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^{19}F MRI of 3D CEM cells to study the effects of tocopherols and tocotrienols

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ABSTRACT

Oximetry of the human T-Lymphoblastoid (CEM) cells was measured using ^{19}F magnetic resonance imaging (^{19}F MRI). The cells were treated with the analogues of vitamin E, α-, γ-, δ-tocopherols and corresponding tocotrienols, *ex vivo* in three-dimensional (3D) cell culture. The study showed that ^{19}F MRI allows to measure the effect of the analogues due to changes of oxygenation, which were detected using MRI. Hexafluorobenzene was used as a ^{19}F MRI probe sensitive to oxygen concentrations. After 72 h of treatment in HFBR with α-, γ-, δ-tocopherols the oxygen concentration was 19.9 ± 0.8%, 19.3 ± 1.4%, 16 ± 3.5%, respectively. The oxygen concentration in cells treated with α-, γ-, δ-tocotrienols was found to be 14 ± 1.5%, 10 ± 1.2% and 8.8 ± 1.1%, respectively whereas for the control cells it was 22.1 ± 1%. The results show that δ-tocopherol and δ-tocotrienol are the most effective treatments in CEM cells among all the tested analogues.

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1. Introduction

Tocopherols and tocotrienols, analogues of vitamin E, are significant sources of antioxidant activity to all living cells thus exhibiting anticancer activity [1]. Oxygen is required for efficient cell functioning and exerts significant effects on the growth of cancer cells. The physiological perturbation of oxygen concentration in cells can be detected by administration of a sufficient probe which is sensitive to oxygen. In order to evaluate the oximetry of cell cultures, multiple and repeated measurements are required to observe dynamic changes. To allow repeated setup of cells for experimentation under controlled conditions, *ex vivo* culture is needed. Moreover, recent studies proved that the cells' growth in three-dimensional (3D) cultures mimics *in vivo* condition [2] through cell–cell and cell–extracellular matrix contacts and morphology [3].

In our study we investigated fluorine (^{19}F) magnetic resonance imaging (MRI) of human T-Lymphoblastoid (CEM) cells grown in the hollow fiber bioreactor (HFBR, FiberCell System Inc., Frederick, MD), as an *ex vivo* method of assessing cell viability after treatment with α-, γ-, δ-tocopherols and corresponding tocotrienols. The fluorine atom provides a tool for cellular imaging applications due to strong MR signal, minimal background signal in tissue and sensitivity to changes in the cells' microenvironment. ^{19}F MRI has already been used to map tissue oxygenation and it was shown that there is a linear relationship between the ^{19}F spin-lattice relaxivity ($R_1 = 1/T_1$) of perfluorocarbon (PFC) and oxygen concentration [4]. Therefore in our study we used T_1 relaxation time of ^{19}F present in hexafluorobenzene (HFB, SynQest Laboratories Inc., Alachua, FL) to assess oxygen concentrations in CEM cell cultures. HFB was found to be a sensitive MR probe of prostatic tumor oxygenation following intratumoral injection [5]. In the previous study [4] we showed the effect of δ-tocopherol on breast cancer cells, where perfluoro-15-crown-5-ether (PFCE) was used as a ^{19}F MR probe. It has recently been proved that T_1 of HFB is highly sensitive to oxygen concentration and minimally to temperature [5], exhibits no toxicity, does not show mutagenicity, fetotoxicity or teratogenicity [6,7]. Both compounds, PFCE and HFB, are suitable probes for MR studies as they provide high signal-to-noise ratio (SNR) due to 20 and 6 equivalents of ^{19}F nuclei, respectively. The major advantage of ^{19}F imaging is lack of any measurable tissue background signal from endogenous ^{19}F nuclei [8]. The aim was to determine which tocopherol and tocotrienol compound was more effective in inhibiting cell growth while growth inhibition was measured indirectly using T_1 of HFB. We measured changes in T_1 caused by variable oxygen concentration in cell culture.

Abbreviations: CEM, human T-Lymphoblastoid; HFB, hexafluorobenzene; HFBR, hollow fiber bioreactor; ms, millisecond.

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2. Experimental

2.1. Cells' culture

CEM cells were obtained from the American Type Cells Collection (Manassas, VA). Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Oakville, ON) with 25 mM glucose, supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 6 mM L-glutamine. When the number of cells in cell flask reached 10^5 cells/ml, the cells were inoculated in perfused HFBR cartridge and cultured over 4 weeks in 3D geometry. The perfusion loop allowed for continuous delivery of nutrients and dissolved oxygen to cells located in the HFBR device. The medium within the perfusion loop circulated continuously at 8 ml/min from the medium reservoir to the HFBR and back to the medium reservoir. The perfusion medium was changed weekly when the glucose level reached 2 g/L as measured with a glucometer. The oxygen concentration in 100 ml of media was 7.6 μg/ml at 37 °C. To confirm MRI measurements the vitality of CEM cells was also determined using the trypan blue exclusion method [9].

