Original Article

Tocotrienol levels in adipose tissue of benign and malignant breast lumps in patients in Malaysia

Kalanithi Nesaretnam PhD[1], Patricia Alison Gomez MD[2], Kanga Rani Selvaduray MS[1] and Ghazali Abdul Razak MS[1]

[1]*Malaysian Palm Oil Board, Kajang*
[2]*Kuala Lumpur Hospital, Kuala Lumpur*

Data on dietary exposure to vitamin E by plasma or adipose tissue concentrations of α-tocopherol (α-T) in observational studies have failed to provide consistent support for the idea that α-T provides women with any protection from breast cancer. In contrast, studies indicate that α, γ, and δ-tocotrienols but not α-T have potent antiproliferative effects in human breast cancer cells. Our aim was to investigate whether there was a difference in tocopherol and tocotrienol concentrations in malignant and benign adipose tissue, in a Malaysian population consuming predominantly a palm oil diet. The study was undertaken using fatty acid levels in breast adipose tissue as a biomarker of qualitative dietary intake of fatty acids. The major fatty acids in breast adipose tissue of patients (benign and malignant) were oleic acid (45-46%), palmitic (28-29%) and linoleic (11-12%). No differences were evident in the fatty acid composition of the two groups. There was a significant difference (p=0.006) in the total tocotrienol levels between malignant (13.7 ± 6.0 μg/g) and benign (20±6.0 μg/g) adipose tissue samples. However, no significant differences were seen in the total tocopherol levels (p=0.42) in the two groups. The study reveals that dietary intake influences adipose tissue fatty acid levels and that adipose tissue is a dynamic reservoir of fat soluble nutrients. The higher adipose tissue concentrations of tocotrienols in benign patients provide support for the idea that tocotrienols may provide protection against breast cancer.

Key Words: tocotrienols, tocopherols, adipose tissue, fatty acid composition, malignant and benign lumps

INTRODUCTION

Breast cancer worldwide affects nearly one million women per year and although current treatments do help many patients, more than 350,000 die from the disease. In the United States 215,990 women were diagnosed with breast cancer in 2004 and 40,110 died from the disease.[1] In Malaysia, breast cancer is the most frequent cancer amongst women. In 2002, the National Cancer Registry recorded 4,337 new cases of breast cancer in Malaysia, which comprised 30.4% of all cancers in women.[2] The established risk factors for breast cancer include a family history of breast cancer, early menarche, late age at first child birth, late age at menopause and history of benign breast disease. With the exception of the genetic predisposition to the disease the rest of the risk factors point to the life time exposure of women to estrogen. Estrogen does not cause the disease but is involved in the progression and development of breast cancer. Anti-estrogens are therefore used as therapy in the control of breast cancer progression.

International variation in breast cancer incidence rates and changes in incidence amongst migrant populations have indicated that breast cancer risk is also influenced by environmental factors, in particular diet, and therefore preventable.[3] A lot of scientific investigations have been performed to discover possible functional properties, antioxidant or otherwise in the diet, which could be efficient in preventing diseases like cancer. One such antioxidant is vitamin E. Previously eight dietary components α-, β-, γ-,δ-tocopherols (T) and α-, β-, γ-,δ- tocotrienols (T3)[4-6] were all considered forms of vitamin E. α-T is thought to be the most biologically important form of vitamin E.[5,6] Recent guidelines have equated α-T with vitamin E, discounting other Tocopherols and the Tocotrienols. [5] Tocopherols and tocotrienols are present in the oil fraction of cereal grains, seeds and nuts. In most food sources, tocopherols are more prevalent than tocotrienols. Palm oil is a particularly rich source of α-, γ-, δ-tocotrienol.[3,7] Palm oil is today the second largest vegetable oil in terms of world production and makes up about 50% of the world's traded oils. Malaysia and Indonesia are the two biggest producers and consumers of the oil. [8]

Observational studies that have assessed exposure to vitamin E by plasma or adipose tissue concentrations of α-T have failed to provide consistent support for the idea that α-T provides any protection against breast cancer. [9,10]

