Nfall) was determined on Day 4. The p-value for dose was significant ($p < .001$), indicating that increasing doses of doxorubicin were associated with decreasing levels of Hct and WBC (data not shown). Statistical analyses showed no important differences among the dietary groups (data not shown).
- Rats fed the standard diet or the same diet supplemented with St. John's wort for 2 weeks were injected with increasing amounts of doxorubicin (12, 15, 18 and 22 mg/kg). These dosages were selected based upon the observed LD50 in the previous experiment with Vitamin E. The LD50 of doxorubicin for rats on the standard diet and the LD50 for rats ingesting the St. John's wort supplemented diet were not significantly different (Table 2). The nadir Hct and WBC, Hctfall and Nfall were not significantly different either (data not shown).
- ii. **Docetaxel.** After 8 weeks on the standard or Vitamin E-supplemented diets, rats were injected with increasing doses of docetaxel (8.5, 12, 18 and 20 mg/kg). As shown in Table 2, these LD50s did not differ significantly from the LD50 of rats fed the standard diet. The nadir WBC count was significantly higher ($p = .004$) in rats fed the diet supplemented with low dose vitamin E than in either the standard or high vitamin E dietary groups (Figure 2). The Nfall was significantly higher ($p = 0.04$) in the high vitamin E group compared to the low vitamin E group, but neither was different from the standard diet group (Figure 3). There was no effect of vitamin E supplementation on Hct or Hctfall after docetaxel treatment (data not shown). In rats fed the standard or St. John's wort diet for 2 weeks, the LD 50s were not significantly different after injection of increasing doses of docetaxel (12, 15, 18, 25, 30 and 40 mg/kg) (Table 2). Hct and WBC measurements were not analyzed after this experiment.
- iii. **Cyclophosphamide.** After 8 weeks on the standard or Vitamin E-supplemented diet, rats were injected with increasing doses of cyclophosphamide (25, 85, 144, and 200 mg/kg). These dosages were selected based upon our prior experience. The LD50s for the standard diet, low supplement Vitamin E diet, and high supplement Vitamin E diet were not significantly different (Table 2). The Nfall was greater ($p = .03$) in the standard and high vitamin E dietary groups than in the low vitamin E group (Figure 4). The nadir WBC, nadir Hct and the Hctfall were not different (data not shown). Rats that were fed the standard diet or the St. John's wort-supplemented diet for 2 weeks were injected with cyclophosphamide (25, 85, 144 and 190 mg/kg). The observed LD50s were not significantly different (Table 2), nor were the differences in nadir Hct and WBC, or in Nfall (data not shown). However, Hctfall was significantly greater ($p = .01$) in rats fed the standard diet compared to those supplemented with St. John's wort (data not shown).

Table 2. Median lethal dose (LD50) for rats receiving dietary supplementations and cancer chemotherapy.

	Doxorubicin		Docetaxel		Cyclophosphamide		
	LD50 (mg/kg)	95% CI ¹ (mg/kg)	LD50 (mg/kg)	95% CI (mg/kg)	LD50 (mg/kg)	95% CI (mg/kg)	CI
Vitamin E							
Standard	13.3	11.2-17.1	18.9	14.6-124.6	135.8	73.1-237.4	
Low Supplement	12.7		20.4		135.2	54.6-670.0	
High Supplement	13.0		16.5	12.9-21.8	134.8	92.9-194.4	
St. John's wort							
Standard	16.3	13.4-20.0	28.0	22.5-40.1	117.0	79.4-151.5	
Supplement	18.2		25.8		144.0		

¹95% CI = 95% confidence intervals; not all 95% confidence intervals could be calculated because of very large, unstable variances.

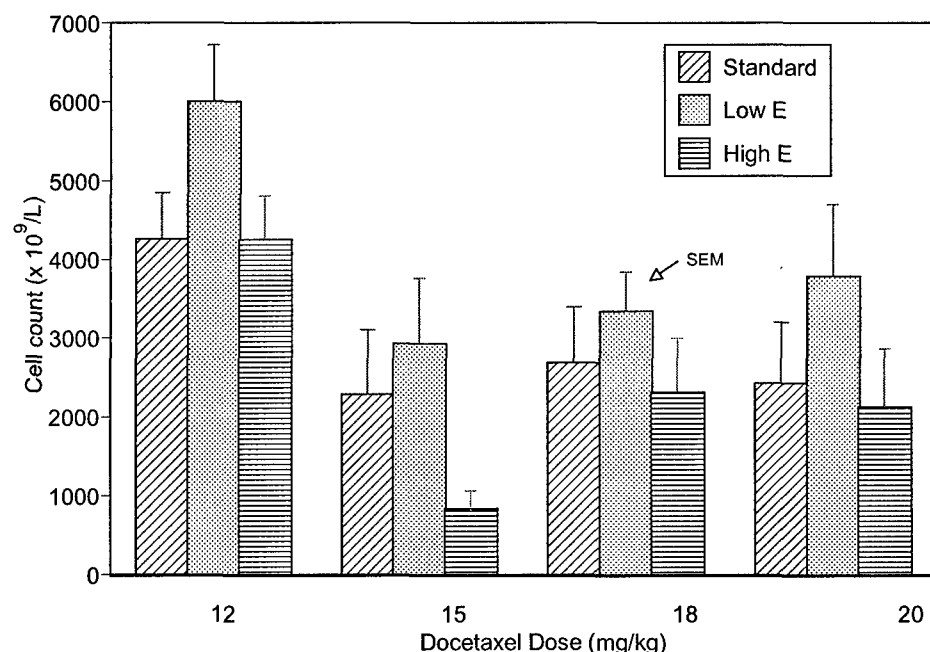


Figure 2. Nadir WBC counts measured 4 days after treatment of rats with docetaxel. The nadir WBC count was significantly higher ($p=0.004$) in rats fed the diet supplemented with low dose vitamin E (stippled bar) than in either the standard (diagonal cross-hatch bar) or high vitamin E (horizontal cross-hatch bar) dietary groups. There were 6 animals in each group. SEM is the standard error of the mean.

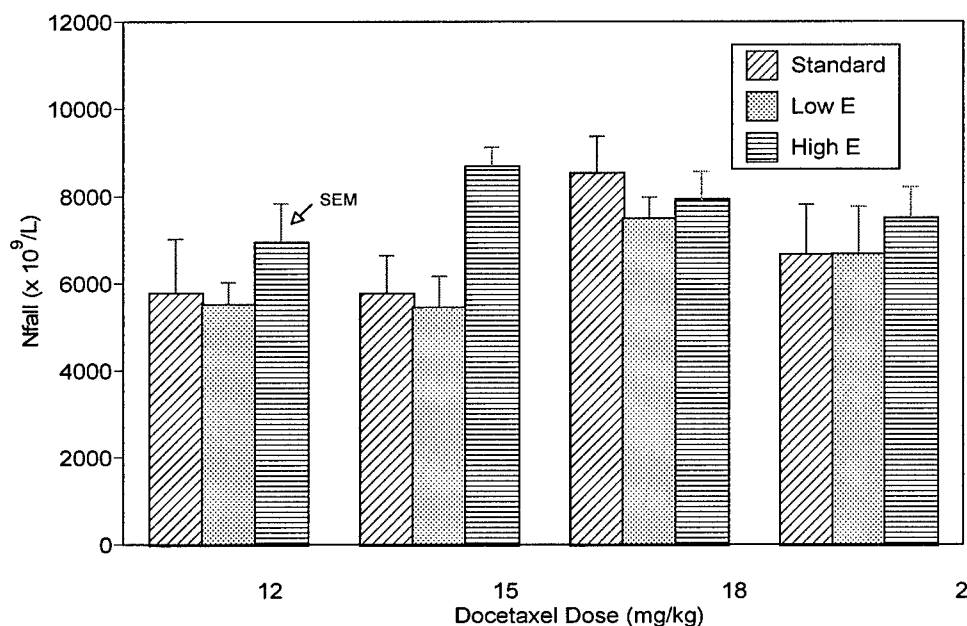


Figure 3. The decrease in WBC count (Nfall) from initial to nadir levels in rats after treatment with docetaxel. The drop was greater ($p=0.04$) in the high dose vitamin E group (horizontal cross-hatched bar) than in the standard diet (diagonal cross-hatched bar) or the low dose vitamin E group (stippled bar). There were 6 animals per group. SEM is the standard error of the mean.

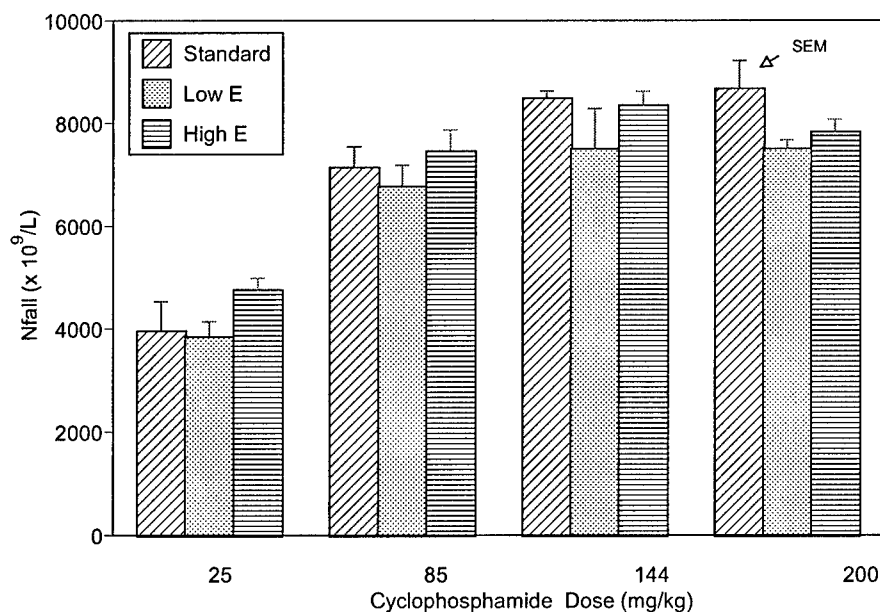


Figure 4. The decrease in WBC count (Nfall) from initial to nadir levels in rats after treatment with cyclophosphamide. The drop was greater ($p=0.03$) in the standard (diagonal cross-hatched bar) and high dose vitamin E (horizontal cross-hatched bar) groups than in the low dose vitamin E (stippled bar) group. There were 6 animals per group. SEM is the standard error of the mean.

Task 3. Study the mechanisms of nutraceutical-chemotherapeutic drug interactions.

- a. Measure hepatic p450 activity where appropriate.
 - b. Measure hepatic P-glycoprotein expression where appropriate.
 - c. Measure glutathione S-transferase activity in liver samples where appropriate.
 - d. Collate and analyze data.
- e. **Mitochondrial DNA deletions and copy number.** A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the rat (9). This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. Table 3 shows the results obtained with this assay at a doxorubicin dose level of 12.8 mg/kg. In this experimental system, a smaller ΔC_T deletion indicates *more* deletions, while a smaller ΔC_T copy number indicates *less* total mitochondrial DNA. The data in Table 3 suggests that doxorubicin does not increase, and in fact may modestly decrease the number of deletions and copy number in cardiac or hepatic mitochondrial DNA. Although most of these differences were not significant, there was a significant decrease ($p=0.01$) in the number of DNA deletions in liver after doxorubicin treatment compared to rats fed the standard diet. The only evidence that vitamin E supplementation modulated mitochondrial DNA damage caused by doxorubicin was an increased DNA copy number in the hepatic samples from rats fed the high vitamin E supplement ($p = 0.03$). We also measured the effect of vitamin E levels and St. John's wort supplementation on mitochondrial DNA damage in rat liver caused by docetaxel (Table 4). None of the treatment means were significantly different from the means of untreated rats fed the standard diet (with adjusted alpha), with the exception of the difference in copy number between the untreated animals and those supplemented with St. John's wort ($p=0.03$). Therefore we conclude that dietary vitamin E levels and St. John's wort supplementation do not have an important effect on the mitochondrial DNA damage caused by either doxorubicin or docetaxel.

Table 3. Mitochondrial DNA deletion and copy number in rats maintained on standard rat chow or supplemented with low or high concentrations of vitamin E. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{Deletion}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg Doxorubicin in the standard diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β -actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{CopyNumber}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg Doxorubicin in the standard diet. Relative Expression indicates the fold difference in deletions or copy number compared to the standard diet.

	Heart			Liver		
	ΔC_T Deletion (n^1)	Rel Exp	ΔC_T Copy Number (n)	Rel Exp	ΔC_T Deletion (n)	Rel Exp
Standard Diet	7.95 $\pm$ 0.62 (6)	1.00	12.33 $\pm$ 0.45 (6)	1.00	7.24 $\pm$ 0.29 (6)	1.00
Doxorubicin (12.8mg/kg)						
Standard Diet	9.84 $\pm$ 2.80 (4)	0.27	12.34 $\pm$ 1.18 (4)	0.99	7.70 $\pm$ 0.97 (4)	0.73
Low Vitamin E	12.45 $\pm$ 3.47 (2)	0.04	12.45 $\pm$ 1.82 (2)	0.92	8.12 $\pm$ 0.04 (2)	0.55
High Vitamin E	9.96 $\pm$ 0.72 (5)	0.25	12.25 $\pm$ 0.40 (5)	1.06	8.08 $\pm$ 0.23 (5)	0.56

¹ n : number of animals

Table 4. Mitochondrial DNA deletion and copy number in liver from rats fed a standard diet alone, or the same diet supplemented with St. John's wort, low supplement Vitamin E, or high supplement Vitamin E. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{Deletion}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg docetaxel in the standard diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β -actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{CopyNumber}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg docetaxel in the standard diet. Relative Expression indicates the fold difference in deletions or copy number compared to the standard diet. n : number of animals

	ΔC_T Deletion (n')	Rel Exp	ΔC_T Copy Number (n)	Rel Exp
Standard Diet	8.69 ± 1.08 (6)	1.0	9.33 ± 0.16	1.0
Docetaxel (18 mg/kg)				
Standard Diet	7.94 ± 0.57 (7)	1.68	9.40 ± 0.19 (7)	0.95
St. John's wort	7.59 ± 0.46 (5)	2.14	9.14 ± 0.12 (5)	1.14
Low supplement Vitamin E	8.16 ± 0.45 (4)	1.44	9.37 ± 0.17 (4)	0.97
High supplement Vitamin E	8.29 ± 1.01 (3)	1.32	9.52 ± 0.31 (3)	0.88

We completed a study of the effect of vitamin B12, folate and dietary supplements on breast cancer chemotherapy-induced mucositis and neutropenia in women. This work was started under a previous DOD grant (DAMD17-981-8345) and completed during this grant period. The results of this study, published in *CANCER*, are directly relevant to the current grant. The full manuscript is appended (10). This clinical study supports the results of our findings in rats, described above, and provides further evidence that dietary supplements can modulate the toxicity of cancer chemotherapy.

