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[Methyl-^{14}C]-choline incorporation into nude mice bearing tumor xenografts-correlation with [methyl-^{3}H]-thymidine: A pilot study

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Background: Many *in vitro* studies suggested that choline incorporation into many types of tumors is related to cell proliferation. Whether *in vivo* choline incorporation is related also to cell proliferation or not was my question. Therefore, the aim of this pilot study was to investigate the relationship of *in vivo* [methyl-^{14}C]-choline incorporation and *in vivo* [methyl-^{3}H]-thymidine incorporation ***Materials and Methods:*** Four female nude mice were inoculated with MCF-7 breast tumor cells. Tumors were allowed to grow to a diameter of 0.5 cm and a diameter of 1 cm. Radioactivity of 370 kBq [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine each was injected. After 10 min, tumors were harvested and extracted with organic and aqueous solvents. The total [methyl-^{3}H]-thymidine and [methyl-^{14}C]-choline were determined and correlated using Spearman's rank (r_s) coefficient at 0.5 cm (n=4) and 1 cm (n=3) tumors. ***Results:*** Spearman's rank (r_s) correlation coefficient showed high r_s=0.8 (p=0.2; n=4) at 0.5 cm tumor compared to low r_s=0.5 (p=0.7; n=3) at 1 cm tumor. ***Conclusion:*** In this preliminary pilot study, *in vivo* [methyl-^{14}C]-choline incorporation may correlate with cell proliferation. More number of investigations is recommended. **Iran. J. Radiat. Res., 2007; 5 (2): 79-83**

Keywords: *Choline, thymidine, tumor, proliferation, MCF-7 cells, nude mice.*

INTRODUCTION

[Methyl-^{11}C]-choline positron emission tomography (PET) has been introduced as a novel tumor-seeking tracer for the imaging of many types of cancers, for example, brain cancers[1], breast cancers[2], and prostate cancer and its metastases[3, 4]. Choline is crucial for both animals and plants. It is important for the structural integrity of cell membranes. Choline is transported into the cell by passive diffusion and carrier-mediated uptake mechanisms[5]. Choline is then phosphorylated to phosphocholine (PCho) for the synthesis of phosphatidylcholine (PtdCho), the most constituent of cell membranes. PCho is a substrate for the rate-limiting step in the synthetic pathways for PtdCho, and it is the first intermediate in the stepwise incorporation of choline into phospholipids. It is produced by phosphorylation of choline by choline kinase[6]. Evidence from clinical [7] and animal nuclear magnetic resonance (NMR) studies [8] and from cell studies [9, 10] suggest that the tumor cell content of choline metabolites is related to the proliferation rate. *In vitro* studies [9-11] using cell culture lines have shown that the PCho content of confluent populations of tumor cells is much lower than in cells undergoing logarithmic growth. The number of transporters (V_{max}) of choline uptake was found to be increased whilst choline affinity (K_m) was decreased in populations of human breast cancer MCF-7 cells with higher proliferative fractions, compared with populations having lower proliferative fractions[5]. This reflects the relationship between choline incorporation and tumor cell proliferation.

After intravenous injection of [methyl-^{11}C]-choline, the radioactivity is rapidly cleared from the blood and stays constant in tumor cells within 5 to 40 min. The mechanism of choline incorporation into tumor cells is still not fully understood. Many clinical and experimental studies suggested that the metabolites of choline are related by somehow to tumor cell proliferation[8, 12]. In a previous study [13] the relationship between

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in vitro [methyl-^{14}C]-choline incorporation and cell proliferation was investigated. Cell proliferation was determined by measurements involve flow cytometry DNA synthesis (S phase) and [methyl-^{3}H]-thymidine incorporation in MCF-7 cells and the study demonstrated a strong correlation.

Data on the *in vivo* methylcholine incorporation in tumors are lacking in the experimental and literature work. Therefore, the aim of this pilot study was to investigate the relationship of *in vivo* [methyl-^{14}C]-choline incorporation and *in vivo* [methyl-^{3}H]-thymidine incorporation.

MATERIALS AND METHODS

This pilot study was conducted at the Nuclear Medicine and Surgery Department, Faculty of Medicine, Kuwait University Health Sciences Center, Safat, Kuwait. The study protocol was approved by our institutional "research administration" review board. In addition, our study was conducted in accordance with the principles of the Good Clinical Practice Guidelines and the Declaration of Helsinki and its amendments.

Material

All chemical reagents used were supplied by Sigma-Aldrich (UK) unless otherwise stated. Trypsin-EDTA was obtained from Gibco (Grand Island, NY, USA). Ultima Gold scintillant fluid was obtained from Meridian (Scotland, UK). A liquid scintillation counter Packard TriCARB LSC-1900CA, Packard Instrument Company (Frankfurt, Germany) was used to count all radioactive samples.

