TECHNICAL NOTE

Can DCEMRI assess the effect of green tea on the angiogenic properties of rodent prostate tumors?

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Abstract The purpose of this research was to test whether dynamic contrast enhanced MRI could assess the effect of green tea on the angiogenic properties of transplanted rodent tumors. Copenhagen rats bearing AT6.1 prostate tumors inoculated in the hind limbs were randomly assigned to cages in which they were allowed to only drink either plain water (control group) or water containing green tea extract (treated group). Assignments were made after a baseline MRI experiment (week 0) was performed on each rat at 4.7 T. All the rats were subsequently imaged at day 7 (week 1) and day 14 (week 2) to follow tumor growth and vascular development. The two-compartment pharmacokinetic model was used to analyze the dynamic contrast Gd-DTPA enhanced MRI data on a pixel-by-pixel basis over the tumor area to obtain the volume transfer constant (K^{trans}) and extravascular extracellular space (v_e). An identity Chi-squared test showed that the distributions of averaged histograms ($n = 6$) of K^{trans} and v_e were significantly different from week 0 to both weeks 1 and 2 ($p < 0.001$) in both the control and the treated rats due to increasing areas of tumor necrosis. However, the tumor growth rate was statistically indistinguishable between control and treated rats. There was no significant difference in the distributions of K^{trans} and v_e between control and treated rats. The results showed that no effects of green tea on tumor micro-vasculature were measurable by dynamic Gd-DTPA enhanced MRI.

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Introduction

Green tea is a popular beverage consumed worldwide and has demonstrated cancer preventative effects [1,2]. Epidemiological studies suggest that consumption of green tea decreases cancer risk, particularly for colon and prostate cancers [3,4].

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Epigallocatechin gallate (EGCG) is an anti-oxidant found in green tea that has attracted strong interest as an anti-cancer agent [5,6]. It has been demonstrated that EGCG inhibits angiogenesis *in vitro* through the inhibition of vascular endothelial growth factor (VEGF) receptor binding [7,8]. Other anti-oxidants in herbal medicines have similar properties.

More than two decades ago, Wang et al. [9,10] demonstrated that green tea was useful as a chemo-preventive agent against cancers in both mice and rats. Recently, polyphenol moieties in green tea have been shown to be able to inhibit prostate and breast cancers in transgenic male mice [11,12] and athymic nude female mice [13], respectively. Although angiogenesis is essential to tumor growth and metastasis, anti-angiogenic effects due to anti-oxidants found in green tea are difficult to quantify with *in vivo* studies; blocking angiogenesis is more likely to slow or stop the tumor growth rather than to cause regression [14]. However, performing accurate measurements of tumor volume and growth rate are difficult. Conventional methods (e.g. calipers) provide inaccurate estimates because they are relatively insensitive to small changes in tumor growth rate, often include surrounding tissue in the measurement, and are prone to measurement error that is not systematic. Widely accepted invasive techniques, such as histopathology [12], cannot be used to access the effects of EGCG on tumor development in serial *in vivo* studies. Thus, measuring anti-angiogenic effects of green tea *in vivo* requires comprehensive studies involving many subjects.

Magnetic resonance imaging (MRI) is routinely used clinically to obtain high-resolution functional and anatomical information of cancers [15]. Dynamic contrast enhanced MRI (DCEMRI) has the potential to quantify the effects of anti-angiogenic drugs on tumor vasculature development by monitoring tumor blood flow and/or capillary permeability [16]. Changes in blood flow and capillary permeability may serve as early indicators of the effects of therapy and, thus, may allow rapid and inexpensive assessment of the therapeutic effects of green tea. Detailed information regarding the physiological effects of green tea *in vivo* may help to guide more effective use of green tea for therapeutic as well as preventative applications.

In this study, we employed MRI to non-invasively monitor tumor growth very accurately and to test whether anti-angiogenic effects of green tea extract on transplanted rodent prostate AT6.1 tumors could be assessed by DCEMRI. The two-compartment pharmacokinetic model for contrast media uptake and washout was used to analyze the DCEMRI data on a pixel-by-pixel basis. Distributions of the histogram of volume transfer constant (K^{trans}) between blood plasma and the extravascular, extracellular space (EES), and the volume of the EES per unit volume of tissue (v_e) were analyzed for control and green tea treated rats. The tumor growth rates were also calculated based on the simple exponential growth model.