BACKGROUND. Although patients with cancer frequently use dietary supplements, the effect of these agents on chemotherapy is unclear. Therefore we investigated the influence of vitamin B₁₂, folate and nutritional supplements on chemotherapy-induced toxicity.

METHODS. Women with breast cancer were asked to complete a questionnaire recording their use of dietary supplements. Blood samples were obtained for serum vitamin B₁₂ and folate levels before and after the first cycle of chemotherapy and for weekly complete blood counts. Toxicity was evaluated by absolute neutrophil counts and by the frequency and severity of oral mucositis.

RESULTS. Of 49 women who submitted questionnaires, 35 (71%) took a total of 165 supplements. Compared to a prior study in 1990, there was a dramatic increase in serum folate levels. Initial neutrophil count, but not type of chemotherapy, patient age or serum vitamin B₁₂ level was predictive of nadir absolute neutropenia and the drop from initial neutrophil count to nadir (Nfall). After adjusting for initial neutrophil count, Nfall was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$). No significant effect of initial neutrophil count, nadir neutrophil count, Nfall, age, vitamin B₁₂ or folate level on oral mucositis was found.

CONCLUSIONS. The drop in neutrophil count caused by cancer chemotherapy may be ameliorated by dietary supplementation with a multivitamin or vitamin E but is increased in association with high serum folate levels.

KEY RESEARCH ACCOMPLISHMENTS EMANATING FROM THIS RESEARCH

- High performance liquid chromatography (HPLC) methodology was adapted and refined for the detection and determination of plasma levels of vitamin E, hyperforin and doxorubicin.
- Vitamin E levels were measured in rat plasma and found to correlate with dietary levels of the vitamin.
- A diet supplemented with St. John's wort was formulated. The diet was palatable to the rats and supported growth at the same rate as the standard rat chow. Hematologic values after 2 weeks on the diet were not significantly different from levels in rats maintained on the standard diet. Measurement of hyperforin levels indicated that the diet resulted in plasma concentrations reported by other investigators to be pharmacologically relevant in rats and humans.
- There was no effect of vitamin E or St. John's wort supplementation on doxorubicin pharmacokinetics.
- Neither low or high vitamin E supplements nor St. John's wort significantly changed the LD50 in rats for doxorubicin, docetaxel or cyclophosphamide.
- The nadir WBC count was significantly higher after docetaxel in rats supplemented with low doses of vitamin E, and the drop in WBC count from initial to nadir was less in animals supplemented with low doses of vitamin E.
- The drop in WBC count from initial to nadir was smaller in rats supplemented with low doses of vitamin E after cyclophosphamide treatment.

- Neither vitamin E nor St. John's wort had an important effect on the mitochondrial damage caused by either doxorubicin or docetaxel.
- In women with breast cancer, the drop in neutrophil count after chemotherapy was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$).

REPORTABLE OUTCOMES

Publications supported by this award:

- Yildirim, Z., Branda, R.F., Powden, C., Brooks, E., McCormack, J.J. Toxicity and pharmacokinetics of doxorubicin in rats treated with vitamin E. ASCO Proceedings 22:155 (2003).
- Branda, R.F., Powden, C., Brooks, E., Yildirim, Z., McCormack, J. Effects of St. John's Wort and Vitamin E on Breast Cancer Chemotherapy. Proc. Era of Hope Department of Defense Breast Cancer Res. Program Meeting (vol. III):P47-1 (2002).
- Nicklas, J.A., Brooks, E.M., Hunter, T.C., Single, R., Branda, R.F., Development of a quantitative PCR (TaqMan®) assay for mitochondrial DNA copy number and the common mitochondrial DNA deletion in the rat. Environ. Mol. Mutagenesis 44:313-320 (2004).
- Branda, R.F., Naud, S.J., Brooks, E.M., Chen, Z., Muss, H. The effect of vitamin B12, folate and dietary supplements on breast cancer chemotherapy-induced mucositis and neutropenia. CANCER 101:1058-64 (2004).
- Branda, R.F., Powden, C., Brooks, E.M., Yildirim, Z., Naud, S.J., McCormack, J.J. Vitamin E but not St. John's wort mitigates neutropenia caused by cancer chemotherapy in rats. Submitted.

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CONCLUSIONS

The experiments described in this Final Report analyze the relationship between dietary supplements and chemotherapeutic drugs used to treat patients with breast cancer. Herbal medicines and dietary supplements are used frequently by patients with cancer, but there is a paucity of information on their interactions with prescribed drugs. Since a majority of women with breast cancer are taking dietary supplements, there is a pressing need to better understand the effects of these supplements on cancer chemotherapy. Our studies in rats suggest that even relatively high doses of vitamin E do not adversely affect the bone marrow or cardiac toxicity of doxorubicin, docetaxel, or cyclophosphamide. However, low dose supplementation with vitamin E mitigated the bone marrow toxicity, as measured by the drop in white blood cell count, after docetaxel or cyclophosphamide treatment. Moreover, studies in women with breast cancer indicate that dietary supplementation with

either a multivitamin or with vitamin E ameliorates the degree of neutropenia after combination chemotherapy. St. John's wort had neither a beneficial nor a detrimental effect on chemotherapy-induced toxicity. Thus dietary supplements such as low doses of vitamin E may be useful to modify toxicity after chemotherapy in patients with breast cancer.

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Appendices

- Appendix I (32 Pages): Branda, R.F., Naud, S.J., Brooks, E.M., Chen, Z., Muss, H. *The effect of vitamin B12, folate and dietary supplements on breast cancer chemotherapy-induced mucositis and neutropenia.* CANCER 101:1058-64 (2004).
- Appendix II (14 Pages): Nicklas, J.A., Brooks, E.M., Hunter, T.C., Single, R., Branda, R.F., *Development of a quantitative PCR (TaqMan®) assay for mitochondrial DNA copy number and the common mitochondrial DNA deletion in the rat.* Environ. Mol. Mutagenesis 44:313-320 (2004).
- Appendix III (21 Pages): Branda, R.F., Powden, C., Brooks, E.M., Yildirim, Z., Naud, S.J., McCormack, J.J. *Vitamin E but not St. John's wort mitigates neutropenia caused by cancer chemotherapy in rats.* Submitted.

**VITAMIN E BUT NOT ST. JOHN'S WORT MITIGATES LEUKOPENIA
CAUSED BY CANCER CHEMOTHERAPY IN RATS ¹**

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ABSTRACT

Dietary supplements are used by the majority of patients with cancer. Since nutraceuticals can interact with many drugs, this study investigated the effect of herbal remedies and vitamins on the toxicity of cancer chemotherapy. Fisher 344 rats were fed a standard cereal-based diet or the same diet with additional vitamin E in low (50 mg/kg) or high (750 mg/kg) concentrations, or with added St. John's wort (400 mg/kg). The LD50 was determined following the administration of chemotherapy drugs. Neither low or high vitamin E supplements nor St. John's wort significantly changed the LD50 for doxorubicin, docetaxel or cyclophosphamide. The nadir white blood cell (WBC) count was significantly higher ($p=0.004$) after docetaxel in rats supplemented with low dose vitamin E, but the drop in WBC count from initial to nadir levels (N_{fall}) was greater in rats fed a diet containing high vitamin E supplementation ($p=0.04$). Similarly the N_{fall} was greater in the standard and high vitamin E dietary groups than in the low vitamin E group after cyclophosphamide ($p=0.03$). There was no effect of vitamin E or St. John's wort supplementation on doxorubicin pharmacokinetics. Neither vitamin E nor St. John's wort had an important effect on the mitochondrial DNA damage caused by either doxorubicin or docetaxel. These data suggest that the decrease in neutrophil count caused by some chemotherapeutic agents can be modified by dietary supplementation with vitamin E, but the effect appears to be dose-dependent. St. John's wort had neither a beneficial nor a detrimental effect on chemotherapy-induced toxicity.

KEY WORDS: chemotherapy, vitamin E, St. John's wort

INTRODUCTION

Many patients begin taking nutritional supplements and herbal remedies after they are diagnosed with cancer. For example, surveys conducted at the National Institute of Health's Clinical Center found that 25-42% of patients enrolled in NIH clinical trials reported taking herbal and other supplements (1). Patients who seek complementary and alternative therapies tend to be better educated, of higher socioeconomic status, female and younger than those who do not (2). Thus it is not surprising that the use of complementary therapies among women with breast cancer appears to be increasing. In a 1999 study, Burstein and colleagues reported that 28.1% of 480 Massachusetts women began to use alternative medicine after surgery for breast cancer (3). A 2004 abstract from the University of Cincinnati noted the usage of one or more complementary/alternative therapies by 64% of 606 breast cancer patients (4). The most commonly used agents were vitamin E, vitamin C, vitamin B6, green tea and selenium (4). In a study conducted at the University of Vermont, we found that 71% of women with breast cancer reported taking one or more dietary supplements (5). The most common supplements were multivitamins, vitamins C and E, and calcium. While the mean number of supplements taken by an individual patient was 3, some patients took as many as 20 supplements per day. Little is known about the value of these dietary supplements to patients with cancer, and even less is known about how these supplements interact with cancer chemotherapy.

A recent American Institute for Cancer Research Cancer Resource Advisory Council concluded that the use of dietary supplements during cancer treatment remains controversial (6). However, there is a growing recognition that concurrent use of nutraceuticals may enhance or oppose the effect of drugs (7). For example, there is decreased bioavailability of irinotecin, theophylline, cyclosporin and phenprocoumon when these drugs are combined with St. John's

wort (8,9). This herbal remedy for depression contains many biologically active compounds that are known to induce P450 enzymes (particularly in the intestinal wall) and P-glycoprotein and resemble polycyclic aromatic hydrocarbons that induce glutathione-S-transferase (10-14). Similarly, there is laboratory evidence to suggest that vitamin E can modulate P-glycoprotein activity, hepatic glutathione S-transferase levels and cytochrome P450 activity (15-17). We investigated the interaction of St. John's wort as an example of an herbal remedy and vitamin E as an example of a dietary supplement with three important chemotherapeutic agents: namely cyclophosphamide, doxorubicin and docetaxel. Since cyclophosphamide is activated by P450 enzymes and metabolized by glutathione S-transferases (18,19), doxorubicin is a substrate for P-glycoprotein (20), and docetaxel is metabolized by hepatic CYP3A4 and is a substrate for P-glycoprotein (21), St. John's wort and vitamin E theoretically could have important interactions with these chemotherapeutic agents that are used in large numbers of patients with cancer.

MATERIALS AND METHODS

Animals and Diets. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. The University of Vermont Animal Care Facility follows procedures that are in accordance with the "Guide to the Care and Use of Laboratory Animals" and are certified by the AAALPC.

Female Fisher 344 rats, 30 days old and weighing approximately 80 gm, were obtained from Charles Rivers Canada (St.-Constant, Quebec) and fed *ad libitum* a standard cereal-based rat diet that supports growth and maintenance (Harlan-Teklad Global 2016, Product # TD 00217, Madison, WI). This diet contains 16% protein, 3.5% fat, 3.9% crude fiber, 61% carbohydrate, 3.3 Kcal/gm digestible energy, and 102 mg/kg Vitamin E (α -tocopherol). This is a fixed

formula diet whose principal ingredients include ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, soybean oil and dried brewers yeast. For the Vitamin E experiments, additional α -tocopherol (dry vitamin E acetate) was added to the Global 2016 diet by Harlan-Teklad. The Low Supplement Vitamin E diet had an additional 50 mg/kg for a total of 152 mg/kg Vitamin E, and the High Supplement Vitamin E diet contained an additional 750 mg/kg for a total of 852 mg/kg Vitamin E. After 8 weeks on this diet, the rats weighed approximately 150 gm.

For the St. John's wort experiments, 4 gm of St. John's wort was added per kg of Global 2016 diet by Harlan-Teklad. The St. John's wort preparation was obtained from Optimal Nutrients (Foster City, CA) product #14772. The preparation contains St. John's wort flowers and tops (*Hypericum perforatum*) extract, standardized to 0.3% hypericin. Excipients include maltodextrin, rice powder, gelatin and magnesium stearate. The goal was to have the animals ingest approximately 400 mg/kg of weight per day of St. John's wort, based upon a calculation that a 150 gm rat will consume about 15 gm of feed per day. This quantity of dietary St. John's wort was calculated to approximate pharmacologically relevant doses of St. John's wort in humans (22). Since the rats were on the vitamin E supplemented diet for 8 weeks but on the St. John's wort diet for only 2 weeks, older rats were ordered, weighing approximately 100 gm, for the St. John's wort experiments. Thus these animals also weighed approximately 150 gm at the time they were treated with chemotherapeutic drugs. Because St. John's wort is light sensitive, the diet was stored in the dark. The rats were housed individually in stainless steel wire-bottomed cages.

