NMR IN BIOMEDICINE
NMR Biomed. 2001;**14**:497–506
DOI:10.1002/nbm.716

Issues in flow and oxygenation dependent contrast (FLOOD) imaging of tumours

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Received 22 March 2001; Revised 4 June 2001; Accepted 6 June 2001

ABSTRACT: The sensitivity of blood oxygenation level dependent (BOLD) contrast techniques to changes to tumour deoxyhaemoglobin concentration is of relevance to many strategies in cancer treatments. In the context of tumour studies, which frequently involve the use of agents to modify blood flow, there are underlying physiological changes different to those of BOLD in the brain. Hence we use the term, flow and oxygenation dependent (FLOOD) contrast, to emphasize this difference and the importance of flow effects. We have measured the R_2* changes in a prolactinoma tumour model for a variety of vasoactive challenges [carbogen, 100% oxygen and 100% nitrogen as different breathing gases, and administration of tumour blood flow modifiers such as calcitonin gene related peptide (CGRP), hydralazine and nicotinamide]. In addition we have measured other relevant physiological parameters, such as bioenergetic status from ^{31}P MRS, and blood pH and glucose, that may change during a vasoactive challenge. Here we discuss how they relate to our understanding of FLOOD contrast in tumours. We frequently observe R_2* changes that match the expected action of the vascular stimulus: R_2* decreases with agents expected to improve tumour oxygenation and blood flow, and increases with agents designed to increase tumour hypoxia. Unlike most normal tissues, tumours have a chaotic and poorly regulated blood supply, and a mix of glycolytic and oxidative metabolism; thus the response to a vasoactive challenge is not predictable. Changes in blood volume can counteract the effect of blood oxygenation changes, and changes in blood pH and glucose levels can alter oxygen extraction. This can lead to R_2* changes that are smaller or the reverse of those expected. To properly interpret FLOOD contrast changes these effects must be accounted for.

KEYWORDS: tumor; oxygenation; MRI; BOLD; perfusion; carbogen

INTRODUCTION

Gradient echo imaging (GRE), which provides blood oxygenation level dependent (BOLD) contrast,[1] is now widely used to study functionality in the brain. As this Special Issue demonstrates, although the overall theory and interpretation are well established, there are still many subtle issues regarding the precise physical mechanisms involved, even in BOLD fMRI of such a structured and well-described organ as normal brain. The BOLD contrast mechanism, dependent on blood deoxyhaemoglobin concentration, can also be used to investigate tumours, particularly with regard to interventions designed to alter tumour oxygenation. In the context of many tumour studies we prefer to designate this as flow and oxygenation dependent (FLOOD) contrast in order to acknowledge that there are underlying physiological changes in tumours that are different to those that cause BOLD effects in the brain, as well as to emphasize the contribution of the flow component to changes in GRE image intensity and blood oxygenation. The use of BOLD/FLOOD contrast in tumours is a more recent area of research[2–10] than BOLD contrast in brain and brings with it new challenges of understanding and interpretation. This topic is beginning to attract much interest, because it offers a potential method for non-invasively monitoring tumour oxygenation.

The chaotic blood supply characteristic of tumours is due to structurally and functionally abnormal vasculature and is of great medical significance. It leads to inadequate drug delivery for chemotherapy and also means that most tumours are hypoxic. The resulting low oxygen tension in tumour tissue reduces the effectiveness of radiotherapy. Cancer treatments often involve strategies to alter tumour blood flow and oxygenation, hence a potential application of the FLOOD technique is to monitor the

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Contract/grant sponsor: Cancer Research Campaign; *contract grant number:* SP1971/0402.

Abbreviations used: [dHb], deoxyhaemoglobin concentration; CGRP, calcitonin gene related peptide; FLOOD, flow and oxygenation dependent; MABP, mean arterial blood pressure; MNU, *N*-methyl-*N* nitrosourea; pH_A, arterial blood pH; pH_I, intracellular pH; p_aO_2, arterial blood oxygen partial pressure; TBF, tumour blood flow.