2.2. Stock solution

The stock solutions of each α-, γ-, δ-tocopherols and -tocotrienols treated media were prepared according to Sylvester et al. [10]. Briefly, 100 μg of each compound was dissolved in 100 μl of 100% ethanol. Once dissolved, solution was added to a 5 mg/ml of bovine serum albumin (BSA) in the water and incubated overnight at 37 °C. The final ethanol concentration was less than 0.1%. We did not observe any decrease in the growth of control cells treated with ethanol concentrations lower than 0.1%.

2.3. Pre-selection of concentrations

To pre-select concentrations of the compounds for cell treatments in the HFBR, the cells were first treated in small volumes on a plate. Approximately 4×10^4 CEM cells were harvested from the HFBR and incubated in media at 37 °C. The media were then removed and replaced with 3 ml of 0, 30, 50, 100, 200, 300, 500, 750, 1000 μM of tocopherols and tocotrienols solutions and incubated for 48, 72, 96 and 120 h.

2.4. Cells' treatments

The fluorine emulsion was prepared using 10 ml of HFB for 5 ml of cells with density 10^9 cells/ml in the media. We did not observe any effect of HFB alone on cell growth. The emulsion was introduced into the perfusion system of the control and treated cells. The fluorine T_1 of HFB in the HFBRs was measured. Oxygen concentration was then calculated on the basis of the calibration curve.

2.5. Calibration curve

The calibration experiments were performed in the HFBRs perfused with 80 ml media containing 40 ml HFB with dissolved oxygen concentrations of 5%, 21%, and 36% in balance with nitrogen, respectively. Twelve HFBRs were used for the calibration experiments, four for each oxygen concentration.

2.6. Magnetic resonance imaging

All MRI experiments were performed using 9.4 T/21 cm magnet (Magnex, UK) equipped with TMX console (NRC-IBD, Canada). To measure the intracellular T_1 we used CEM cells cultured and treated in the HFBR for 72 h with the following pre-selected concentration: 250 μM of α-tocopherol, 200 μM of γ-tocopherol, 150 μM of δ-tocopherol, 13 μM of α-tocotrienol, 11 μM of γ-tocotrienol, 9 μM of δ-tocotrienol ($n = 4$ for each concentration). The pre-selection was done in 4×10^4 cells harvested from HFBR cells and pre-selected concentrations were the IC_{50} values found after 72 h treatment. ^{19}F MR images were acquired using ^{19}F tuned transmit/receive radiofrequency (RF) volume coil operating at 376 MHz corresponding to ^{19}F Larmour frequency at 9.4 T. The inversion recovery (IR) spin-echo (SE) method with inversion time (IT) equal to 100, 200, 400, 800, 1000, 2000, 4000, 8000, 18,000 ms, repetition time (TR) 20 s, echo time (TE) 16.5 ms, 0.5 mm slice thickness, 3 cm × 3 cm field of view, 256 × 256 matrix size, 2 signal averages and spectral width 50,000 Hz were used. T_1 was calculated within the region-of-interest (ROI) using MAREVISI (NRC-IBD) software. Dissolved oxygen concentration was calculated (in percent) based on the calibration curve. All statistical analyses were conducted using Sigma Stat (Chicago, IL) software.

Table 1
Viability (%) after exposure 4×10^4 CEM cells to α-, γ-, δ-tocopherols$^{(1)}$ and α-, γ-, δ-tocotrienols$^{(2)}$.

Exposure time	Compounds					
	α^1	α^2	γ^1	γ^2	δ^1	δ^2
48 h	57 ± 3	32 ± 1	47 ± 3	23 ± 3	42 ± 4	9 ± 2
72 h	52 ± 4	23 ± 4	45 ± 2	18 ± 2	38 ± 2	6 ± 1
96 h	49 ± 7	20 ± 5	40 ± 6	16 ± 6	32 ± 2	5 ± 2
120 h	46 ± 5	19 ± 3	39 ± 8	15 ± 7	30 ± 3	5 ± 1

3. Results

This study demonstrated that α-, γ-, δ-tocopherols as well as corresponding tocotrienols inhibit the growth of CEM cells as compared to controls

The use of the concentrations of analogues in range of 0–1000 μM for 48, 72, 96 and 120 h of treatment allows to select the time and concentrations at which cells reached the maximum of their treatment effect. The viability which described the use of analogues (1000 μM) to known numbers of cells (4×10^4 cells) is presented in Table 1. After 72 h of treatment the cells reached the maximum uptake of analogues used for treatments for 250 μM of α-tocopherol, 200 μM of γ-tocopherol, 150 μM of δ-tocopherol, 13 μM of α-tocotrienol, 11 μM of γ-tocotrienol, 9 μM of δ-tocotrienol. Thus we selected these concentrations for 72 h of treatment in the HFBR device.