Radiolabelled compounds

[Methyl-^{14}C]-choline chloride (specific activity 2.22 GBq/mmol) and [methyl-^{3}H]-thymidine (specific activity 2.96 TBq/mmol) were obtained from American Radiolabelled Chemicals (USA).

Cell culture and culture media

Human breast cancer (MCF-7) cells were obtained from the American Type Culture Collection, Manassas, Virginia. MCF-7 cells were cultured into 75 cm^2 flasks in RPMI-1640 medium supplemented with 20 U/ml penicillin, 20 µg/ml streptomycin, and 10% foetal calf serum (Gibco, Grand Island, NY, USA). Cells were incubated at 37°C in 5% CO_2:95% air. When cultures reached 70% confluency, cells were washed with phosphate buffer saline (PBS), trypsinized with trypsin-EDTA for 5 min. Cells were then centrifuged at 1000 g for 5 min at 4°C and PBS was decanted. Cells pellet was then resuspended with PBS.

Animals

Female nude mice approximately 8 to 10 weeks of age were inoculated subcutaneously with MCF-7 cells in 0.1 ml (approximately 10^7 cells/mouse) in the left flank. Tumors in each nude mouse were allowed to grow to: a) within 1 week to a diameter of 0.5 cm and b) within 2-3 weeks to a diameter of 1 cm.

[Methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation

Nude mice were injected under light anesthesia simultaneously with 370 kBq [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine each into a femoral vein for 10 min. This time is to simulate the incorporation of [methyl-^{11}C]-choline in tumor cells during a PET scan. After 10 min, mice were terminated by cervical dislocation, and dissected. Tumors were harvested, weighed, homogenized and extracted with organic and aqueous solvents [14] to determine the distribution of tracer between phospholipid and water-soluble metabolite pools. The radioactivity of ^{14}C and ^{3}H were counted in a liquid scintillation Packard TriCARB LSC-1900CA counter and expressed as counts per minute (C.P.M.) per tumor weight (g). Then total [methyl-^{3}H]-thymidine and [methyl-^{14}C]-choline were determined and correlated.

Presentation of data and statistical analysis

All data -unless otherwise stated- was

expressed as mean ± standard deviation of the mean (SD). Nonparametric correlation (Spearman's rank; rs) coefficient was applied to correlate in vivo [methyl-^{14}C]-choline incorporation and [methyl-^{3}H]-thymidine incorporation at 0.5 cm (n=4) and 1 cm (n=3) tumors. The Mann-Whitney test was used to determine statistical differences between groups of [methyl-^{14}C]-choline incorporation and [methyl-^{3}H]-thymidine incorporation into 0.5 cm and 1 cm tumors.

RESULTS

[Methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation

Table 1 shows individual total counts expressed as counts per minute (C.P.M.) per tumor weight (g), (CPM/g) of in vivo [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation. The results were expressed as mean (±SD) [methyl-^{14}C]-choline incorporation and [methyl-^{3}H]-thymidine incorporation at 0.5 (n=4) and at 1 cm tumors (n=3).

Table 1. Individual total [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation.

0.5 cm Tumor (n=4)		1 cm Tumor (n=3)	
[Methyl-^{14}C]-choline	[Methyl-^{3}H]-thymidine	[Methyl-^{14}C]-choline	[Methyl-^{3}H]-thymidine
111529	472167	370	1100
104999	299373	471	848
110000	398877	419	1104
106283	429355	dead	dead

Table 2 shows nonparametric correlations for *in vivo* [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation at 0.5 and 1 cm tumors respectively.

The results were expressed as mean (±SD) total [methyl-^{14}C]-choline incorporation and [methyl-^{3}H]-thymidine incorporation. The results showed high Spearman's rank (r_s) correlation coefficient (r_s=0.8) and no significant difference (p=0.2; n=4) between [methyl-^{14}C]-choline incorporation and [methyl-^{3}H]-thymidine incorporation at 0.5 cm, whilst at 1 cm tumor, r_s showed lower value (r_s=0.5) with no significant *p* value (*p*=0.7; n=3).