Blood samples were obtained from the saphenous veins of the animals. Both lower hind legs on each animal were shaved with clippers. A thin layer of silicone grease was applied as the

selected leg was held tightly at the hip. The pressure applied is sufficient to cause the saphenous vein to become clearly visible. Using a Microlance blood lancet a small puncture wound was made in the saphenous vein. The blood was collected in a StatSpin heparinized capillary tube (300 μ L), mixed and centrifuged at 12,000 rpm for 10 minutes. The plasma was removed and stored at -80°C in a microcentrifuge vial. Blood for white blood cell (WBC) counts was collected using the Unopette System (Becton Dickinson). WBC counts were performed manually after dilution of blood in the Unopette System. Blood for hematocrit (Hct) measurements was collected in micro-hematocrit capillary tubes. Cardiac and liver tissues were flash frozen with liquid nitrogen and stored at -80°C.

Euthanasia was performed by pentobarbital sodium injection (60 mg/kg IP) followed by exsanguination via cardiac puncture. Criteria used to determine humane euthanasia included: 1) >20% weight loss within 3 days after chemotherapy without rebound within 2 days; 2) >20% weight loss in any animal that persists more than 2 days; 3) a white blood cell (WBC) count <500/mm³ on Day 4 after chemotherapy; 4) Diarrhea >2 days or bloody diarrhea at any time; 5) dehydration >2 days (treated with 10 ml of sterile saline IP); 6) walking on tiptoe; 7) cold to touch; 8) unable to stand; and 9) for cyclophosphamide-treated animals only: cystitis > 2 days. Gross necropsies were performed.

Chemotherapy drugs. Doxorubicin Hydrochloride injection was obtained from GensiaSicor Pharmaceuticals (Irvine, CA 92618) supplied as 10 mg/5 mL. Cyclophosphamide was obtained as Lyophilized CYTOXAN (Mead Johnson/Bristol Myers Squibb Co, Princeton, NJ) and was reconstituted with sterile water for injection, USP (Abbott Laboratories, North Chicago, IL). Docetaxel (Taxotere®) was a gift from Aventis Pharmaceuticals Products Inc., (Bridgewater, NJ 08807). The concentrate for injection form of the drug was diluted for use.

We had tried reconstituting the powdered form of docetaxel with ethanol and Tween 80, but this preparation was poorly tolerated by the rats and caused rapid death in some animals.

Vitamin E assay. α -Tocopherol (vitamin E) concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography (HPLC) method using UV detection as previously reported by Julianto *et.al.* (23). Briefly plasma samples were deproteinized by treating 100 μ L plasma with 200 μ L acetonitrile-tetrahydrofuran (3:2 [vol/vol]). The mobile phase consisted of 6% tetrahydrofuran in methanol with a flow rate of 1mL/min through an Econosphere C18 5 μ column (Alltech, Deerfield, IL) and an Econosphere C18 5 μ guard column (Alltech). The detector was operated at a wavelength of 292nm and the samples were quantified using peak area. A good concentration-peak area relationship was obtained with a correlation coefficient of 0.96.

St. John's wort assay. Because recent studies have identified hyperforin as an important contributor to the pharmacological actions of this herbal product (24), we employed an HPLC analytical method that accurately detects and determines hyperforin. Hyperforin concentrations in plasma were measured by reverse-phase high-performance liquid chromatography with UV detection. Using a modification of a previously reported method (25), hyperforin was extracted from plasma by solid-phase extraction on 3mL Strata C18E columns (Phenomenex, Torrance, CA). The columns were first conditioned with 3mL methanol followed by 3mL water. The samples were loaded onto individual Strata columns and washed with 2mL of water and hyperforin was eluted from the columns with 1mL of methanol. The eluents were evaporated to dryness under nitrogen and the residues were reconstituted in 75% acetonitrile in water containing 3mL phosphoric acid per liter. The mobile phase consisted of acetonitrile-0.01M ammonium phosphate (pH 2.5) (85:15 [vol/vol]) with a flow rate of 1.5 mL/min through a

Symmetry C18 5 μ column with a matching guard column (Waters, Milford, MA). The detector was operated at a wavelength of 272nm and the samples were quantified using peak area.

Doxorubicin assay. Doxorubicin concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using fluorescence detection as previously reported by Warren *et al.* (26) and as modified by us. Briefly, plasma samples were deproteinized by treating with 4 volumes of ethanol and the supernatant evaporated under nitrogen and redissolved in the mobile phase. The mobile phase consisted of 0.4M ammonium formate pH 4.0-acetonitrile (79:21 [vol/vol]) with a flow rate of 2mL/min through a μ -Bondapak phenyl column (Waters Corporation, Milford, MA) with a PS-GU phenyl 5 μ guard column (Thomson Instrument Company, Springfield, VA). An excitation wavelength of 480nm and emission wavelength of 595nm was used for fluorescence detection and the samples were quantified using peak area. Excellent concentration-peak area relationships (correlation coefficients > 0.99) were obtained for samples of pooled rat plasma containing doxorubicin.

Mitochondrial DNA assays. Total cardiac and hepatic DNA was isolated using the Qiagen DNeasy Tissue kit (Valencia, CA). Primers and probes for the rat D-loop and the rat mitochondrial deletion from the rat mitochondrial genome (GenBank accession X14848) and rat β -actin primers (GenBank V01217) were designed using Primer Express software (Applied Biosystems). Primers and probes were synthesized and HPLC purified by the Oligo Factory (Applied Biosystems). Sequence for the primers and probes can be found in Table 1.

Mitochondrial deletion expression was quantified with a 5' VIC reporter and a 3' TAMRA quencher dye and D-loop expression with a 5' 6-FAM reporter and 3' TAMRA labeled quencher dye. PCR amplification was carried out as previously reported (27). Briefly, the reaction concentrations for the common mitochondrial deletion/D-loop assays (CD/DL) (50 μ l

reaction) consisted of 1X TaqMan Universal mix, 200nM each mitochondrial deletion forward and reverse primers, 100nM each D-loop forward and reverse primers, and 100nM each mitochondrial deletion and D-loop probe and ~50ng of sample DNA. For the D-Loop/ β -actin (DL/BA) assays, conditions (50 μ l reaction) consisted of 1X TaqMan Universal mix, 200nM each β -actin forward and reverse primers, 50nM each D-loop forward and reverse primers, and 100nM each β -actin and D-loop probe and ~50ng of sample DNA. The cycling conditions include an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C. Each sample was assayed in duplicate and the fluorescence spectra was continuously monitored by the 7700 Sequence Detection System (Applied Biosystems) with sequence detection software version 1.6.3.

Statistical Analyses. Pharmacokinetics parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats analyzed by Win-Nonlin (Pharsight Corporation, Mountain View, CA.). Statistical analyses were performed using SAS version 8.02 (SAS Institute, Cary, NC). For the pharmacokinetic and mitochondrial DNA data, the control group was compared to each of the treatment groups using the t-test procedure with the alpha adjusted by the number of comparisons (Bonferroni method). For the analyses of the WBC counts and Hct, parametric analyses of variance (ANOVA) were run without any data transformation. Confirmatory nonparametric analyses were run using rank scores; all significant results were confirmed with similar p-values. The post-hoc mean comparisons were done using the LSD procedure. The median lethal dose levels (LD50) were calculated using Proc Probit.

RESULTS

Preliminary studies were performed with a preparation of St. John's wort extract (HBC St. John's wort) that is standardized to 0.3% Hypericin and 4% Hyperforin. The herb was suspended in water at a dose level of 100 mg/kg. This converts to approximately the same concentration that is available to humans ingesting these tablets (22). The suspension was administered daily to rats by gavage, and a 14 day course was planned. Unfortunately, all of the animals lost weight, and most died before the completion of 14 days. Rats in the control group that were given water by gavage survived, indicating that the deaths were due to the St. John's wort preparation rather than to faulty gavage technique. Post-mortem examination by the veterinarian identified esophageal irritation with abscess formation in some animals, and intestinal obstruction in most of the rats. As is the case for other commercially available herbal preparations, this St. John's wort preparation contained a variety of components other than hyperforin and hypericin, the putative active agents. A review of the ingredients of this St. John's wort preparation indicated that the excipients included silicon dioxide. We believe that this ingredient may have caused the intestinal complications. However, several other St. John's wort preparations also caused toxicity when given by gavage. Further exploratory studies suggested that the stress of gavage in young rats contributed importantly to the observed mortality. Therefore St. John's wort was incorporated into the diet rather than administered by gavage. On this diet the rats grew at the same rate and ingested the same quantity of food as rats maintained on a control diet (data not shown). The hematocrit and white blood cell counts measured after 2 weeks on the diet were not significantly different from rats maintained on the standard diet (data not shown). After 2 weeks on the diet, the rats were found to have hyperforin levels of $2.50 \pm 0.69 \times 10^{-6}$ M. This drug concentration is comparable to levels measured in rats that were given St. John's wort by gavage ($1.38 \pm 0.32 \times 10^{-6}$ M) and similar to levels of hyperforin

reported in the literature by other laboratories (22). A lower dietary intake of 1 g of St. John's wort/kg of feed (1 mg/g) resulted in a hyperforin level of $2.04 \pm 0.69 \times 10^{-6}$ M.

The plasma levels of vitamin E were measured and confirmed that dietary vitamin E content influenced plasma vitamin E levels. Thus, rats on the diet containing 102 I.U./kg vitamin E had plasma levels of 15.4 uM, those on the diet containing 152 I.U./kg vitamin E had plasma levels of 20.0 uM, and those on the diet containing 750 I.U./kg vitamin E had plasma levels of 26.2 uM. These values are similar to levels reported in the literature for rodents maintained on diets enriched for vitamin E (28).

a. *Doxorubicin pharmacokinetics.* In preliminary experiments rats were injected intravenously with doxorubicin at a dose of 5 mg/kg, but plasma concentrations at later time points were at or below the limit of quantitation. Therefore the dose was increased to 7.5 mg/kg. Blood samples were obtained prior to injection of the drug and at 10, 20 and 30 min, and 1, 3, 5, 7, 24 and 48 hrs. For all conditions there was a biexponential elimination pattern, with rapid distribution and slow elimination typically described for doxorubicin in rodents with doses comparable to the 7.5 mg/kg dose used (data not shown). The results for doxorubicin are shown in Table 2. Estimated pharmacokinetic parameters were comparable for rats fed control, low supplement vitamin E and high supplement vitamin E diets. For example, in animals receiving the control diet the elimination half-life was in the 2 hour range and volume of distribution and clearance were 4.5 liters and clearance was 32 ml/min/kg respectively; these parameters were closely similar to those estimated recently for doxorubicin in rats in a study of the effects of a P-glycoprotein inhibitor on the pharmacokinetics of doxorubicin (29). Estimated "peak" plasma concentrations of doxorubicin 10 minutes after intravenous administration were in the 500- 600 nanomolar

range for rats in each study group (data not shown). Estimated plasma concentrations of doxorubicin 24 hours after intravenous administration were also comparable in rats on the high and low vitamin E diets. For example the mean doxorubicin concentrations at 24 hours were 35 and 39 nanomolar respectively for the low and high vitamin E diets. (data not shown). Pharmacokinetic parameters for St. John's wort treated rats was similar to parameters in rats fed a standard diet and those supplemented with vitamin E (Table 2). None of the treatment means were significantly different from the control means.

b. Toxicity studies.

- i. **Doxorubicin.** After 8 weeks on the cereal-based diet, or that diet supplemented with a low or high dose of Vitamin E, the rats were injected with increasing amounts of doxorubicin (5.0, 7.5, 9.8, 12.8 and 17.9 mg/kg). The LD50 of doxorubicin for rats on the standard diet and that for rats ingesting the supplemented diets were not significantly different, and there was no dose-response relationship for Vitamin E (Table 3). Measurements of Hct and WBC counts on Days 4, 9 and 14 following injection of doxorubicin were obtained. Since the nadir for the Hct and WBC count was on Day 4, the nadir Hct and WBC and the drop from the initial to the nadir level of the Hct (Hctfall) and WBC (Nfall) was determined on Day 4. The p-value for dose was significant ($p < .001$), indicating that increasing doses of doxorubicin were associated with decreasing levels of Hct and WBC (data not shown). Statistical analyses showed no important differences among the dietary groups (data not shown).

Rats fed the standard diet or the same diet supplemented with St. John's wort for 2 weeks were injected with increasing amounts of doxorubicin (12, 15, 18 and 22 mg/kg). These dosages were selected based upon the observed LD50 in the previous

experiment with Vitamin E. The LD50 of doxorubicin for rats on the standard diet and the LD50 for rats ingesting the St. John's wort supplemented diet were not significantly different (Table 3). The nadir Hct and WBC, Hctfall and Nfall were not significantly different either (data not shown).

ii. Docetaxel. After 8 weeks on the standard or Vitamin E-supplemented diets, rats were injected with increasing doses of docetaxel (8.5, 12, 18 and 20 mg/kg). As shown in Table 3, these LD50s did not differ significantly from the LD50 of rats fed the standard diet. The nadir WBC count was significantly higher ($p=.004$) in rats fed the diet supplemented with low dose vitamin E than in either the standard or high vitamin E dietary groups (Figure 1). The Nfall was significantly higher ($p=0.04$) in the high vitamin E group compared to the low vitamin E group, but neither was different from the standard diet group (Figure 2). There was no effect of vitamin E supplementation on Hct or Hctfall after docetaxel treatment (data not shown). In rats fed the standard or St. John's wort diet for 2 weeks, the LD 50s were not significantly different after injection of increasing doses of docetaxel (12, 15, 18, 25, 30 and 40 mg/kg) (Table 3). Hct and WBC measurements were not analyzed after this experiment.

ii. Cyclophosphamide. After 8 weeks on the standard or Vitamin E-supplemented diet, rats were injected with increasing doses of cyclophosphamide (25, 85, 144, and 200 mg/kg). These dosages were selected based upon our prior experience (30). The LD50s for the standard diet, low supplement Vitamin E diet, and high supplement Vitamin E diet were not significantly different (Table 3). The Nfall was greater ($p=.03$) in the standard and high vitamin E dietary groups than in the low vitamin E

group (Figure 3). The nadir WBC, nadir Hct and the Hctfall were not different (data not shown). Rats that were fed the standard diet or the St. John's wort-supplemented diet for 2 weeks were injected with cyclophosphamide (25, 85, 144 and 190 mg/kg). The observed LD50s were not significantly different (Table 3), nor were the differences in nadir Hct and WBC, or in Nfall (data not shown). However, Hctfall was significantly greater ($p=.01$) in rats fed the standard diet compared to those supplemented with St. John's wort (data not shown).