The ^{19}F MR images showed regions of cells with emulsion distribution after 72 h of treatments. Moreover, no ^{19}F signal was observed from extracellular media, for instance compounds that have not been taken up by cells because fresh media was flushed right after treatment. As shown in Fig. 1A, the ^{19}F signal of cell culture after 72 h of treatment was obtained mainly from the region of the tumor, while the media showed no ^{19}F signal. The use of α-, γ-, δ-tocotrienols showed faster and higher uptake in cell cultures. The linear least-square regression was used to find the T_1 dependence on the oxygen content. Fig. 1B shows the calibration curve used to estimate various oxygen concentrations in cells. The oximetry was evaluated on the basis of the following calibration curve: $y = 0.013 \times x + 0.070$, where "$y$" is a relaxivity ($1/T_1$) in ms^{-1} and "x" is oxygen concentration (% O_2) in %. The ^{19}F MRI untreated (control) cells ($n = 4$ HFBR) showed $T_1 = 2808 \pm 18$ ms ($22 \pm 1\%$ O_2) at the same time. ^{19}F MR measurements showed that treatments with α-tocopherol ($n = 4$ HFBR) increased the mean value of T_1 cells from 2824 ± 14 ms ($21.9 \pm 0.7\%$ O_2) to 3048 ± 12 ms ($19.9 \pm 0.8\%$ O_2). Treatment with γ-tocopherol ($n = 4$ HFBR) showed that the mean value of T_1 in cells' regions increased from 2808 ± 9 ms ($22 \pm 1.5\%$ O_2) to $T_1 = 3125 \pm 11$ ms ($19.3 \pm 1.4\%$ O_2). T_1 values of cells treated

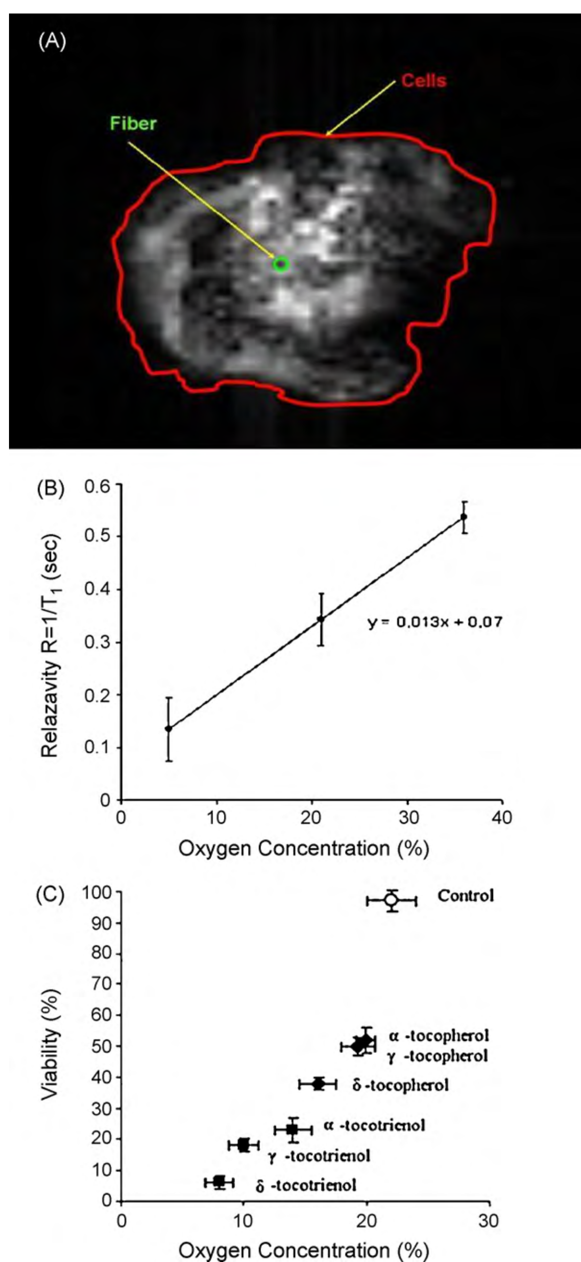

Fig. 1. (A) An example of ^{19}F MRI (TR/TE/TI = 20 s/16.5 ms/18,000 ms) with 3 cm × 3 cm field of view, 256 × 256 matrix size and 0.5 mm slice thickness. This image shows tumor after 72 h of treatment with δ-tocotrienol: red solid line indicated total volume of cells and green solid line showed the fiber; (B) the calibration curve; (C) the correlation between viability and oxygen concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with δ-tocopherol showed a significant increase from 2762 ± 19 ms (22.5 ± 1% O_2) to 3597 ± 31 ms (16 ± 3.5% O_2).