Corresponding Author: Dr. Kalanithi Nesaretnam, Malaysian Palm Oil Board, 6 Persiaran Insitusi, Bandar Baru Bangi, 43000 Kajang, Malaysia
Tel: 603-89282847; Fax: 605-89221742
Email: sarnesar@mpob.gov.my
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In contrast, studies in human breast cancer cells indicate that of α-, γ-, δ-T3 have potent anti-proliferative and pro-apoptotic effects that would be expected to reduce the risk of breast cancer [11-15] whilst α-T had no effect. Galli and co-workers [16] recently demonstrated that γ-T when compared with α-T showed a much stronger inhibitory effect on prostate cancer cell growth. However, when tested further with the T3 homologues [17] he found that γ-T3 had greater anti-proliferative effects than γ-T. Thus it seems plausible that the modest protection from breast cancer associated with dietary vitamin E may be due to the effects of the other T and T3 in the diet.

Information regarding the prognosis of women with breast cancer in relation to breast adipose tissue concentrations of tocopherols and tocotrienols would provide insight into the roles that tocopherols and tocotrienols might play in preventing or reducing the risk of breast cancer. In the current study we investigated vitamin E levels in Malaysian women where palm oil is the main fat consumed in the diet and a particularly rich source of tocotrienols. Among all biological markers of qualitative composition of dietary intake of fatty acids, adipose tissue fatty acid composition is particularly advantageous because it reflects qualitative dietary intake of fatty acids on a long term basis [18-20] thereby avoiding the potential bias derived from the disease on the measured biochemical parameters. Additionally, because the ability of tocopherol and tocotrienols to reduce risk of breast cancer is likely to be determined by their delivery to the breast, it will be important to determine concentrations of these dietary constituents in the breast adipose tissue, and to relate these concentrations to dietary intake.

MATERIALS AND METHODS

Subjects

The study population was derived from Kuala Lumpur Hospital and was approved by the Ethics Committee of the Ministry of Health, Malaysia. Of the 75 women studied, 40 had breast cancer and 35 had benign breast pathologies. Both groups of women presented with breast lumps. Both groups had imaging and needle biopsies done to prove the pathology i.e. benign vs. malignant prior to surgery and the adipose tissue from the breast was obtained at surgery. The tissue submitted was not the actual pathology but from the surrounding adipose tissue. After the initial pathological evaluation of the specimen, 0.2-1.0 g of adipose tissue was washed with saline, frozen immediately in liquid nitrogen, and stored at -70°C in vials until analysis.

Adipose tissue preparation and fatty acid analysis

Total lipids were extracted from adipose tissue with chloroform-methanol 2:1 (v:v).[21] The extract was washed with sodium chloride and the mixture was allowed to separate into two phases. The chloroform layer was transferred to a tube and evaporated to dryness under nitrogen. The lipid extract was dissolved in 200 µl of chloroform-methanol 2:1 (v:v) and directly used for column procedure.

Triacylglycerol was purified by adsorption chromatography on silica tubes (Supelco, France) as follows: the column was pretreated with 2 ml chloroform-methanol 2:1 (v:v). Then the lipid sample was applied to the column and the tube treated with 100 µl of this same system solvent added to the column. Triacyglycerols were eluted with 20 ml of chloroform. Chloroform fractions were collected in screw-cap tubes with a Teflon seal. Fractions obtained were evaporated to dryness. Three hundred microlitres of sodium methoxide 2 N and 500 µl of boron trifluoride were added to convert the fatty acids to their methyl esters. The mixture was incubated and shaken 15 min at room temperature. Fatty acid methyl esters were extracted twice into hexane.

Fatty acid methyl esters (FAME) composition was determined by capillary gas chromatography. The system was composed of a GC 800 series chromatograph (Perkin Elmer, USA) equipped with a cold on-column injector, and a flame ionization detector. A 60 m long x 0.22 mm internal diameter fused silica column (Varian, USA) was used. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The detector temperature was 280ºC. One microlitre was injected through the cold on-column injector (60ºC). The oven temperature was programmed to rise from 60-170ºC at a rate of 15ºC/min and kept constant for 20 min, from 170-190ºC at a rate of 5ºC and kept constant for 10 min, and from 190-215ºC at a rate of 1ºC/min and kept constant for 10 min. Identification of FAME was obtained by comparison of their relative retention times with those pure standard mixtures. The relative amount of each fatty acid was quantified by integrating the peak at baseline and dividing the results by the total area for all fatty acids. All integrations were performed by the same laboratory worker. Peaks accounting for less than 1% of total area were well detected and quantified.