- c. **Mitochondrial DNA deletions and copy number.** A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the rat (27). This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. Table 4 shows the results obtained with this assay at a doxorubicin dose level of 12.8 mg/kg. In this experimental system, a smaller ΔC_T deletion indicates *more* deletions, while a smaller ΔC_T copy number indicates *less* total mitochondrial DNA. The data in Table 4 suggests that doxorubicin does not increase, and in fact may modestly decrease the number of deletions and copy number in cardiac or hepatic mitochondrial DNA. Although most of these differences were not significant, there was a significant decrease ($p=0.01$) in the number of DNA deletions in liver after doxorubicin treatment compared to rats fed the standard diet. The only evidence that vitamin E supplementation modulated mitochondrial DNA damage caused by doxorubicin was an increased DNA copy number in the hepatic samples from rats fed the high vitamin E supplement ($p = 0.03$). We also measured the effect of vitamin E levels and St. John's wort supplementation on mitochondrial DNA damage in rat liver caused by docetaxel (Table 5).

None of the treatment means were significantly different from the means of untreated rats fed the standard diet (with adjusted alpha), with the exception of the difference in copy number between the untreated animals and those supplemented with St. John's wort ($p=0.03$).

Therefore we conclude that dietary vitamin E levels and St. John's wort supplementation do not have an important effect on the mitochondrial DNA damage caused by either doxorubicin or docetaxel.

DISCUSSION

These results suggest that dietary supplementation of rats with vitamin E can mitigate the neutropenia caused by docetaxel or cyclophosphamide. This observation is consistent with our previous study in women with breast cancer, wherein the decrease from initial neutrophil count to nadir (Nfall) was lower for women taking multivitamins or vitamin E (5). In the present study this beneficial effect appears to be dose-dependent, because it was observed with low but not with high concentrations of vitamin E supplementation. Thus there may be an optimal level of vitamin E that will reduce chemotherapy-induced bone marrow toxicity. The mitigating effect was not seen with all chemotherapeutic agents, since neither high nor low levels of supplemental vitamin E influenced the white count after doxorubicin. While neutropenia is a major and sometimes life-threatening toxic effect of chemotherapy, its amelioration by vitamin E was not sufficient to improve survival in treated rats as determined by the LD50. In addition there was no favorable effect of vitamin E supplementation on anemia caused by chemotherapy. Therefore the benefit of vitamin E to these animals in reducing toxicity was limited. In contrast to vitamin E, there was no positive or negative effect of dietary supplementation with St. John's wort with

respect to neutropenia or survival in rats, but anemia was somewhat less severe in supplemented animals.

The risks and benefits of dietary supplementation with vitamin E in both the general population and in cancer patients are controversial. Oxidative injury has been implicated in the pathogenesis of the two leading causes of death in humans, namely atherosclerosis and cancer. Therefore it is not surprising that vitamin E is often used to reduce oxidative stress. For example, vitamin E is taken as a specific supplement by 24% of U.S. adults undergoing a periodic health examination (31) and by almost half of cancer patients enrolled onto Phase I clinical trials (32). It has been suggested that the addition of antioxidant vitamins is a valuable or even an essential addition to cancer chemotherapy (33). Vitamin E as α -tocopheryl succinate has been described as inducing differentiation, inhibition of proliferation and apoptosis in cancer cells while protecting normal cells (34). However, a recent meta-analysis of the dose-response relationship between vitamin E supplementation and total mortality found that high-dosage vitamin E (≥ 400 IU/d) showed increased risk for all-cause mortality (35). And in patients with vascular disease or diabetes mellitus, long-term vitamin E supplementation did not prevent cancer or major cardiovascular events but did increase the risk of heart failure (36). Recent reviews of the role of antioxidants in cancer therapy have concluded that inconsistencies in trial design preclude definitive conclusions, and that at present there is insufficient evidence to support attenuation of chemotherapy toxicity by vitamin E (37, 38). Thus, although our studies in rodents and humans suggest that, like chemotherapy-induced neuropathy (39), neutropenia may be mitigated by vitamin E, high doses of the vitamin probably should be avoided. In addition, patients with cancer who are receiving chemotherapy should discuss the use of vitamin E with their physicians because vitamin E can contribute to a bleeding tendency (40).

An important component of the toxicity profile of some chemotherapeutic agents such as doxorubicin is cardiac toxicity (41). Vitamin E has been studied extensively as a protective agent against the acute and chronic forms of doxorubicin cardiotoxicity with variable results (42, 43). We studied the modulatory effect of dietary vitamin E and of St. John's wort on mitochondrial DNA damage caused by doxorubicin and docetaxel. Surprisingly, doxorubicin decreased the number of deletions and copy number in cardiac and hepatic mitochondrial DNA. Vitamin E supplementation had little effect except to increase DNA copy number in the livers of rats in the high vitamin E group. The addition of St. John's wort to the diets of rats was associated with an increased hepatic DNA copy number after docetaxel treatment. The factors that control mitochondrial DNA copy number are poorly understood (44). Mammalian cells tend to maintain a constant mass of mitochondrial DNA rather than a constant number of mitochondrial genomes (44). Paradoxically, an increase in mitochondrial DNA copy number has been reported as a response to oxidative stress (45). Therefore it is unclear why the combination of chemotherapy and either St. John's wort or a high dose of vitamin E was associated with increased DNA copy number in these experiments.

In summary, the studies reported here investigated the possible modulation of several manifestations of chemotherapy-induced toxicity by vitamin E and St. John's wort. These included lethal toxicity (LD50), bone marrow toxicity (neutropenia and anemia) and cardiac toxicity associated with doxorubicin, docetaxel and cyclophosphamide. Neither dietary supplement exacerbated these toxicities. Low dose supplementation with vitamin E was associated with higher nadir neutrophil counts after docetaxel and a smaller drop in WBC count after cyclophosphamide, but high dose vitamin E supplementation caused a greater drop in WBC count after docetaxel. Thus at least in rodents neither St. John's wort nor low dose

supplementation with vitamin E has an adverse effect on chemotherapy-induced toxicity, and low dose supplements may mitigate post-chemotherapy neutropenia.

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LEGENDS TO FIGURES

1. Nadir WBC counts measured 4 days after treatment of rats with docetaxel. The nadir WBC count was significantly higher ($p=0.004$) in rats fed the diet supplemented with low dose vitamin E (stippled bar) than in either the standard (diagonal cross-hatch bar) or high vitamin E (horizontal cross-hatch bar) dietary groups. There were 6 animals in each group. SEM is the standard error of the mean.
2. The decrease in WBC count (Nfall) from initial to nadir levels in rats after treatment with docetaxel. The drop was greater ($p=0.04$) in the high dose vitamin E group (horizontal cross-hatched bar) than in the standard diet (diagonal cross-hatched bar) or the low dose vitamin E group (stippled bar). There were 6 animals per group. SEM is the standard error of the mean.
3. The decrease in WBC count (Nfall) from initial to nadir levels in rats after treatment with cyclophosphamide. The drop was greater ($p=0.03$) in the standard (diagonal cross-hatched bar) and high dose vitamin E (horizontal cross-hatch bar) groups than in the low dose vitamin E (stippled bar) group. There were 6 animals per group. SEM is the standard error of the mean.

Table 1. Sequences of the TaqMan primers and probes

<i>Primer/Probe</i>	<i>Sequence (5'-3')</i>
Mitochondrial D-loop	
Forward	GGTTCTTACTTCAGGGCCATCA
Reverse	GATTAGACCCGTTACCATCGAGAT
Probe	6FAM-TTGGTTCATCGTCCATACGTTCCCCTTA-TAMRA
Mitochondrial deletion	
Forward	AAGGACGAACCTGAGCCCTAATA
Reverse	CGAAGTAGATGATCCGTATGCTGTA
Probe	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
β-actin	
Forward	GGGATGTTTGCTCCAACCAA
Reverse	GCGCTTTTGACTCAAGGATTAA
Probe	VIC-CGGTCGCCTTCACCGTTCCAGTT-TAMRA

Table 2. Pharmacokinetics parameters of doxorubicin in control, vitamin E or St. John's wort-treated rats analyzed by Win-Nonlin.

<i>Diet</i>	<i>AUC</i> ¹	<i>Cl (ml/min)</i>	<i>T</i> _{1/2} (<i>min</i>)	<i>V</i> _{ss} (<i>liters</i>)	<i>N</i> ²
Control	6.1 ± 2.5 ³	32.5 ± 18.9	96.5 ± 28.0	4.5 ± 1.6	4
High E	7.4 ± 1.1	22.0 ± 4.5	132.3 ± 30.1	4.6 ± 1.0	5
Low E	8.5 ± 4.1	22.5 ± 9.6	86.2 ± 44.7	3.5 ± 0.7	4
St. John's wort	9.8 ± 3.9	21.7 ± 7.5	108.1 ± 31.5	3.7 ± 2.7	6

¹AUC x 10 = micromoles/liter x minutes

² N = number of animals

³ mean ± SD

Table 3. Median lethal dose (LD50) for rats receiving dietary supplementations and cancer chemotherapy.

	Doxorubicin		Docetaxel		Cyclophosphamide	
	<i>LD50</i>	<i>95% CI¹</i>	<i>LD50</i>	<i>95% CI</i>	<i>LD50</i>	<i>95% CI</i>
	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Vitamin E						
<i>Standard</i>	13.3	11.2-17.1	18.9	14.6-124.6	135.8	73.1-237.4
<i>Low Supplement</i>	12.7		20.4		135.2	54.6-670.0
<i>High Supplement</i>	13.0		16.5	12.9-21.8	134.8	92.9-194.4
St. John's wort						
<i>Standard</i>	16.3	13.4-20.0	28.0	22.5-40.1	117.0	79.4-151.5
<i>Supplement</i>	18.2		25.8		144.0	

¹95% CI = 95% confidence intervals; not all 95% confidence intervals could be calculated because of very large, unstable variances.

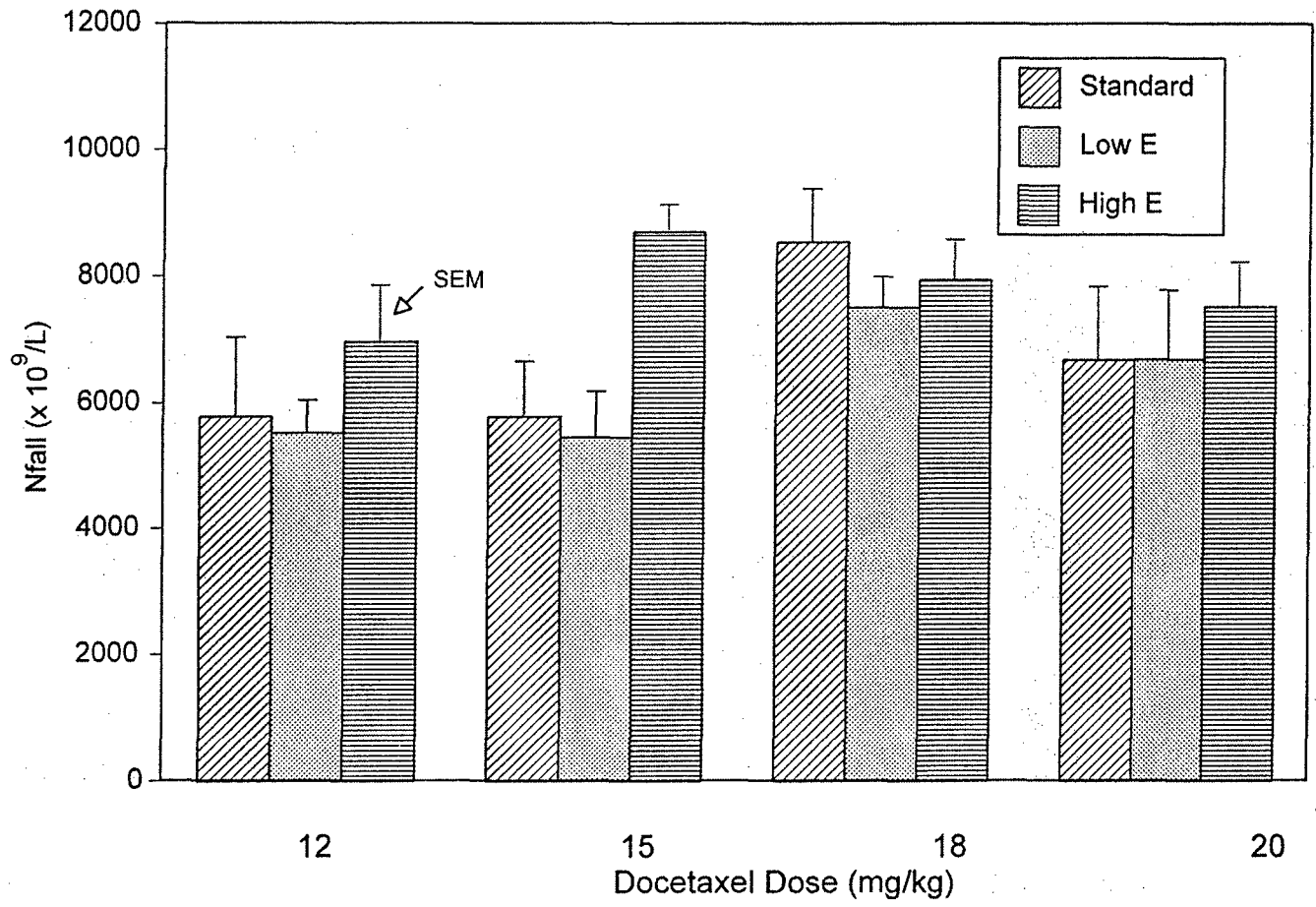
Table 4. Mitochondrial DNA deletion and copy number in rats maintained on standard rat chow or supplemented with low or high concentrations of vitamin E. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{Deletion}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg Doxorubicin in the standard diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β -actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{CopyNumber}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg Doxorubicin in the standard diet. Relative Expression indicates the fold difference in deletions or copy number compared to the standard diet.