After 72 h exposure to α-, γ-, δ-tocotrienols the T_1 values of cells were 3968 ± 15, 5000 ± 12, 5747 ± 14 ms, respectively and corresponded to oxygen concentrations of 14 ± 1.5% O_2, 10 ± 1.2% O_2, 8 ± 1.1% O_2, respectively. The above data show that δ-tocopherol and δ-tocotrienol were the most effective in CEM cells' treatments among all studied compounds. Comparison of ^{19}F images showed that the cells occupied central regions of HFBR device. The total time for the setup of MR experiment and to obtain the image was less than 3 h. Due to the perfusion the HFBR absorbed sufficient oxygen from the reservoir with fresh media to keep cells alive. Oxygen concentrations (%) in the cultured and treated cells for 72 h treatment are presented in Table 2. Fig. 1C presents the correlation between the viability and oxygen concentrations. Each point is an average of 4 independent experiments.

Table 2
Oxygen concentration (%) in the cultured and treated cells for 72 h treatment with α-, γ-, δ-tocopherols$^{(1)}$ and α-, γ-, δ-tocotrienols$^{(2)}$.

Control cells	Treated cells					
	α^1	α^2	γ^1	γ^2	δ^1	δ^2
22 ± 1	19.9 ± 3	14 ± 1.5	19.3 ± 1.4	10 ± 1.2	16 ± 3.5	8 ± 1.1

4. Discussion

Ex vivo cell culture in HFBR device provides numerous opportunities and benefits to be derived from studies relying on human organs. This 3D system evaluates controlling condition and adjusting experimental variables for optimization of tumor cell cultures [11]. It has been already established that 3D culture in the HFBR simulates *in vivo* an environment and recapitulates particularly well the spatial organization of solid tumor [12]. It was shown that breast cancer cells cultured in two-dimensional (2D) cultures do not recapitulate the differential structures observed *in vivo*. Moreover, it was noted that biochemical signals from the surrounding microenvironment of cells in 2D culture are lost, causing deregulation of the susceptibility of cells [13]. Therefore, the use of cells cultured in 3D geometry may accelerate the progress in design and development of cancer therapies. Here, 3D culture of cancer cells, provides perspectives for MRI by offering measurements in high density 3D cancer tissue.

Many studies have been already conducted on the role of tocopherols and tocotrienols in cancer research development [14,15]. Despite of an increasing number of reports on the influence of analogues of vitamin E on cancer cells, the question about which isomer is the most potent inhibitor, has not been answered yet. However, previous studies associated with vitamin E have been mostly focused on the role of α-tocopherol in breast cancer development [16]. Some authors demonstrated that 24 h exposure to 0–250 μM of α- or γ-tocopherol and 0–250 μM of δ-tocopherol had effect on cell viability in highly malignant cells [17]. In particular for MCF-7 cells, the order of the concentrations required to induce 50% of cells viability was as follow: δ-tocopherol > γ-tocotrienol > α-tocotrienol > δ-tocotrienol whereas for MDAMB-435 cells was α-tocotrienol > δ-tocopherol > γ-tocotrienol > δ-tocotrienol [18].

Our cell-specific MR imaging method is useful for living cells. What is most important, this method can be used to visualize any cell after treatment with any antioxidative agent. ^{19}F MR relaxation measurement is one of few techniques which may allow repetitive measurements of tissue O_2 levels *in vivo*. Therefore, the cells oxygenation assay could be applied to measure tocopherols and tocotrienols efficacy. This study shows how viability of cells corresponds to dissolved oxygen concentration in the HFBR device and demonstrates that NMR can play an important role in defining biochemical engineering parameters required for optimization of cell culture and treatment design. We observed that the intracellular oxygen concentrations after treatment were significantly lower than those before treatment in all performed experiments. During treatments, when the number of viable cells in the HFBR decreases, lower oxygen consumption in the culture was also observed. We found that viability and oxygen concentrations are related to the type and time of treatments. We measured 2–8% decreases in oxygen content for cells treated with α–γ-tocopherols and 6–14% decreases for cells treated with α–γ-tocotrienols as compared to control. In conclusion, the use of HFB for ^{19}F MRI *ex vivo* allows

investigations of tumor oxygenation after treatment with analogues of vitamin E.

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