Analysis of tocotrienols and tocopherols in breast adipose tissue

The tocopherols and tocotrienols were analysed by HPLC. The system used was a Shimadzu LC-10AT HPLC coupled with a Shimadzu Model RF-10AXL fluorescence spectrophotometer, Shimadzu Class VP data acquisition software, and silica column (YMC A-012/ 150 x 6 mm I.D, 5µm). The eluting solvent was hexane/isopropyl alcohol (99.5/0.5 v/v) at a flow rate of 2.0 ml/min. The detector was set at excitation wavelength of 295 nm and emission wavelength of 325 nm. 500 mg adipose tissue was homogenized with 4:1:1 mixture of hexane:ethanol and 0.9% sodium chloride at 10000 rpm for 5 minutes. The homogenate was then centrifuged at 2000 rpm for 5 min. The resulting supernatant was filtered and evaporated using a rotary evaporator. A known amount of lipid sample was dissolved in a 10 ml volumetric flask using the eluting solvent and 10 µl of the solution was injected into the HPLC system. A standard solution of a mixture of α, γ, and δ-tocotrienols was also injected accordingly. Quantification of the major components of vitamin E was carried out by comparing the peak areas of the components with those of the standards.

Statistics analysis

Mean and standard deviations of individual fatty acids were calculated, and the total sum of fatty acids in each family was determined. Statistical analysis was performed using the SPSS program. Two-sided Student's t-tests

were used to compare the mean differences between women with and without breast cancer in relation to fatty acid composition (FAC) and vitamin E levels in adipose tissue. Results were considered significant at $p<0.05$.

RESULTS

Fatty acid composition in breast adipose tissue

Table 1 shows the fatty acid composition of breast fat in benign and malignant lumps. The major fatty acids in breast adipose tissue were oleic acid (18:1 n–9c), palmitic acid (16:0), linoleic acid (18:2 n–6) and stearic acid (18:0). These fatty acids accounted for about 90% of total area under the chromatographic curve. There was no significant difference in the FAC of breast adipose tissue from benign and malignant lumps.

Vitamin E composition in breast adipose tissue

Table 2 shows the T and T3 measured in the lipid extract of breast adipose tissue obtained from 40 patients with malignant and 35 with benign breast lumps. The content of T and T3 was expressed in µg/g adipose tissue. In malignant lumps, the mean content of α-T in breast adipose tissue was 126µg/g adipose tissue whilst in the benign lumps it was 147µg/g. There was a large variability between patients especially for α-T. This could be due to different dietary intake patterns as tocopherols are prevalent in many food items. The mean of α-T value in adipose tissue was however not significantly different in malignant cancer patients than in benign subjects (p=0.44). The mean γ-T (7.51 vs. 6.18) and δ-T (1.26 vs. 0.65) levels were also reduced in malignant patients as compared to benign. Whilst it was not significant for γ-T (p=0.36), the reduced level of δ-T was significant (p=0.03).

In breast cancer patients, the mean content of α-, γ-, and δ-T3 in breast adipose tissue was 13.7 µg/g adipose tissue. In patients with benign lumps it was 20.0µg/g adipose tissue. Mean T3 value was significantly (p=0.01) lower in breast cancer patients than in subjects with benign lumps. There was a decrease in α-T3 (p=0.01), γ-T3 (p=0.05), and δ-T3 (p=0.02) in malignant tissue compared to benign. The distribution of tocotrienols with α and γ-T3 being higher also reflects closely the composition of tocotrienols in palm oil.

DISCUSSION

The evaluation of long-term nutritional vitamin E status in breast cancer patients using adipose tissue concentrations has definite advantages over measurement of plasma

Table 1. Fatty acid composition of breast adipose tissue in benign and malignant breast lumps