	Heart			Liver		
	ΔC_T Deletion (n^1)	Rel Exp	ΔC_T Copy Number (n)	Rel Exp	ΔC_T Copy Number (n)	Rel Exp
Standard Diet	7.95 $\pm$ 0.62 (6)	1.00	12.33 $\pm$ 0.45 (6)	1.00	10.01 $\pm$ 0.22 (6)	1.00
Doxorubicin (12.8mg/kg)						
Standard Diet	9.84 $\pm$ 2.80 (4)	0.27	12.34 $\pm$ 1.18 (4)	0.99	7.70 $\pm$ 0.97 (4)	0.73
Low Vitamin E	12.45 $\pm$ 3.47 (2)	0.04	12.45 $\pm$ 1.82 (2)	0.92	8.12 $\pm$ 0.04 (2)	0.55
High Vitamin E	9.96 $\pm$ 0.72 (5)	0.25	12.25 $\pm$ 0.40 (5)	1.06	8.08 $\pm$ 0.23 (5)	0.56

¹ n : number of animals

Table 5. Mitochondrial DNA deletion and copy number in liver from rats fed a standard diet alone, or the same diet supplemented with St. John's wort, low supplement Vitamin E, or high supplement Vitamin E. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{Deletion}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg docetaxel in the standard diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β -actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean $\pm$ SD. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ equals $\Delta C_{T\text{CopyNumber}} - \Delta C_{T\text{Calibrator}}$ where the calibrator is the ΔC_T of 0 mg/kg docetaxel in the standard diet. Relative Expression indicates the fold difference in deletions or copy number compared to the standard diet. 1n : number of animals

	ΔC_T Deletion (n^1)	Rel Exp	ΔC_T Copy Number (n)	Rel Exp
Standard Diet	8.69 $\pm$ 1.08 (6)	1.0	9.33 $\pm$ 0.16	1.0
Docetaxel (18 mg/kg)				
Standard Diet	7.94 $\pm$ 0.57 (7)	1.68	9.40 $\pm$ 0.19 (7)	0.95
St. John's wort	7.59 $\pm$ 0.46 (5)	2.14	9.14 $\pm$ 0.12 (5)	1.14
Low supplement Vitamin E	8.16 $\pm$ 0.45 (4)	1.44	9.37 $\pm$ 0.17 (4)	0.97
High supplement Vitamin E	8.29 $\pm$ 1.01 (3)	1.32	9.52 $\pm$ 0.31 (3)	0.88



Research Reports

Development of a Quantitative PCR (TaqMan) Assay for Relative Mitochondrial DNA Copy Number and the Common Mitochondrial DNA Deletion in the Rat

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Changes in mitochondrial DNA copy number and increases in mitochondrial DNA mutations, especially deletions, have been associated with exposure to mutagens and with aging. Common deletions that are the result of recombination between direct repeats in human and rat (4,977 and 4,834, bp, respectively) are known to increase in tissues of aged individuals. Previous studies have used long-distance PCR and Southern blot or quantitative PCR to determine the frequency of deleted mitochondrial DNA. A quantitative PCR (TaqMan) assay was developed to detect both mitochondrial DNA copy number and deletion frequency in the

rat. This methodology allows not only the determination of changes in the amount of mitochondrial DNA deletion relative to total mitochondrial DNA but also to determine changes in total mitochondrial DNA relative to genomic DNA. As a validation of the assay in rat liver, the frequency of the common 4,834 bp deletion is shown to increase with age, while the relative mitochondrial DNA copy number rises at a young age (3–60 days), then decreases and holds fairly steady to 2 years of age. *Environ. Mol. Mutagen.* 44:000–000, 2004. © 2004 Wiley-Liss, Inc.

Key words: rat; mitochondrial DNA; quantitative PCR; mitochondrial deletion; aging

INTRODUCTION

Somatic mutations in mitochondrial DNA (mtDNA) are hypothesized to be a (or the) cause of aging and age-related disease [Harman, 1972; Linnane et al., 1989; Gadaleta et al., 1998]. According to the free radical theory of aging, mtDNA damage leads to free radical production that in turn causes further damage. Numerous studies have found increases in mitochondrial mutations in a variety of tissues from aged individuals. While point mutations of mtDNA have been shown to vary in both normal and malignant tissues and increase with age [Munscher et al., 1993; Yowe and Ames, 1998; Zhang et al., 1998; Fliss et al., 2000; Jones et al., 2001; Kirches et al., 2001; Penta et al., 2001; Lin et al., 2002; Khaidakov et al., 2003], most studies have focused on the induction of large deletions in mtDNA. In humans, a 13 bp direct repeat (at 8,470–8,482 and 13,447–13,459) in the mtDNA leads to the frequent occurrence of a 4,977 bp deletion by recombination between the repeats [Holt et al., 1989; Schon et al., 1989; Tang et al., 2000]. Heteroplasmic inheritance of this deletion causes Pearson

syndrome or Kearns-Sayre syndrome [Holt et al., 1989, 1989; Schon et al., 1989; Rotig et al., 1991], but this deletion also occurs frequently during aging.

A similar common mtDNA deletion occurs in the rat due to a 16 bp repeat at 8,103–8,118 and 12,937–12,952; recombination between these repeats leads to a 4,834 bp deletion [Gadaleta et al., 1992; Edris et al., 1994]. This

Abbreviations: CD/DL, common mitochondrial deletion/mitochondrial D-loop; C_T , cycle threshold; ΔC_T , C_T experimental gene – C_T control gene; $\Delta\Delta C_T$, ΔC_T of unknown sample – ΔC_T of reference sample; DL/BA, mitochondrial D-Loop/ β -actin.

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deletion also increases in aged rats [Gadaleta et al., 1992; Filburn et al., 1996; Seidman et al., 1997; Kang et al., 1998; Yowe and Ames, 1998; Nagley et al., 2001]. It can be increased by some drugs [Nagley et al., 2001; Suliman et al., 2002] or stress [Sakai et al., 1999] and decreased by dietary restriction in at least some tissues in rats [Kang et al., 1998]. Other mtDNA deletions also are seen in aged rats [Van Tuyle et al., 1996].

Quantitative PCR (qPCR) generally is used to detect changes in gene expression (RT-qPCR), but also can be employed to detect amplifications or deletions in genomic DNA. Quantitation studies have been performed either with Northern blots, Western blots, Southern blots, or quantitative PCR blots, all of which are tedious, technically demanding, and involve the use of radioisotopes. Furthermore, these PCR methods use an endpoint determination that may not be truly quantitative because of plateau effects.

Several methods have been developed using real-time PCR (either TaqMan or molecular beacons) to monitor either mtDNA copy number or the amount of the common deletion in human cells [Heid et al., 1996; Steuerwald et al., 2000; Gahan et al., 2001; Lim et al., 2001; Reynier et al., 2001; Rodriguez-Santiago et al., 2001; Szuhai et al., 2001; He et al., 2002; Miller et al., 2003]. Rodriguez-Santiago et al. [2001] determined mtDNA copy number in the brains of Alzheimer patients using qPCR of the mitochondrial *ND2* gene vs. the nuclear 18S gene. Wong and Bai [2002] determined copy number in patients with mitochondrial disease. Reynier et al. [2001] quantified absolute mtDNA copy number in oocytes. Gahan et al. [2001] used molecular beacons of the mitochondrial cytochrome b and nuclear *CCR5* genes to measure mtDNA copy number in human monocytes and in the fat of AIDS patients. Szuhai et al. [2001] also used molecular beacons for the mitochondrial tRNA *LYS* gene and the nuclear globin gene to determine heteroplasmy in MERRF syndrome patients using a $\Delta\Delta C_T$ method. Heid et al. [1996] and Lim et al. [2001] used the mtDNA and nuclear β -actin gene in a TaqMan relative curve method to quantitate mtDNA copy number, while Steuerwald et al. [2000] quantitated mtDNA copy number in oocytes using a standard curve method and SYBR green. He et al. [2002] used TaqMan probes against the commonly deleted *ND4* gene and the rarely deleted *ND1* gene to determine mtDNA copy number. Miller et al. [2003] determined the ratio of mtDNA (*CYTb* gene) vs. a nuclear gene (β -globin) as compared to plasmid standards. Lastly, a number of groups are now using real-time PCR to quantitate human mtDNA in patients treated with antiviral drugs for HIV infection [Cossarizza et al., 2003; Gourelain et al., 2003; Miura et al., 2003; Pace et al., 2003; Lopez et al., 2004].

Two groups have reported TaqMan methods to measure the human common deletion. Koch et al. [2001] used a nondeleted region probe and a probe specific for the deletion to quantitate the deletion in human keratinocytes, while

Meissner et al. [2000] used similar probes to detect the deletion in human blood.

We wished to develop a fast and inexpensive method to measure changes in mtDNA copy number and the frequency of the common mtDNA deletion in rats. This report details the optimization of such a method quantitating rat mtDNA as compared to a nuclear gene (β -actin) as well as quantitating the amount of the common deletion relative to the number of copies of the D-loop region. The reproducibility of the method is demonstrated and the method is used to determine the changes in relative mtDNA copy number and the frequency of the rat common deletion in 3-day-old to 2-year-old animals. This assay has also been used to show increases in mtDNA copy number and deletion frequency in sialodacryoadenitis virus-infected rats and also that diet can affect the frequency of mtDNA deletions after chemotherapy [Branda et al., 2002a, 2002b].

MATERIALS AND METHODS

Animals and DNA Extraction

A control female Fisher 344 rat (#6776) was obtained from Charles River Canada (St.-Constant, Quebec, Canada) and fed ad libitum a standard cereal-based rat chow (LM-485 Harlan Teklad, Madison, WI). For the aging experiments, Sprague-Dawley rats from newborn to 26 months of age (3-day-old, 10-day-old, 30-day-old, 45-day-old, 60-day-old, 120-day-old, 240-day-old, 365-day-old, and 735-day-old) were utilized. All time points used three animals except the 735-day point, where only two animals could be procured (others died in transit). The animals younger than 3 months were a gift from Dr. Deborah Damon, who obtained the mothers from Harlan Teklad (Madison, WI). The older animals were also obtained from Harlan Teklad at the indicated age, housed 1 week, and then euthanized. They were also fed the standard chow as above. The Sprague-Dawley rats that were old enough for gender to be determined (30-day-old) were all male. The rats were sacrificed and the liver tissue removed and stored at -80°C until processing. Total hepatic DNA was isolated using the Qiagen DNeasy Tissue kit (Valencia, CA).

All animal experiments were approved by the University of Vermont Institutional Animal Care and Use Committee and American Veterinary Medical Association guidelines for euthanasia were followed. The rats were euthanized by lethal injection of pentobarbital followed by exsanguination. The University of Vermont Animal Care Facilities follow procedures that are in accordance with the Guide to the Care and Use of Laboratory Animals and are certified by the Association for Assessment and Accreditation of Laboratory Animal Care.

Oligonucleotide Primers and TaqMan Probe Design

Primers and probes for the rat D-loop and the rat mitochondrial deletion from the rat mitochondrial genome (Genbank accession X14848) and rat β -actin primers (Genbank V01217) were designed using Primer Express software (Applied Biosystems, Foster City, CA). Primers and probes were synthesized and HPLC-purified by the Oligo Factory (Applied Biosystems). The sequences of the primers and probes can be found in Table I.

Real-Time PCR

Mitochondrial deletion expression was quantified with a 5' VIC reporter and a 3' TAMRA quencher dye and D-loop expression with a 5' 6-FAM

TABLE I. Sequences of the TaqMan Primers and Probes

Primer/probe	Sequence (5'-3')
Mitochondrial D-loop	
Forward	GGTTCTTACTTCAGGGCCATCA
Reverse	GATTAGACCCGTTACCATCGAGAT
Probe	6FAM-TTGGTTCATCGTCCATACGTTCCGCTTA-TAMRA
Mitochondrial deletion	
Forward	AAGGACGAACCTGAGCCCTAATA
Reverse	CGAAGTAGATGATCCGTATGCTGTA
Probe	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
β-actin	
Forward	GGGATGTTTGCTCCAACCAA
Reverse	GCGCTTTTGACTCAAGGATTAA
Probe	VIC-CGGTCGCTTCACCGTCCAGTT-TAMRA

TABLE II. Effects of Altering Primer Concentrations on C_T and ΔC_T

β -actin primer	D-loop primer	β -actin C_T	D-loop C_T	D-loop C_T - β -actin C_T (ΔC_T)
200 nM	100 nM	27.55	18.54	-9.01
200 nM	50 nM	27.09	19.03	-8.06
200 nM		27.45		
100 nM	200 nM	28.48	18.38	-10.1
50 nM	200 nM	29.95	18.45	-11.5
	200 nM		18.42	
Common deletion primer	D-loop primer	Common deletion C_T	D-loop C_T	Common deletion C_T - D-loop C_T (ΔC_T)
200 nM	200 nM	23.54	18.43	5.11
200 nM	100 nM	22.48	18.31	4.17
200 nM	50 nM	22.82	18.74	4.08
200 nM		23.32		
100 nM	200 nM	25.36	18.36	7.00
50 nM	200 nM	28.41	18.22	10.10

reporter and 3' TAMRA-labeled quencher dye. PCR amplification was carried out in a 50 μ L reaction consisting of 1 $\times$ TaqMan Universal Master Mix (Applied Biosystems) and varied concentrations of probe and varied concentrations of forward and reverse primers to optimize reaction conditions (Table II). Optimal reaction conditions were those concentrations that gave the maximum ΔR_n and minimum C_T . The final optimal reaction concentrations for the common mitochondrial deletion/D-loop assays (CD/DL; 50 μ L reaction) consisted of 1 $\times$ TaqMan Universal mix, 200 nM each mitochondrial deletion forward and reverse primer, 100 nM each D-loop forward and reverse primer, 100 nM each mitochondrial deletion and D-loop probe, and $\sim$ 50 ng of sample DNA. For the D-Loop/ β -actin (DL/BA) assays, final conditions (50 μ L reaction) consisted of 1 $\times$ TaqMan Universal mix, 200 nM each β -actin forward and reverse primer, 50 nM each D-loop forward and reverse primer, and 100 nM each β -actin and D-loop probe, and $\sim$ 50 ng of sample DNA. The cycling conditions included an initial phase of 2 min at 50°C; followed by 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was assayed in duplicate and fluorescence spectra were continuously monitored by the 7700 Sequence Detection System (Applied Biosystems) with sequence detection software version 1.6.3.