Materials and methods

Animal, tumor model and green tea treatment

AT6.1 metastatic prostate tumors [17] were inoculated in the hind limbs of Copenhagen rats. They metastasized

relatively quickly to the lung about three weeks after inoculation. A total of 16 rats were used in this study. All surgical procedures were performed in accordance with the University of Chicago Institutional Animal Care and Use Committee guidelines. We selected rats with tumors of similar size at the beginning of this study. The first MRI at day 0 (Week 0) was performed on tumors about one to two weeks after inoculation. Then, the rats were separated into two groups ($n = 8$ each) – ‘control’ and ‘treated with green tea’. For the control group, rats were fed a standard diet with untreated drinking water and for the treated group, rats were fed a standard diet with treated drinking water (green tea extract added). Additional MRI experiments were performed on the same tumors at day 7 (Week 1) and day 14 (Week 2). Since four rats died before the third MRI experiment, only six rats were left in each group for the final analysis.

The green tea (TeGreen 97) capsules were obtained from Pharmanex LLC (Provo, UT, USA). Each capsule contains approximately 95 mg of EGCG, which is equivalent to the polyphenol content of approximately seven cups of green tea without the caffeine. Three capsules were dissolved in ~300 ml water in each bottle for treated rats. Water bottles for both the control and the treated groups were replaced three times per week.

Tumor growth rate

After baseline MRI experiments (Week 0), rats were monitored every other day for any overt signs of distress, such as dehydration, body weight change, or gastrointestinal disturbance. The following simple model was chosen to describe the tumor growth rate [18]:

$$V_t = V_0 e^{\rho t}, \quad (1)$$

where V_0 is the initial size, t is time in days, and ρ is the growth rate. By this model, the time required to double the tumor size (t_d) is given by $t_d = \ln(2)/\rho$. The Eq. (1) was fitted with the tumor volumes V_t determined for each rat based on the three MRI experiments.

MRI experiments

Animals were anesthetized using Isoflurane gas (~2%) mixed with medical air (2 l/min) and oxygen (0.2 l/min) flowing through a mask. Exhausted gases were eliminated through an F-Air canister attached to the mask system. All animals were monitored for heart rate, respiration rate, blood pressure, and temperature using a system from SA Instruments (Stony Brook, NY, USA) developed for use in MRI. Temperature was controlled by blowing warm air through the bore of the magnet. Rats had 24 gauge angi catheters placed in their tail vein for contrast agent injection. To minimize motion artifacts, rats were wrapped in Co-Flex Vet Wrap and taped to a Plexiglas board. The leg was secured through the coil in a plastic tube and taped to the cradle. Approximately 30 min were required to prepare each animal and position it in the magnet.

MR studies were performed on a 33 cm, horizontal bore, 4.7 T scanner with a Bruker console (Bruker-Biospin, Billerica, MA, USA) equipped with a 20 cm shielded gradient set

with a maximum strength of 200 mT/m. The tumor-bearing leg was placed in a Helmholtz coil, 3.0 cm in both diameter and length. To delineate tumor boundary, multi-slice spin-echo images covering the whole tumor along the long axis of the leg were acquired first with the following parameters: TR/TE = 1500/40 ms, field of view = 40 mm × 40 mm, matrix = 256 × 256, slice thickness = 1 mm, and number of averages = 1. These images provided reference anatomical information as well as estimation of tumor volume. The tumor areas were manually traced from these images and multiplied by the slice thickness to determine the volume for the slice, and then the sum of all slices was computed to estimate the final tumor volume. The MRI measured tumor volumes in each rat at weeks 0, 1, and 2 were used to calculate its growth rate by fitting it to Eq. (1) using a logarithmic plot.

Previous studies have demonstrated that T_1 -weighted dynamic Gd-DTPA enhanced MRI technology is a promising non-invasive investigational tool for the detection of angiogenesis [19–21]. Therefore, we adapted the method to assess the angiogenic effects of green tea treatment. For DCEMRI experiments, the middle three slices covering the largest cross-sectional area of tumor were selected. T_1 -weighted gradient echo images (TR/TE = 40/5 ms, field of view = 40 mm × 40 mm, matrix = 128 × 128, slice thickness = 1.0 mm, flip angle = 30°, number of averages = 1) were acquired for ~10 min with 5 s temporal resolution. Contrast agent Gd-DTPA (Omniscan®, GE Healthcare, Piscataway, NJ, USA) was injected after ~30 s from the start of the acquisition at a typical dose of 0.2 mmol/kg of body weight over the course of approximately 5 s. An approximate time of 60 min was required for the entire *in vivo* MRI protocol.