The Mann-Whitney test was used to determine statistical differences between [methyl-^{3}H]-thymidine incorporation at 0.5 cm and [methyl-^{3}H]-thymidine incorporation at 1 cm, and between [methyl-^{14}C]-choline incorporation at 0.5 cm and 1 cm. The Mann-Whitney test for both [methyl-^{3}H]-thymidine incorporation and [methyl-^{14}C]-choline incorporation at 0.5 cm and 1 cm showed not significant *p* value (*p*=0.034). However, [methyl-^{3}H]-thymidine incorporation at 0.5 cm has bigger median value ± interquartile range (IQR): 414115.94 ± 137215.04 than at 1 cm tumor: 1100.41 ± 255.78. Similarly, [methyl-14_C]-choline incorporation has bigger median value ± IQR at 0.5 cm: 108141.38 ± 5826.81 compared to: 419.43 ± 100.69 at 1 cm tumor.

Table 2. Nonparametric correlations of in vivo [methyl-^{14}C]-choline and [methyl-^{3}H]-thymidine incorporation at 0.5 and 1 cm tumors.

			Tymidine 0.5cm	Choline 0.5cm	Tymidine 1cm	Choline 1cm
Spearman's rho	Tymidine 0.5cm	Correlation Coefficient	1.000	.800	.500	-1.000**
		sig. (2-tailed)	.	.200	.667	.000
		N	4	4	3	3
	Choline 0.5cm	Correlation Coefficient	.800	1.000	.500	-1.000**
		sig. (2-tailed)	.200	.	.667	.000
		N	4	4	3	3
	Tymidine 1cm	Correlation Coefficient	.500	.500	1.000	-.500
		sig. (2-tailed)	.667	.667	.	.667
		N	3	3	3	3
	Choline 1cm	Correlation Coefficient	-1.000**	-1.000**	-.500	1.000
		sig. (2-tailed)	.000	.000	.667	.
		N	3	3	3	3

** Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

[Methyl-^{11}C]-choline was introduced in 1997 as a tumor-seeking PET tracer [15, 16], especially for imaging and staging prostate cancer[16, 19, 4]. Choline is essential for new membrane formation (phospholipids)[20, 21]. Many studies reported the relationship between choline, DNA synthesis, and cell proliferation[13, 20, 22, 23]. Here, the results suggest the presence of an association between the *in vivo* incorporation of choline and thymidine at tumors (0.5 cm) but not at 1 cm tumors. Although the time of 10 min incorporation is not the standard for [methyl-^{3}H]-thymidine (4-6 h), the results suggest a relationship.

The fact that the rapidly proliferating cells, as indicated in this study by 0.5 cm diameter, need to synthesize phospholipids more than the confluent cells, as indicated in this study by 1 cm diameter, may explain the high choline incorporation in these cells in xenografts in nude mice and the presence of an association with thymidine.

[Methyl-^{14}C]-choline was used instead of [methyl-^{11}C]-choline because of its ^{14}C long half-life (5568 y) compared to the short half-life (20.4 min) of ^{11}C and suitability for metabolic studies. Here, [methyl-^{3}H]-thymidine incorporation into DNA was investigated and it was in agreement with many *in vitro* studies, which reported strong correlation between [^{14}C]-choline, [^{3}H]-thymidine incorporation, and DNA synthesis [13, 24] and in agreement with one *in vivo* clinical study [25] using [methyl-^{11}C]-choline and proton magnetic resonance spectroscopy (^{1}H-MRS), which reported that both choline uptake and the content of choline-containing metabolites in gliomas correlated with proliferative fraction, and that the difference between choline concentration determined in 1H-MRS and [^{11}C]choline uptake measured with PET was not significant indicating that both ^{1}H-MRS and [11C]choline PET can be used to estimate proliferative activity of human brain tumors [25], which supports our current pilot study.

Some studies suggest that [methyl-^{11}C]-choline used during PET might accumulate in tumor cells via increased phospholipid synthesis and phosphocholine (PCho) trapping with highly proliferating cells[26, 27]. Hara [26] suggested that the only metabolic fate of [methyl-^{11}C]-choline is conversion into PCho within tumor cells and integration into phospholipids, resulting in the production of phosphatidylcholine (PtdCho). Once [methyl-^{11}C]-choline has been phosphorylated within tumor cells, it remains there, yielding ^{11}C-PCho and constituting a chemical "trap". Hara also suggested that [methyl-^{11}C]-choline might be useful for indicating the rate of membrane synthesis and, therefore, the rate of replication of tumor cells. This is in agreement with the current pilot study that indicates the presence of an association between the *in vivo* incorporation of choline and thymidine into 0.5 cm tumor cells. In this pilot study, the results suggest that proliferation (DNA) is responsible for the incorporation of [methyl-^{14}C]-choline into tumors in nude mice and that the *in vivo* incorporation of [methyl-^{14}C]-choline reflects tumor cell proliferation as determined by [methyl-^{3}H]-thymidine at the highly proliferating cells (0.5 cm) while not at the slowly proliferating cells (1 cm) in nude mice bearing tumor xenografts.

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