Analysis of Data

Data analysis was based on measurement of the cycle threshold (C_T), which is the PCR cycle number when the fluorescence measurement reaches a set value. Two types of experiments were performed. The first

measured mtDNA copy number vs. nuclear DNA copy number by amplification of the mitochondrial D-Loop vs. the nuclear β -actin gene (DL/BA), while the second measured the amount of the mitochondrial deletion vs. the mitochondrial D-Loop (CD/DL). The difference in C_T values was used as the measure of relative abundance, i.e., in DL/BA experiments, $C_T(\text{DL}) - C_T(\text{BA})$ was used as the abundance of the mitochondrial genome, and in the MD/DL experiments, $C_T(\text{CD}) - C_T(\text{DL})$ was used as the abundance of the mitochondrial deletion. Descriptive statistics and plots were used to explore possible trends in ΔC_T values based on the day that the experiment was performed. Linear regression was used to characterize the strength of any trends to the extent that they were linear.

Analysis of variance (ANOVA) was used for comparisons of relative expression levels for different rats. Dunnett's [1955] method for multiple comparisons was used to compare results for older rats to those from the 3-day-old rats.

RESULTS

The objective was to develop an assay that could detect both changes in the amount of mtDNA in a cell (as compared to a known nuclear gene, which has two copies/diploid genome) and in the amount of the common mtDNA deletion as compared to the number of mitochondria. We chose to use a sequence in the rat β -actin gene as our

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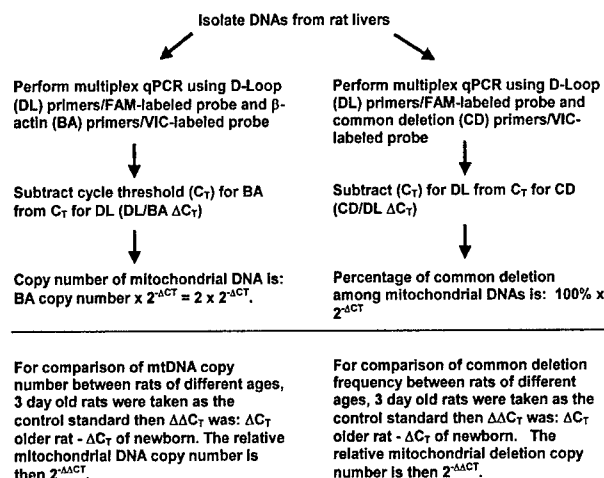


Fig. 1. Flow chart for the experiments and the calculation of results.

nuclear gene control, a sequence in the mitochondrial D-loop as the measure of the number of mitochondria, and a sequence that represented the new fusion sequence present only in deleted mtDNA as a measure for the common deletion. Figure 1 is a flow chart of the experimental design and result calculations.

TaqMan primers and probes were designed for the rat mitochondrial D-loop region, across the common deletion, and for the rat β -actin gene (Table I). PCR reactions were performed with these primers using liver DNA isolated from a single control F344 rat. The specificities of all three sets of primers were tested by demonstrating the correct size product on an agarose gel and by DNA sequencing, which revealed the correct sequence (data not shown).

For qPCR, it is important that the efficiencies of the PCR reactions be close to 100% [i.e., the slope of the graph of C_T vs. $\log$ (DNA) is -3.33]. Figure 2 plots the results for the β -actin, D-loop, and common deletion PCR reactions. All the slopes are close to the optimal -3.33 , with values of -3.346 , -3.241 , and -3.314 for the nuclear β -actin, mitochondrial D-loop, and mitochondrial common deletion reactions, respectively.

Several experiments were performed changing primer and probe concentrations to optimize the assay and to allow utilization of as little primer/probe per assay as possible for cost considerations. Table II shows the results of these experiments. Use of one primer at 50 nM while the other at 200 nM did decrease the C_T ; however, use of 100 nM vs. 200 nM probes did not greatly affect the results.

TaqMan experiments can be quantitated comparatively in the same reaction to an endogenous control. To compare different experimental conditions, the ΔC_T of the experimental sample is subtracted from the ΔC_T of the control sample; the DNA concentration difference between the two experiments is given by $2^{-\Delta\Delta C_T}$ (the $\Delta\Delta C_T$ method). We chose to use the $\Delta\Delta C_T$ method because it affords savings in reagents and time. However, in order to utilize this method,

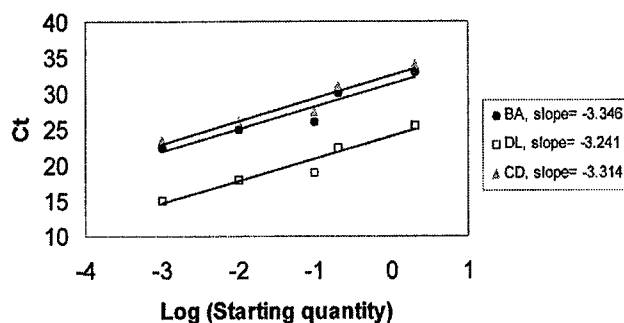


Fig. 2. Slopes of C_T vs. $\log$ (starting DNA). C_T was measured for five dilutions of input DNA for each primer and probe set separately [β -actin (BA), D-loop (DL), and common deletion (CD)]. C_T is plotted vs. the $\log$ of the input DNA. Slopes of the trend line for each probe were determined. A primer/probe set with 100% efficiency would have a slope of -3.33 .

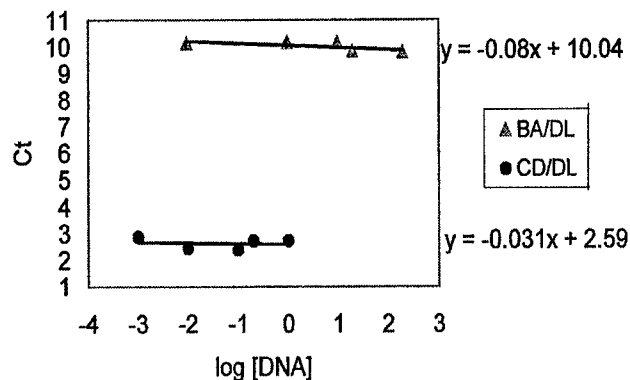


Fig. 3. Slopes of ΔC_T vs. input DNA for the multiplex PCR reactions. The multiplex reactions of β -actin with D-loop probe/primers to measure mtDNA copy number (DL/BA) and common deletion with D-loop probe/primers to measure the frequency of the common mitochondrial deletion (CD/DL) were performed on five dilutions of input DNA. The ΔC_T is plotted vs. the $\log$ of input DNA. Slopes should be < 0.1 for a good probe/primer set.

it is necessary to demonstrate that the ratio of the two C_T s stays constant over a wide concentration range. In practical terms, this means that the absolute value of the slope of the ΔC_T vs. $\log$ amount of input DNA is less than 0.1. Figure 3 shows the plots for the D-Loop/ β -actin (DL/BA) and common deletion/D-loop (CD/DL) results. For confirmation, experiments were performed with each PCR separately and in multiplex. These reactions gave similar results at a given concentration whether the PCR was a singleplex or multiplex (Table II).

In order to demonstrate the reproducibility of the assay, the DL/BA and CD/DL assays were performed a total of 23 times over a period of 12.5 months on the same concentration of a single DNA sample from a 15-week-old rat (#6776). The mean ΔC_T ($\pm$ standard deviation) for the DL/BA value was -5.25 ± 0.93 while for mean CD/DL value was 8.13 ± 1.00 . There was a significant linear trend in the ΔC_T values for the DL/BA measurement based on the day the experiment was done, with higher values (absolute

values) associated with experiments done on later dates. This linear trend explained roughly 23% of the total variability in the β -actin ΔC_T measurements. The trend is characterized as an average 0.0039 unit decrease in the ΔC_T values for each additional day after the first. For the CD/DL measurements, the trend in ΔC_T vs. day of the experiment had a more pronounced nonlinear trend. However, the linear trend still explains roughly 14% of the overall variability in the ΔC_T values. The linear trend shows an average 0.0033 unit increase in the ΔC_T values for each additional day. The amount of overall variability in the ΔC_T values was higher for the second half of the experiments as compared with the first half. For both measurements, the standard deviation of the results from the second half of the experiment was nearly twice as large as that of the first half. This could indicate some degradation or problems with uniform mixing in the later experiments. Furthermore, differential changes in the nuclear vs. mtDNA will have a significant effect on the DL/BA experiments. Measurements of the D-loop were made for both the DL/BA and CD/DL experiments. The correlation between the two different sets of measurements of D-loop was 0.903. This indicates good reproducibility on a single day and between the two types of assays, i.e., whether the D-loop was multiplexed with BA or CD, the same result was still obtained. The correlation between ΔC_T for DL/BA and ΔC_T for CD/DL is -0.409 .

Finally, a series of livers from Sprague-Dawley rats of different ages (3 days to 23 months) was studied to determine changes in mtDNA copy number and changes in the frequency of the mitochondrial deletion with age. Figures 4 and 5, respectively, show the results of these experiments based on $2^{-\Delta\Delta C_T}$. From Figure 4, the mtDNA copy number appears to rise steeply at 30 days ($P = 0.003$ comparing the 30-day result to the 3-day result using Dunnett's procedure), then decreases slowly to 120 days, before holding fairly steady in old age. The frequency of the mitochondrial deletion rose until 60 days of age ($P = 0.007$ comparing the 60-day result to the 3-day result), then plateaued, before rising again until about 2 years of age ($P = 0.007$ comparing the 2-year result to the 3-day result).

DISCUSSION

Recently, several methods have become available to measure RNA or DNA quantitatively using fast and sensitive real-time (nonendpoint) nonradioactive methods. These involve PCR monitored continuously by fluorescence as the reaction proceeds. The most popular method uses the TaqMan system of Applied Biosystems (ABI). In TaqMan technology, a probe specific for the gene of interest is created with a fluorescent dye at the 5' end and a quencher at the 3' end. As the specific PCR for the gene of interest progresses, the 5' exonuclease of the polymerase frees more and more fluorescent dye from the probe that is then quantified. The amount of mRNA or DNA for a specific gene in

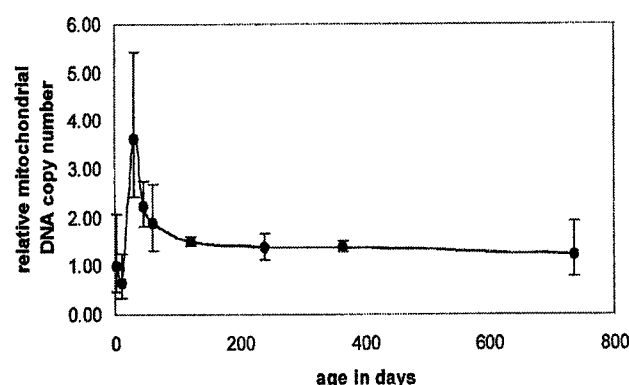


Fig. 4. Changes in mtDNA copy number with age. DNA was isolated from the liver of rats from newborn to 735-day-old (3-day-old, 10-day-old, 30-day-old, 45-day-old, 60-day-old, 120-day-old, 240-day-old, 365-day-old, and 735-day-old). All time points used three animals except the 735-day point, where only two animals could be procured (others died in transit). The 3-day-old DL/BA ΔC_T (mitochondrial copy number) was taken as the reference point for a $\Delta\Delta C_T$ to calculate $2^{-\Delta\Delta C_T}$ (relative mtDNA copy number) that is plotted vs. the age (in days) of the rats.

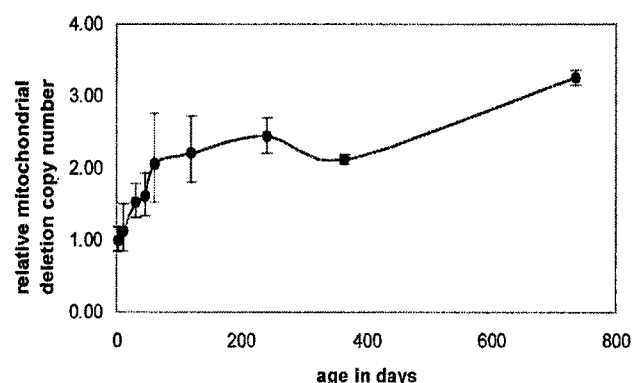


Fig. 5. Changes in the frequency of mitochondrial deletion with age. DNA was isolated from the liver of rats from newborn to 735-day-old as in Figure 4. The 3-day-old CD/DL ΔC_T (mitochondrial deletion frequency) was taken as the reference point for a $\Delta\Delta C_T$ in order to calculate $2^{-\Delta\Delta C_T}$ (relative mitochondrial deletion copy number) that is plotted vs. the age (in days) of the rats.

the experimental sample can be quantified absolutely using a standard curve made with solutions having a known number of copies, quantified relative to a known sample, or quantified comparatively in the same reaction to another gene (an endogenous control). In the first two cases, separate amplifications of an endogenous control are needed to control for PCR or reverse transcriptase efficiency and the amount of cDNA or DNA added to the reaction, while in the latter, the reactions for the control and experimental gene can be multiplexed. Our method simultaneously quantifies mtDNA and the common deletion rapidly and economically.