Table 1. Comparison of brain and tumour characteristics in relation to BOLD imaging

Brain	Tumour
Blood flow regulated	Poor blood flow regulation leading to hypoxia
Normal metabolism is oxidative	Mixed glycolytic and oxidative metabolism
Well-structured vascular architecture and capillary network	Chaotic architecture of structurally and functionally abnormal blood vessels
Uniform capillary and tissue density	Heterogeneous capillary and cell density
Healthy grey and white matter	Viable tumour tissue, oedema, stroma and necrosis
Inter-subject response to an activation task or vascular challenge is reproducible	Variable response to vascular challenge both intra-tumourally and between different tumour types
Response can be characterized using known models of cerebral perfusion. Data from many studies may be averaged to improve sensitivity	Difficult to model, quantify and validate because of heterogeneity of response. However, this heterogeneity may also be the basis of providing useful diagnostic/prognostic information

effectiveness of vascular modifiers on individual tumours and so provide a method for optimizing cancer treatments. One vascular intervention currently being assessed for its ability to improve tumour oxygenation prior to radiotherapy is carbogen (95% CO_2/5%O_2) breathing and nicotinamide in combination.[11] Conversely, hypotensive agents such as calcitonin gene related peptide (CGRP)[12] and hydralazine[13] have been used to further increase tumour hypoxia in order to enhance the action of bioreductive agents.

For the purposes of this discussion we shall write the contribution of blood deoxyhaemoglobin in small vessels to the R_2^* ($=1/T_2^*$) relaxation rate of the surrounding tissue as:

$$R_{2i}^* = k_i V_i (1 - Y_i) \qquad (1)$$

where V_i is the blood volume, Y_i the blood haemoglobin saturation and k_i depends on field strength, fractional haematocrit, blood vessel orientation and morphology.[1] The subscript i corresponds to arterioles (a), capillaries (c) and venules (v), and within a single voxel in a GRE image the total contribution to R_2^* relaxation will generally contain three terms of the form in eqn (1). With the assumption that within each voxel there are a large number of randomly oriented blood vessels of each type the total relaxation rate contribution of blood is simply a linear sum of the individual rates:[14]

$$R_{2i}^* = \sum_{i=a,c,v} k_i V_i (1 - Y_i) \qquad (1a)$$

Note that eqn (1) is derived from the average signal dephasing over the extravascular spins in a voxel and arises from the product of the integrated field inhomogeneities produced by all vessels.[14] This is different to the case of BOLD-induced R_2 relaxation for which the signal is the average of intravascular spins in separate non-exchanging blood vessel compartments.[15] The form of eqn (1a) is also validated by experimental data, which show that the BOLD-induced R_2^* change in rat brain shows a better correlation with the sum of the contributions of the changes in arterial and venular blood oxygen saturation, weighted according to vascular fraction, than with the individual changes.[16]

The theoretical description of BOLD of the type given by eqn (1) is generally applied to normal brain, and experimental studies have shown a linear correlation between changes in R_2^* and either oxygen saturation[16] or deoxyhaemoglobin concentration.[17] Determining a quantitative relationship between the MR measurable parameter R_2^* and physiological ones such as vascularity (V_i) and oxygenation (Y_i) is confounded by the very heterogeneous nature of tumour tissue, and the poor regulation of tumor blood flow relative to metabolic requirements. Similarly, the functional form of the contribution of all blood vessels to the apparent R_2^* relaxation rate will not be as easy to determine for tumours as for brain because blood vessel size, density and distribution are not well characterized in tumours. The assumptions used in models to describe BOLD contrast of fMRI in brain cannot therefore be applied directly to tumours without allowing for the differences between normal and abnormal tissue structure (see Table 1).

To investigate the use of the FLOOD technique to study tumour oxygenation we have compared the effects of various vasomodulators in subcutaneously implanted tumours in the rat using GRE and SE imaging, in conjunction with ^{31}P MRS and blood analysis.[2–8] In this paper we report on these observations, and describe how they relate to our understanding of the FLOOD phenomenon in tumours.

EXPERIMENTAL

We shall review the results of experiments performed over several years on rat tumours.[2–8] MR data were acquired using a 4.7 T Varian instrument with either, a single-(^{1}H), or double-tuned (^{1}H/^{31}P) solenoid, surrounding the tumour. The experimental techniques are summarized below, and more complete details of the methods described above can be found in the references given.