Fatty acids		Benign Mean value % (± SD) n=35	Malignant Mean value % (± SD) n=40
Saturates			
	12:0	1.2 (0.35)	0.9 (0.44)
	14:0	2.3 (0.46)	2.2 (0.55)
	16:0	30.0 (0.95)	28.4 (1.97)
	18:0	3.0 (1.29)	4.8 (0.97)
	20:0	0.2 (0.09)	0.5 (0.30)
	Total	36.6 (0.63)	36.9 (0.84)
Monounsaturates			
	16:1 n–7c	5.5 (2.11)	3.4 (1.19)
	18:1 n–9c	46.3 (0.93)	45.6 (2.34)
	20:1 n–9	0.5 (0.04)	0.5 (0.18)
	Total	52.3 (1.02)	49.6 (1.24)
n–6 PUFA			
	18:2 n–6	10.8 (0.89)	12.4 (1.05)
	18:3 n–6	0.3 (0.06)	0.3 (0.11)
	Total	11.1 (0.48)	12.7 (0.58)

Table 2. Tocopherol and tocotrienol content in breast adipose tissue of malignant and benign breast lumps

Parameters measured		Benign Mean value (µg/g) (± SD) n=35	Malignant Mean value (µg/g) (± SD) n=40
Tocopherols			
	α	147 (61.9)	126 (80.9)
	β	2.7 (1.57)	2.81 (1.53)
	γ	7.5 (3.79)	6.18 (4.17)
	δ	1.3 (0.85)	0.6 (0.31)
	Total	159 (65.4)	36 (85.1)
Tocotrienols			
	α	11.4 (3.31)	7.2 (4.28)
	γ	7.90 (2.61)	6.0 (2.47)
	δ	0.82 (0.29)	0.5 (0.31)
	Total	20.1 (6.02)	13.7 (6.09)

levels. Plasma levels of tocopherol and tocotrienols change very rapidly in humans following modifications in the dietary intake, reaching new steady state levels within a few days.[22] The content of α-T in adipose tissue has been evaluated in humans in relation to dietary intake of vitamin E and it has been shown that α-T content reflects long-term dietary intake of vitamin E.[23]

Several studies that carefully collected adipose tissue have investigated the relationship between adipose tissue tocopherols and breast cancer risk. [24,25] Overall, studies of the association of vitamin E with breast cancer risk suggest the possibility that increased dietary exposure to vitamin E may slightly reduce breast cancer risk. [26] However, there is no evidence that supplemental vitamin E, most, if not all of which is in the form of α-T, confers any protection at all. [26] Furthermore, cell culture data has showed that α-T combined with tamoxifen increased the IC_{50} for tamoxifen in MCF-7 cells more than 1000 fold [27] and in a further study α-T completely blocked the potent growth inhibitory effects of tamoxifen on MDA-MB-231 cells. [28] In contrast, we have shown that α-, γ-, and δ-T3 and the tocotrienol rich-fraction of palm oil inhibited proliferation of MCF-7[12] and in ZR-75-1 cells, both in the absence and the presence of estradiol and tamoxifen.[13] The inhibitory effect on cell growth was more pronounced with γ and δ-T3. The mechanism of action is unknown, with previous data suggesting action does not reside in antagonism of estrogen action or in alterations to growth inhibitory insulin-like growth factor binding proteins in MCF-7 human breast cancer cells.[12] Tocotrienols are also reported to have a pro-apoptotic effect on several tumour cell lines.[14,28,29,30] However, McIntyre and coworkers [29] have also shown that highly malignant cells are more sensitive to the anti-proliferative and apoptotic effects of tocotrienols in comparison with pre-neoplastic cells.

The fatty acid composition data reflects closely the intake of a palm oil diet in Malaysia. This is not surprising as it is the cheapest and most readily available oil in the country and is reported to constitute the major fat in the diet of the majority of Malaysians. [31] While most vegetable oils provide mainly α- or γ-T, palm oil is unique in the sense that it contains relatively large concentrations of T3. The distribution of the various T and T3 fractions in palm oil are as follows: α-T 32%, α-T3 25%, γ-T3 29% and δ-T3 14%. In the United States, analyses of balanced diets ranging from 2,000 to 3,000 kcal per day indicated that the average daily intakes of vitamin E range from 7 to 11 mg. [32] Malaysian data on vitamin E intakes are presently limited. However, it is envisaged that for a 2,000 to 3,000 kcal per day diet one would be consuming between 10 to 15 mg of tocotrienols per day. [33]

The most significant finding of the present study was the higher, 65%-more tocotrienol (α-T3, γ-T3 and δ-T3) concentrations in the adipose tissue of the benign lumps in comparison to the women with malignant breast lumps. There was no difference in the α-T and γ-T content. However, δ-T showed a significant reduction in malignant vs benign adipose tissue. The depletion in tocotrienols in patients with malignant breast lumps compared to patients with benign lumps could be due to its role as an antioxidant in quenching free radicals and regulating peroxidation reactions.