These experiments show that for control liver DNA, the mean DL/BA ΔC_T was -8.0 , which indicates that (assuming 100% efficiency of both PCRs) the mitochondrial se-

TABLE III. Liver Mitochondrial Deletion Frequency in Rats

Deletion frequency in young rats	Deletion frequency in older rats	Reference
0.18% (3 months)	0.59% (23 months)	This study
0.0005% (7 months)	0.02% (27 months)	Gadaleta et al. [1992]
0.05% (6 months)	0.32% (24 months)	Kang et al. [1998]
0.001% (6 months)	0.5% (24 months)	Edris et al. [1994]
ND	1.7% (20 months)	Filser et al. [1997]
ND	1.88% (~24 months)	Yowe and Ames [1998]

quence is present in ~ 256 more copies than the β -actin sequence ($= 2^{-\Delta CT} = 2^8 = 256$) or at 512 copies/diploid nuclear genome, assuming the β -actin sequence is present in two copies. Liver has an average of 1,300 mitochondria per cell, with 8–10 copies of mtDNA/mitochondria, giving 13,000 mitochondrial DNA copies/cell [Loud, 1968]. However, calculating copy number from several published reports gives lower numbers. For example, Gadelata et al. [1992] report that rats have 2.30 μ g mtDNA per mg of genomic DNA; this results in a copy number of ~ 860 in rat. Filser et al. [1997] reported 0.039% mtDNA, which converts to 146 copies/cell. Our calculated value is quite low compared to the value reported for liver but near those in Gadelata et al. [1992] and Filser et al. [1997]. The low values reported here for copy number could be a result of β -actin pseudogenes in the rat genome; our calculation assumes two copies/diploid nuclear genome but if there are four or six nuclear copies, then the result should be multiplied by two or three. Locus Link lists six actin-like genes in the rat. A Blast search, however, came up with a match to only the β -actin sequence. Further sequencing of the rat genome could reveal additional pseudogenes in the future. It must be pointed out that this comparative C_T method is not meant to give absolute quantitation but only relative quantitation between experimental conditions. Lastly, multinucleate liver cells accumulate in older livers and may result from exogenous treatment; these multinucleate cells will affect determinations of mitochondria/cell. As a result, the results in this study are expressed above as mitochondria per diploid genome; however, the figures of other researchers could include these multinucleate cells.

The results in Figure 4 show interesting changes in mtDNA copy number with age. At a young age (3–60 days), mtDNA copy number rises and then decreases; these changes in the mtDNA copy number could coincide with sexual maturity. The mtDNA copy number then holds fairly steady to 2 years of age (although a small increase cannot be ruled out). Others usually have found a slow rise with aging in mammals [Heerdt and Augenlicht, 1990; Barrientos et al., 1997; Lee et al., 1998; Wong and Bai, 2002]. For example, Lee et al. [1998] found an increase of mtDNA copy number with aging in human lung, and Barrientos et al. [1997] found a 1.6-fold increase in 80-year-olds vs. 20-year-olds. Heerdt and Augenlicht [1990] re-

ported an increase in human mtDNA with development. Using hybridization to the *COIII* gene of mtDNA from liver, they found a level of ~ 3 at 22 weeks of gestation, ~ 5.5 at 32 weeks of gestation, and ~ 12 in two adults. Wong and Bai [2002] also report an increase in mtDNA from birth to 5 years of age in human muscle. However, Barazzoni et al. [2000] found that 27-month-old rats had only 50% of the mtDNA as 6-month-old rats. Based on in vitro studies, Tang et al. [2000] speculated that mtDNA copy number is inversely proportional to the size of the mtDNA, i.e., cells keep a constant mass of mtDNA, not a constant number of genomes.

In terms of an absolute value for the frequency of the mtDNA deletion, the ΔC_T for the CD/DL experiment of 9.11 indicates that the common deletion is 0.0018 (0.18%) of the total mitochondrial sequences for 3-month-old rats [$1/2^{\Delta C_T} = 1/2^{9.11} = 1/553$], while the ΔC_T for the 23-month-old rats of 7.41 indicates that the common deletion is 0.0059 (0.59%) of the total mitochondrial sequences at this older age [$1/2^{\Delta C_T} = 1/2^{7.41} = 1/170$]. The findings of other groups with regard to the frequency of mtDNA deletions are very variable, undoubtedly a result of the many experimental methods utilized as well as the tissues studied (Table III). The numbers found here are higher than most of those reported, although similar to Filser et al. [1997] and Edris et al. [1994]. In human liver, Wei et al. [1996] found a frequency of 0.00076% for 20- to 29-year-olds and a frequency of 0.076% in 70- to 79-year-olds, while Lee et al. [1994] found a frequency of 0.0076% in 70-year-olds. Our results (Fig. 5) clearly confirm the reported increase in deletions in older animals.

In conclusion, we have developed an assay to detect both relative mtDNA copy number and mtDNA deletion frequency in the rat. We have shown that the assay is robust and reproducible and have demonstrated the expected results with aging. This assay should be useful to study the effects of drugs, diet, or environmental changes on mtDNA.

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**THE EFFECT OF VITAMIN B₁₂, FOLATE AND DIETARY SUPPLEMENTS ON
BREAST CANCER CHEMOTHERAPY-INDUCED MUCOSITIS AND NEUTROPENIA**

Running Title: Diet Supplements and Drug Toxicity

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ABSTRACT

BACKGROUND. Although patients with cancer frequently use dietary supplements, the effect of these agents on chemotherapy is unclear. Therefore we investigated the influence of vitamin B₁₂, folate and nutritional supplements on chemotherapy-induced toxicity.

METHODS. Women with breast cancer were asked to complete a questionnaire recording their use of dietary supplements. Blood samples were obtained for serum vitamin B₁₂ and folate levels before and after the first cycle of chemotherapy and for weekly complete blood counts. Toxicity was evaluated by absolute neutrophil counts and by the frequency and severity of oral mucositis.

RESULTS. Of 49 women who submitted questionnaires, 35 (71%) took a total of 165 supplements. Compared to a prior study in 1990, there was a dramatic increase in serum folate levels. Initial neutrophil count, but not type of chemotherapy, patient age or serum vitamin B₁₂ level was predictive of nadir absolute neutropenia and the drop from initial neutrophil count to nadir (Nfall). After adjusting for initial neutrophil count, Nfall was less in women taking supplements versus no supplements ($p=.01$), and in those taking multivitamins ($p=.01$) or vitamin E ($p=.03$). Women with serum folic acid levels <20 ng/ml had a smaller drop in neutrophil count after chemotherapy than those with higher folate levels ($p=.04$). No significant effect of initial neutrophil count, nadir neutrophil count, Nfall, age, vitamin B₁₂ or folate level on oral mucositis was found.

CONCLUSIONS. The drop in neutrophil count caused by cancer chemotherapy may be ameliorated by dietary supplementation with a multivitamin or vitamin E but is increased in association with high serum folate levels.

Key Words: diet, folate, vitamins, chemotherapy, toxicity

INTRODUCTION

Relatively little is known regarding the effects of dietary components and nutritional supplements on cancer chemotherapy (1). Nevertheless, many patients alter their diets and begin taking nutritional supplements and vitamins after they are diagnosed with cancer, and this practice appears to be increasing (2,3). In a seminal 1984 paper, Dr. Barrie Cassileth reported that 37% of cancer patients on conventional treatment also used diet therapy and 30% used megavitamin therapy (4). More recently Cassileth reviewed 26 surveys of cancer patients from 13 countries, including 5 performed in the United States, and found a 31% average prevalence of use of complementary/alternative cancer medicine (5). However, a 1999 survey of 8 clinics at the M.D. Anderson Cancer Center found that 83% of patients used complementary/alternative therapy, and that the most frequently used approach (62%) was herbal and/or vitamin supplementation (6).

Our interest in this area began with studies of the effect of folic acid supplementation on chemotherapy in breast cancer. In 1998 we reported that nutritional folate status influenced the efficacy and toxicity of chemotherapy in rats (7). We found that cyclophosphamide and doxorubicin were 183% and 244% as effective, respectively, against a rat mammary tumor, with less host toxicity, in folate-supplemented rats compared to folate-deficient animals (7). Exploratory studies in our laboratory of the mechanism underlying this interaction of folate metabolism and cancer chemotherapy suggested a previously under-appreciated relationship between folate status and glutathione levels (8). Glutathione levels are an established determinant of chemotherapy toxicity (9). More recent studies in rats provided additional evidence for the influence of diet on cancer chemotherapy. We found that rats on a cereal-based

diet were far more resistant to the toxic effects of cyclophosphamide than rats on a purified diet (8).

Following the FDA-mandated fortification of cereal foods with folic acid, there has been a remarkable shift toward high blood folic acid levels in the general population (10,11). While our animal studies suggest that elevated folic acid levels may be beneficial to patients undergoing chemotherapy, the relevance of these studies to humans is uncertain. Recently, Sellers and colleagues found that high-folate diets did not have a significant adverse effect on survival after chemotherapy for breast cancer (12). In this report we describe the influence of vitamin B₁₂ and folate status and the use of dietary supplements on the toxicity of chemotherapy in a pilot study of women with breast cancer.

MATERIALS AND METHODS

Population Studied. Women with histologically proven breast cancer were asked to participate in this study. After informed consent was obtained, following procedures approved by the University of Vermont Committee on Human Research, blood samples were obtained and the women completed a questionnaire. Any woman with breast cancer regardless of stage was eligible.

Sample Collection. Weekly blood counts (hemoglobin, hematocrit, white blood cell count with differential, and platelet count) were obtained during the first cycle of chemotherapy, typically 3 or 4 weeks. Serum was collected before the first and second cycles of chemotherapy and cryopreserved in the dark for subsequent analysis of vitamin levels.

Assays of Vitamin Levels. Serum vitamin B₁₂ and folate levels were measured using the Quantaphase II B₁₂ and Folate Radioassay (Bio-Rad Diagnostics Group, Hercules, CA 94547).

The assays were performed by combining a serum sample with vitamin B₁₂ (⁵⁷Co) and/or folate (¹²⁵I) in a solution containing dithiothreitol and cyanide. The mixture was combined with immobilized, affinity-purified porcine intrinsic factor and folate binding protein. Labeled and unlabeled vitamins binding to the immobilized binding proteins were concentrated in a pellet, and the radioactivity of the pellet was counted. Standard curves were prepared using vitamin B₁₂ and folate standards in a human serum albumin base.

Toxicity Assessment. Neutropenia was analyzed in three ways. The first method was to measure neutrophil decrease (Nfall) as the difference between the neutrophil count immediately before chemotherapy and the nadir count during the first cycle (13). The second method was to identify the nadir absolute neutrophil count. The third method was to categorize neutrophils by grade, with grade 4 = $\leq 0.100 \times 10^9/L$, 3 = 0.101 to $0.500 \times 10^9/L$, 2 = 0.501 to $1.000 \times 10^9/L$, and 0-1 = $> 1.000 \times 10^9/L$. The nadir absolute neutrophil count was highly correlated with the neutrophil count 2 weeks after treatment ($r = 0.94$) (Figure 1). Only 2 patients were admitted to the hospital for evaluation and treatment of febrile neutropenia.

The patient's physician was asked to grade the degree of oral mucositis semi-quantitatively by a modification of the National Cancer Institute common toxicity criteria (version 2.0) (Grade 0=none; Grade 1=mild; painless ulcers, erythema, or mild soreness in the absence of lesions; Grade 2=moderate; painful erythema, edema or ulcers but can eat or swallow; Grades 3 and 4=severe; painful erythema, edema or ulcers preventing swallowing or requiring hydration or parenteral support or requiring intubation).

Statistical Analysis. The statistical analyses were run using SAS version 6.12 (SAS Institute Inc., Cary, NC: STAT Institute Inc., copyright 1989-1996). Continuous outcomes were analyzed using t-test, regression, ANOVA or ANCOVA. The folate values and initial and nadir

neutrophil counts had skewed distributions so the natural log transformation was used. Binomial outcomes were analyzed with logistic regression. If both variables could be considered as being ordinal, then the gamma statistic was used. In 7 patients, there was no blood sample collected before the first cycle of chemotherapy for vitamin assay, but a sample was obtained prior to the second cycle. Analysis indicated that the vitamin B₁₂ and folic acid values from these samples could be substituted for statistical purposes.

RESULTS

Seventy-nine patients were enrolled in this study; of these, 68 patients met the exclusion criteria of having an initial neutrophil count and at least 2 neutrophil counts in the first 3 weeks (Table 1). Their ages were a mean of 48.1 ± 9.3 years with a range of 30-75 years. Fifty-four patients received doxorubicin/cyclophosphamide, 3 received doxorubicin with docetaxel, 6 patients received cyclophosphamide, methotrexate and 5-fluorouracil, 2 received single agent doxorubicin, and 3 received single agent taxane (2 paclitaxel, 1 docetaxel) (Table 1).

Supplement questionnaire. Forty-nine women who met the above exclusion criteria submitted questionnaires that listed their dietary supplement use at the time of enrollment (Table 2). Of these, 14 (29%) took no supplements. The remaining 35 women (71%) took a total of 165 supplements. Usage ranged from a single agent (8 patients) to 20 different substances taken daily by one patient. The mean number of supplementation substances taken was 3 and the median was 2. Twenty patients (41%) took supplements other than vitamins and minerals, usually complex mixtures of herbal extracts. Thirty-two patients took 1 or more vitamin supplements; 17 of the 32 also took mineral supplements – most commonly calcium (53%). There were no differences in initial neutrophil count, nadir or Nfall between the group taking

only vitamin supplements and the patients taking both mineral and vitamin supplements (t-test, $p>.40$). Therefore, mineral supplements did not add any beneficial effect independent of vitamins.