Kinetic model of dynamic MRI

A simple two-compartment model (TCM) of the tumor (the intravascular vs. the extravascular, extracellular space (EES)) was used to describe the redistribution of contrast agent following a bolus injection. Following the conventional method as proposed by Tofts and Kermode [22], the model predicts the change in the contrast agent concentration in tissue $C(t)$ as a function of time:

$$\frac{dC(t)}{dt} = K^{\text{trans}} \cdot (C_p(t) - C(t)/v_e), \quad (2)$$

where K^{trans} (min^{-1}) is the volume transfer constant between blood plasma and EES, v_e is the volume of EES per unit volume of tissue, and $C_p(t)$ is the tracer concentration in blood plasma. The contrast agent concentration vs. time curves $C(t)$ were calculated from DCEMRI data using a previously described approximation formula [23]. $C_p(t)$ was calculated by using a reference tissue method [24] from $C(t)$ in normal muscle far from the edge of the tumor with K^{trans} (0.11 min^{-1}) and v_e (0.20) obtained from the literature values for Gd-DTPA [24].

Data analysis and statistics

The raw MR data was analyzed using IDL software (Research Systems, Inc., Boulder, CO, USA). The contrast concentration

as a function of time was calculated in each pixel and analyzed by the TCM. For each tumor, the histograms of K^{trans} and v_e were calculated for all three slices and then averaged histograms were calculated over the six different experiments at weeks 0, 1, and 2 for both control and treated rats. Chi-squared tests were performed to evaluate whether there were differences in the histogram distributions of K^{trans} and v_e from different weeks, as well as between control and treated groups. For all statistical analyses, a confidence interval of 95% or greater ($p < 0.05$) was considered statistically significant.

Results

Fig. 1 (first column) shows typical gradient echo MR images of a green tea treated transplanted rodent prostate tumor at weeks 0, 1, and 2. Post-contrast minus pre-contrast difference images at 10 s, 1 min, and 10 min after contrast agent injection are also shown for each week. The images demonstrate that the contrast agent uptake at the tumor rim is much more rapid than in the tumor center. The cores of the tumor centers barely enhanced and were believed to be areas of necrotic tissue. The volumes of necrotic areas grew significantly at each week for both control and treated rats.

The average tumor volumes measured by MRI and the growth rate determined from Eq. (1) for the control and the treated groups are given in Table 1. Tumors grew at an average rate of ~0.14 cm^3 per day (or $t_d = \sim 5$ days) and tumor volumes were found to be statistically the same ($p > 0.80$) between these two groups for each time point. Therefore, the experimental green tea treatment did not have any measurable effect on the progression of transplanted rodent prostate tumors.

Fig. 2 shows histogram distributions of K^{trans} for control and treated rats at weeks 0, 1, and 2. The identity Chi-square test showed no statistical difference in the shape of the K^{trans} histogram distributions between the control and treated rats ($p > 0.80$) for each week. However, the distributions changed from right (positively) skewed in week 0 to a reverse J-shaped in weeks 1 and 2 ($p < 0.0002$), with almost no difference between weeks 1 and 2 ($p > 0.90$) in either control or treated rats. For both control and treated groups, the median of K^{trans} was reduced about half from week 0 (control/treated = 0.22/0.25 min^{-1}) to week 1 (0.10/0.12 min^{-1}) and remaining almost the same for week 2 (0.07/0.08 min^{-1}). The percentage of pixels with K^{trans} values close to zero were significantly increased after week 0, suggesting an increased volume of necrosis.

Similarly, the identity Chi-squared test showed that there were no statistical differences in the shapes of the v_e histogram distributions between the control and the treated rats ($p > 0.80$) for the same week, as illustrated in Fig. 3. If one neglects the values near 1.0 (outliers), the shape of v_e histogram distribution changed from almost normal in week 0 to right (positively) skewed in weeks 1 and 2 for both the control ($p < 0.10$) and the treated ($p < 0.03$) rats. There was no significant change in distributions between weeks 1 and 2 ($p > 0.90$) for both the control and the treated rats. For both control and treated groups, the median of v_e was reduced about one-fourth from week 0 (control/treated = 0.50/0.41) to week 1 (0.38/0.32) and remained almost the same for week 2 (0.35/0.26).

Figure 1 A typical MRI of green tea treated rat leg tumor for weeks 0, 1 and 2. The farthest left column contains reference gradient echo images. The difference images before and after the contrast injection at 10 s, 1 min, and 10 min are shown for each week in the second, third and fourth column, respectively.

Discussion

The results of this MRI study showed significant changes in patterns of contrast media uptake/washout during tumor development. Changes in the distribution of K^{trans} and v_e in control and treated rats demonstrate that the percentage of tumor tissue accessible to contrast media decreased dramatically over time. However, there were no measurable differences in tumor growth (based on MRI volume measurements) or significant differences in the distributions of K^{trans} and v_e between control and treated rats. Therefore, MRI was unable to detect anti-angiogenic effects of green tea in this tumor model.