Tocotrienols possess powerful antioxidant-, anticancer- and cholesterol-lowering properties. Some studies have confirmed that tocotrienol activity as an antioxidant-, anticancer- and cholesterol-reducing substance to be stronger than tocopherols. [34] Tocotrienols are thought to have more potent antioxidant properties than α-T. [35,36] The unsaturated side-chain of tocotrienol allows far more efficient penetration into tissues, such as the adipose, brain and liver, that have saturated fatty layers. [37] Experimental research examining the antioxidant, free radical scavenging effects of tocopherols and tocotrienols revealed that tocotrienols appear superior because of their better distribution in the fatty layers of the cell membrane. [37]

Oxidative stress has also been implicated in breast cancer [38-40] and may influence breast cancer by altering gene expression [41] or by promoting oxidative DNA damage. We however did not measure the hydroperoxide levels in the breast adipose tissue but other authors [24] demonstrated higher conjugated dienes and hydroperoxides in breast adipose tissue of breast cancer patients than in control patients.

In Malaysia we are fortunate that palm oil contains high amounts of tocotrienols and we have previously demonstrated *in vitro* [11-13] and *in vivo* [42] that tocotrienols protect against breast cancer. Thus it seems plausible that the modest protection from breast cancer associated with dietary vitamin E maybe due to the effects of tocotrienols in the diet.

The low tocotrienols in the adipose tissue of malignant breast lumps needs to be further investigated as to whether their intake of tocotrienols was low or whether it was used up because they had cancer. Studies have shown that tocotrienol supplementation up to 240 mg for 16-months duration does not have any apparent adverse effect.[43] Furthermore, it is likely that breast adipose tissue concentrations will be five- to 10-fold those in plasma. This indicates that lower levels of tocotrienol supplementation might be adequate to reach breast adipose tissue tocotrienol concentrations similar to those that inhibit proliferation and promote apoptosis in breast cancer cells. Further studies will be needed to determine whether individuals that achieve such plasma and tissue tocotrienol concentrations are protected from development of breast cancer. A further extension to this project would also look at the tocotrienol levels in adipose tissue of normal subjects as in this paper we determined levels in subjects with benign and malignant breast lumps only. It could well be that benign and control subjects may reflect the same amounts. The potentially protective effect of tocotrienols against breast cancer and the mechanism by which these dietary constituents protect women needs to be further investigated.

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[1]*Malaysian Palm Oil Board, Kajang*
[2]*Kuala Lumpur Hospital, Kuala Lumpur*

馬來西亞良性及惡性乳房腫塊病人脂肪組織三烯生育醇濃度

觀察性研究中對受飲食維生素 E 暴露影響的血漿或是脂肪組織的 α-生育醇(α-T)濃度，是否提供婦女任何乳癌保護作用，無法提供一致的支持。反之，研究指出 α、 γ 及 δ-三烯生育醇而非 α-T 對人類乳癌細胞有潛在抗增生效應。我們的目的為研究在攝取大量棕櫚油飲食的馬來西亞族群中，惡性及良性脂肪組織的生育醇及三烯生育醇濃度是否有差異。本研究採用乳房脂肪組織的脂肪酸量當作飲食攝取的脂肪酸之定性生物標記。在病人（良性及惡性）乳房脂肪組織中主要的脂肪酸為油酸(45-46%)、棕櫚油酸(28-29%)及亞麻油酸(11-12%)。兩組的脂肪酸組成沒有差異。在惡性(13.7 ± 6.0 μg/g)及良性(20±6.0 μg/g)脂肪組織樣本，總三烯生育醇量有顯著差異(p=0.006)。然而，兩組的總生育醇並未見顯著差異(p=0.420)。本研究顯示飲食攝取會影響脂肪組織的脂肪酸量，而脂肪組織是脂溶性維生素的動態儲存槽。良性病患的脂肪組織較高的三烯生育醇濃度，支持三烯生育醇可能對乳癌具有保護作用的想法。

關鍵字：三烯生育醇、生育醇、脂肪組織、脂肪酸攝取、惡性及良性腫塊。