Comparisons were made between the no-supplement group and the subjects taking any kind of supplement. After adjusting for initial neutrophil count, the supplement group had a significantly lower Nfall than the no-supplement group (ANCOVA $p=.01$), but the difference in the nadir count was not significant. There were three vitamin supplements taken by more than 10 of the subjects, namely multivitamins (47%), and vitamins C (22%) and E (37%). None of these was significantly associated with nadir count ($.09<p<.28$), but multivitamins and vitamin E were significantly associated with Nfall ($p=.01$ and $.03$ respectively) (Figure 2). The significant associations corresponded to a smaller Nfall that decreased by 625 to 750 $\times 10^9/L$ less for the supplement group (after adjusting for differences in initial neutrophil count). That is, patients taking these supplemental vitamins had a smaller drop in absolute neutrophil count after chemotherapy than women who did not take them. This pattern of results was the same when the analyses were repeated for the subset of subjects who had doxorubicin/cyclophosphamide treatment (11 in the no-supplement group, 29 taking supplements). The differences in Nfall were larger in magnitude (742 to 909 $\times 10^9/L$). There was a suggestion of an interaction of supplement use, but because of the small numbers, no clear picture emerged. Multivitamin or vitamin E supplement use versus no supplement use was associated with more grade 0-1 (30%, 22% and 7%, respectively) and less grade 3 neutropenia (26%, 33% and 57%, respectively).

Serum levels. Serum vitamin B₁₂ and folate levels were measured at the beginning and/or the end of the first cycle of chemotherapy in 63 patients. The first vitamin B₁₂ level was 526 ± 330 pg/ml (mean $\pm$ SD), and the second was 628 ± 560 pg/ml. The two vitamin B₁₂ levels were

correlated ($r=0.64$, $p<0.0001$). The mean $\pm$ SD for the first and second folate levels were 17.8 ± 9.6 ng/ml and 18.6 ± 9.9 ng/ml, respectively. The two folate levels were correlated as well (0.56 , $P = 0.002$).

In 1990 we measured serum folate levels in 47 women with breast cancer from the same community and socio-economic group using a similar assay system (14,15). Table 3 shows the results of that study compared with folate levels observed in the current study. It can be seen that there has been a dramatic shift toward higher serum folate levels in this population over the past decade.

Regression analyses were performed with nadir absolute neutrophil count and Nfall as outcomes and the initial absolute neutrophil count, patient age, the serum vitamin B₁₂ level, the serum folate level and the type of chemotherapy (dichotomous variables: cyclophosphamide/doxorubicin or doxorubicin alone versus any other chemotherapy--- 5-fluorouracil, docetaxel, methotrexate or paclitaxel) separately as predictors. The only significant predictor of both outcomes was the initial neutrophil count ($p<0.01$). The type of chemotherapy was not significant for either outcome ($p>0.20$). Repeating the regressions when limiting the analysis to the subset of patients who received cyclophosphamide and doxorubicin produced the same pattern of results: only the initial neutrophil count was significant. The predictors were transformed into categorical variables (age: <40 , 40 to <50 , >50 years old), vitamin B₁₂ (<400 , 400 to <800 , >800 pg/ml) and folate (20 or less, >20 ng/ml). Because the initial neutrophil count was significant in the regressions, it was included in the ANCOVA models as a covariate. Neither the type of chemotherapy, age, nor vitamin B₁₂ level was a significant predictor for nadir neutrophil count or Nfall ($p>0.15$). Folate category was significant ($p=0.04$) for Nfall but not for nadir count ($p=0.11$). The model estimates that the Nfall is $426 \times 10^9/L$ lower for the subjects

with folate levels less than 20 ng/ml, after adjusting for initial neutrophil count (Figure 2). For the doxorubicin/cyclophosphamide subset, the significance of the folate group declined somewhat ($p=0.06$). Thus patients with relatively lower serum folate levels had a smaller decrease in neutrophil count after chemotherapy compared to women with higher folate levels (Figure 2).

Mucositis. The distribution of oral mucositis was Grade 0=51 patients, Grade 1=11 patients, Grades 2 and 3=6 patients, and 4=0 patients. Logistic regression was used to evaluate which variables can be used to predict mucositis (0 versus any value). Initial neutrophil count, nadir neutrophil count, Nfall, initial vitamin B₁₂ and folate levels, and age were not found to be significant (all $p>0.10$). These predictors were then tested as categorical variables, and no significant effect was found. There was no effect of supplementation with multivitamins or vitamin E on the severity of mucositis.

DISCUSSION

Our study in women with breast cancer confirms prior investigations that found that the majority of cancer patients are taking one or more dietary supplements or herbal remedies (2-6). The most commonly used agents in our study were multivitamins, vitamin E and calcium, but some patients took as many as 20 different substances. Because vitamins and other nutritional supplements are natural products, patients often consider them less toxic alternatives or additions to conventional cancer chemotherapy. However, there is growing evidence that these dietary supplements can mimic, increase or decrease the effects of drugs (16,17). Unfortunately there are relatively few studies of the effects of alternative medicine, which often includes dietary supplements and herbal medicines, on cancer chemotherapy, and many of these give conflicting

results (1). For example, a few small trials of antioxidant supplements in patients with breast, lung and squamous cancer have hinted at a survival benefit, but comparisons were with historical controls (18,19). Vitamin users with non-small cell lung cancer had a longer median survival compared to nonusers, but the number of subjects was too small to identify the specific micronutrient responsible for the beneficial effect (20). Conversely, a comparison of women with breast cancer who were prescribed mega-doses of beta-carotene, vitamin C, niacin, selenium, coenzyme Q10 and zinc with matched controls found that breast-cancer specific survival and disease-free survival times were shorter for the vitamin/mineral treated group (21). Supplementation with β -carotene or α -tocopherol did not alter the mortality rate from pancreatic cancer (22). Burstein and colleagues reported that the use of alternative medicine by women with breast cancer was a marker of greater psychosocial distress and worse quality of life (3). In this study, women who received chemotherapy were more likely to begin using alternative medicine (3). Finally, a recently published report showed striking reductions in the active metabolite of irinotecan in patients who were taking St. John's Wort (23). Thus the beneficial effect of vitamin supplementation or herbal remedies, if any, on the efficacy of chemotherapy and on patient survival remains uncertain.

Dietary supplementation with vitamins, particularly vitamin E, has been recommended to decrease the toxicity of chemotherapy. Since reactive oxidant species have been implicated in doxorubicin-induced cardiomyopathy, bleomycin-induced pulmonary fibrosis, and cisplatin-induced neuropathy and nephrotoxicity, antioxidants have been used to reduce or prevent these side effects (24 and reviewed in 25). Several studies support the possibility that vitamin E supplementation may increase the efficacy and reduce the toxicity of cancer chemotherapy, particularly of doxorubicin-containing regimens (25). There has been little or no evidence,

however, to suggest that vitamin E supplementation influenced chemotherapy-induced myelosuppression, mucositis, nausea or vomiting (25). A recent review of the literature by Seifried and colleagues concluded that current knowledge makes it premature to generalize and make specific recommendations about antioxidant use during cancer chemotherapy (26). In our study, we found that vitamin E influenced myelosuppression caused by chemotherapy by reducing the drop in absolute neutrophil count from initial to nadir levels. It did not, however, affect the nadir counts.

Oral mucositis is a distressing side-effect of chemotherapy and contributes to the morbidity and mortality of high-dose chemotherapy (27). A peak oral mucositis score such as the one used in this study is a useful index of gastrointestinal toxicity and correlates with clinically significant events such as bacteremia (27). In prior studies, diagnosis, treatment protocol, rate of neutrophil recovery, and patient age were found to influence the severity of mucositis (27). Oral L-glutamine has been shown to decrease the duration and severity of oral mucositis, particularly when used with chemotherapy regimens that contain doxorubicin or methotrexate (28). In our study, initial neutrophil count, Nfall, nadir absolute neutrophil count and serum vitamin B₁₂ and folate levels were not significantly related to the risk of developing mucositis. In contrast to the results of a prior study (27), we did not find that younger patients were more likely to experience oral mucositis.

In view of the remarkable overall increase in blood folate levels both in the general population (10,11) and in our patients with breast cancer, it was of interest to determine if elevated folate levels modulated myelosuppression by chemotherapy. Our investigations in rats suggested that the interaction between folate levels and cancer chemotherapy is complex. Initial studies indicated that cyclophosphamide was less effective at inhibiting the growth of a rat

mammary tumor in folate-deficient rats (7). In addition, toxicity from cyclophosphamide was increased in folate-deficient rats and ameliorated in folate-supplemented animals compared to rats that ingested the standard amount of folic acid (7). The toxicity of 5-fluorouracil was significantly greater in folate-deficient rats than in folate-replete or supplemented rats, but there was no relationship between folate status and doxorubicin toxicity (7). Thus the effects of folate status on toxicity varied with the chemotherapeutic agent. A subsequent study indicated that the chemotherapy schedule also may influence the interaction. When the drugs were given as a single bolus instead of in divided doses, high folate rats developed more severe anemia, azotemia and leukopenia after 5-fluorouracil but no different toxicity with cyclophosphamide (8). In the case of both drugs, rats on a cereal-based diet were more resistant to toxicity than animals on a purified diet. The cereal-based diet appeared to protect against severe renal damage caused by the combination of 5-fluorouracil and a purified diet supplemented with folic acid (8). The women in the current study were predominantly treated with bolus cyclophosphamide and doxorubicin. We found no significant association between serum vitamin B₁₂ level and neutropenia. Serum folic acid levels influenced the Nfall but not the absolute neutrophil nadir. Women with serum folate levels <20 ng/ml had a smaller decrease from initial to nadir absolute neutrophil counts than women with higher folate levels. There were too few women treated with 5-fluorouracil to determine if chronically high levels of folic acid lead to renal damage or more severe neutropenia with this drug (5 of 6 patients had serum folate levels <20 ng/ml).

The major limitation of this study is its small sample size. The trial was designed as a pilot study in a single institution to identify nutritional components, particularly vitamin B₁₂ and folate, that might affect chemotherapy-induced toxicity. There is sufficient power to indicate that blood levels of vitamin B₁₂ are unlikely to have a major effect on neutropenia or mucositis,

but relatively small effects may have been missed. Given the sizeable number and diversity of dietary supplements taken by these patients, a much larger clinical trial will be required to measure the effect on toxicity of any but the most commonly used specific agents. A second limitation of the study is the heterogeneous chemotherapeutic regimens that were used. Although the majority of the patients received doxorubicin/cyclophosphamide as adjuvant chemotherapy, and 87% of the patients received doxorubicin either as a single agent or in combination, it is likely that some drug regimens are more likely to cause neutropenia or mucositis than other drugs. Finally, the study included patients with both newly diagnosed and advanced breast cancer. It is possible that patients who were previously treated may have been more susceptible to neutropenia or mucositis.

In conclusion, we found a high prevalence of dietary supplement use in women with breast cancer. As is the case in the general population (10,11), many of these women had elevated blood folate levels. There was evidence that the decrease in neutrophil count after chemotherapy was ameliorated by ingestion of multivitamins or vitamin E. However, women with high serum folate levels were more likely to have greater drops in their neutrophil counts after chemotherapy. In view of the small sample size in this pilot study, these results will need to be confirmed in a larger number of patients.

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LEGENDS

Figure 1. Total white blood cell count (stipled bars) and absolute neutrophil counts (open bars) measured before and weekly during the first course of cancer chemotherapy in 68 women with breast cancer. The nadir absolute neutrophil count was highly correlated with the neutrophil count 2 weeks after treatment ($r = 0.94$). Error bars represent standard error of the mean.

Figure 2. The effect of vitamin supplementation and serum folic acid levels on neutropenia following cancer chemotherapy. Neutropenia was assessed by measuring the difference between the initial absolute neutrophil count and the nadir count (Nfall). Brackets show the standard error of the mean. There were 14 patients who took no supplements, 18 who took vitamin E, and 23 who took supplemental multivitamins. There were 39 women with serum folic acid levels <20 ng/ml and 24 with levels >20 ng/ml. The comparisons were significantly different ($p=.03$ for vitamin E, $p=.01$ for multivitamins, and $p=.04$ for folic acid).

Table 1. Number of patients treated with chemotherapy regimens in the adjuvant, neoadjuvant or metastatic disease settings.

Total patients (number)	68	
Adjuvant Chemotherapy	56	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		47
cyclophosphamide/methotrexate/5-fluorouracil (600/40/600 mg/m ²)		6
doxorubicin/docetaxel (45/75 mg/m ²)		2
doxorubicin (60 mg/m ²)		1
Neoadjuvant Chemotherapy	6	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		6
Metastatic Disease	6	
doxorubicin/cyclophosphamide (600/60 mg/m ²)		1
doxorubicin/docetaxel (45/75 mg/m ²)		1
doxorubicin (75 mg/m ²)		1
paclitaxel (175 mg/m ²)		2
docetaxel (40 mg/m ² weekly x3)		1

Table 2. Dietary supplement use by women with breast cancer.

Supplement Use	N*	%						
None	14	29						
Single Supplement	8	16						
2-5 Supplements	18	37						
6-10 Supplements	5	10						
>10 Supplements	4	8						
<u>Supplements Taken</u>								
<u>Vitamins</u>	<u>N</u>	<u>%</u>	<u>Minerals</u>	<u>N</u>	<u>%</u>	<u>Nutraceuticals</u>	<u>N</u>	<u>%</u>
Multivitamin	23	47	Mineral complex	3	6	Ginko	7	12
Vitamin A	5	10	Calcium	13	27	Echinacea	3	6
Folic acid	3	6	Iron	1	2	Co-enzyme Q	8	14
Vitamin B ₁₂	2	4	Magnesium	2	4	Pectin	2	3
Vitamin B complex	3	6	Selenium	3	6	Glutamine	3	5
Vitamin C	11	22	Zinc	1	2	Other	18	37
Vitamin D	3	6						
Vitamin E	18	37						

*N = number of patients

Table 3. Serum folate levels measured ten years apart in two different groups of women with breast cancer from the same community.

Year	1990		2000	
<u>Serum Folate Level</u>	<u>Number</u>	<u>%</u>	<u>Number</u>	<u>%</u>
Deficient (<3.0 ng/ml)	12	26	0	0
Indeterminate (3.1-6.0 ng/ml)	18	38	4	6
Sufficient (6.1-20 ng/ml)	17	36	35	56
High (>20 ng/ml)	0	0	24	38