DCEMRI analysis suggested that the prostate tumor rim was better perfused than the center with faster contrast agent uptake and greater signal enhancement. The histogram distributions of K^{trans} with reduced median after week 0 clearly demonstrated that the necrotic areas in tumor centers increased dramatically during tumor development.

This is consistent with the histology study of the area of necrosis at the center of prostate tumors performed by Zhao et al. [25].

DCEMRI is considered to be an indirect method of assessing tumor angiogenesis [26] compared with direct methods, such as microvessel density counts. The pharmacokinetic model parameter K^{trans} is influenced by blood volume and flow and the permeability surface area product of capillaries. Therefore, high values of K^{trans} indicate high permeability and probably high perfusion, and this marker provides important information regarding the angiogenic properties of tumors [27]. The advantage of DCEMRI is that it is non-invasive and can be performed *in situ* rather than on specimens. However, the sensitivity and the accuracy of the DCEMRI method is limited by its spatial and temporal resolutions.

A number of previous studies based on both *in vitro* and *in vivo* measurements have shown that green tea extract and EGCG have anti-angiogenic effects in several tumor

Table 1 Calculated average tumor volumes for each week and predicted tumor growth rates for control and green tea treated rats.

	Tumor volume (cm ³)			Growth rate (1/day)
	Week 0	Week 1	Week 2	
Control (<i>n</i> = 6)	0.20 ± 0.12	0.49 ± 0.26	1.17 ± 0.42	0.139 ± 0.026
Treated (<i>n</i> = 6)	0.20 ± 0.19	0.54 ± 0.39	1.16 ± 0.60	0.137 ± 0.025

Figure 2 The averaged histogram distributions of K^{trans} for control and treated rats at weeks 0, 1, and 2. The distributions changed from right (positively) skewed in week 0 to a reverse J-shaped in weeks 1 and 2 in either control or treated rats. The median of K^{trans} for control/treated rats was 0.22/0.25 min^{-1} , 0.10/0.12 min^{-1} , and 0.07/0.08 min^{-1} for week 0, 1, and 2, respectively.

models [11,28–31]. These effects appeared to be associated with inhibition of VEGF production and VEGF receptor activity. *In vivo* studies using mice showed that EGCG inhibited tumor growth significantly in several human tumor models [12,13,28,29,31,32]. Unfortunately, our results did not provide evidence that green tea extract inhibited transplanted rodent tumor growth. In contrast to previous studies – the present work relied on MRI to measure the tumor volume. This approach is more accurate than calipers since the tumor boundary may not be clearly defined by palpation alone, and MRI can delineate the tumor boundary clearly in 3-dimensions.

This study had a number of potential weaknesses. The AT6.1 prostate tumor is an extremely aggressive, highly metastatic tumor line. This may explain why DCEMRI and volumetric MRI measurements did not detect an effect of green tea in these tumors. This cell line, inoculated into Copenhagen rats, may not represent the optimal model for a study of therapeutic effects of green tea. In addition, administration of green tea extracts by drinking water does not allow us to establish the exact amount of the active compound administered and retained by each animal. Moreover, the current administration method does not

Figure 3 The averaged histogram distributions of v_e for control and treated rats at weeks 0, 1, and 2. If the values near 1.0 (outliers) are neglected, the distribution changed from almost normal in week 0 to right (positively) skewed in weeks 1 and 2 in either control or treated rats. The median of v_e for control/treated rats was 0.50/0.41, 0.38/0.32, and 0.35/0.26 for week 0, 1, and 2, respectively.

allow for recording the exact timing of green tea extract intake over the three weeks treatment period. Finally, in the present experiments, histopathology was not performed at each time point for correlation with MRI measurements.

Despite these weaknesses, the present study is a useful model for non-invasive, serial measurements of effects of green tea *in vivo*. The DCEMRI and volumetric measurements described here could be applied to study other tumor models and other therapeutic agents. In addition, the present study provides valuable information concerning growth patterns and changes in hemodynamic function over time in AT6.1 tumors. This information can guide the design of future studies involving this tumor line.

In summary, DCEMRI did not detect effects of green tea on the angiogenic properties of transplanted rodent AT6.1 prostate tumors. Larger effects of green tea may be detectable in more realistic, transgenic rodent models of tumors [33], and/or in the early stages of prostate cancer [34] development. In addition, effects of green tea might be more easily detected if it is administered prior to tumor inoculation.

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