Table 2. MRI and physiological changes in the rat GH3 prolactinoma after vascular challenge. Data are mean and standard deviations over all studies reported in References 2–8 and dashes indicate no data was available

Vascular challenge	ΔR_2^* (s^{-1})	ΔSE (%)	βNTP/P_i	ΔpH_I	ΔpH_A	p_AO_2 (mmHg)	p_ACO_2 (mmHg)	Glucose (mmol/l)
CGRP	11 ± 5	—	0.5 ± 0.2	-0.1 ± 0.1	—	—	—	—
Hydralazine	8 ± 1	-8 ± 3	0.7 ± 0.6	-0.3 ± 0.15	—	—	—	—
Air (21% O_2)	0	0	1.0 ± 0.1	0	0	100 ± 10	55 ± 10	6.6 ± 0.3
100% O_2	-6 ± 8	—	—	—	0	350 ± 50	63 ± 10	—
Carbogen (95% O_2)	-19 ± 7	15 ± 2	1.3 ± 0.3	0.03 ± 0.1	-0.15 ± 0.1	400 ± 100	67 ± 5	15.6 ± 0.6
Nicotinamide	-3.8 ± 1.3	0 ± 4	1.8 ± 0.2	0.1 ± 0.04	—	—	—	11.4 ± 0.7

Tumours

Data were acquired from tumours grown in rats, either from cells transplanted subcutaneously for the GH3 prolactinomas[2–6,8] and Morris H9618a hepatomas,[7] or chemically induced in the case of MNU-induced mammary carcinomas.[7]

MRI

GRE images through the centre of all tumours were acquired with $TE/TR/\alpha = 20$ ms/80 ms/45°.[2–8] GRE images at multiple *TE*s were acquired to enable absolute T_2^* images to be calculated[4,6–8] and SE images with $TE/TR = 20$ ms/300 ms to evaluate flow effects.[4,5] Dynamic contrast-enhanced imaging was performed using an adiabatic-inversion recovery multiple FLASH sequence with $TE/TR/\alpha = 2.6$ ms/5 ms/7° and 0.5 mmol/kg Gd-DTPA (Magnevist) was delivered via a tail vein.[8]

MRS

Non-localized ^{31}P spectra were acquired with repetition time $TR = 3$ s.[2,5] Tumour energetic status was then assessed from the ratio of βNTP/P_i and intracellular pH (pH_I) was calculated from the chemical shift difference between P_i and αNTP.

Vascular challenges

Breathing gases were administered via a nose-piece with integral scavenger at 2 l/min. For all studies baseline data were acquired during air breathing and the other gases used were: 100% O_2,[3,6] carbogen (95% O_2/5% CO_2),[2–8] 100% N_2.[7] The hypotensive agents CGRP (39 μg/kg)[2] and hydralazine (5 mg/kg)[5] were administered via a tail vein cannula, as was nicotinamide (1000 mg/kg)[5], a potential agent for reducing hypoxia.

Blood analysis

The iliac artery was cannulated in separate cohorts of tumour-bearing rats to those investigated by MR and sequential blood samples taken during air breathing and after a vascular challenge. Changes in blood p_AO_2, p_ACO_2, pH_A were determined following 100% O_2 and carbogen breathing.[3,6] Blood glucose was determined after challenges with nicotinamide or carbogen.[5]

RESULTS

In Table 2 we summarise the results by giving mean data from our studies using a variety of vascular challenges with one tumor type, the GH3 prolactinoma. Calculated T_2^* maps were obtained from gradient echo images from which the average R_2^* ($=1/T_2^*$) could be calculated. The changes in R_2^* ($\Delta R_2^* = R_2^*_{base} - R_2^*_{challenge}$) were as expected for the action of each vascular challenge. After breathing high oxygen content gases arterial oxygenation (p_AO_2) increased and the tumour R_2^* relaxation rate decreased suggesting improved blood oxygenation and reduced deoxyhaemoglobin concentration ([dHb]). Conversely, administration of the hypotensive agents CGRP and hydralazine, which are expected to reduce tumour blood flow (TBF) by the steal effect, thus further desaturating the blood, resulted in an increase in tumour R_2^* rate. The small reduction in R_2^* with nicotinamide (and implied decrease in [dHb]) is discussed later. Changes in the βNTP/P_i ratio are also consistent with improved or worsened substrate (oxygen or glucose) supply and show qualitative correlation with the ΔR_2^*. For challenges with hydralazine and carbogen we acquired SE images for which the image intensity was qualitatively sensitive to blood flow and, in Table 2, ΔSE% is the percentage change in SE signal intensity following vasoactive challenge. The ΔSE% change is consistent with the expected action of hydralazine to decrease, and carbogen to increase tumour blood flow (see Fig. 1). [We point out that, although an SE image has some sensitivity to T_2 changes, we expect this to be small. For carbogen breathing, the vascular challenge that

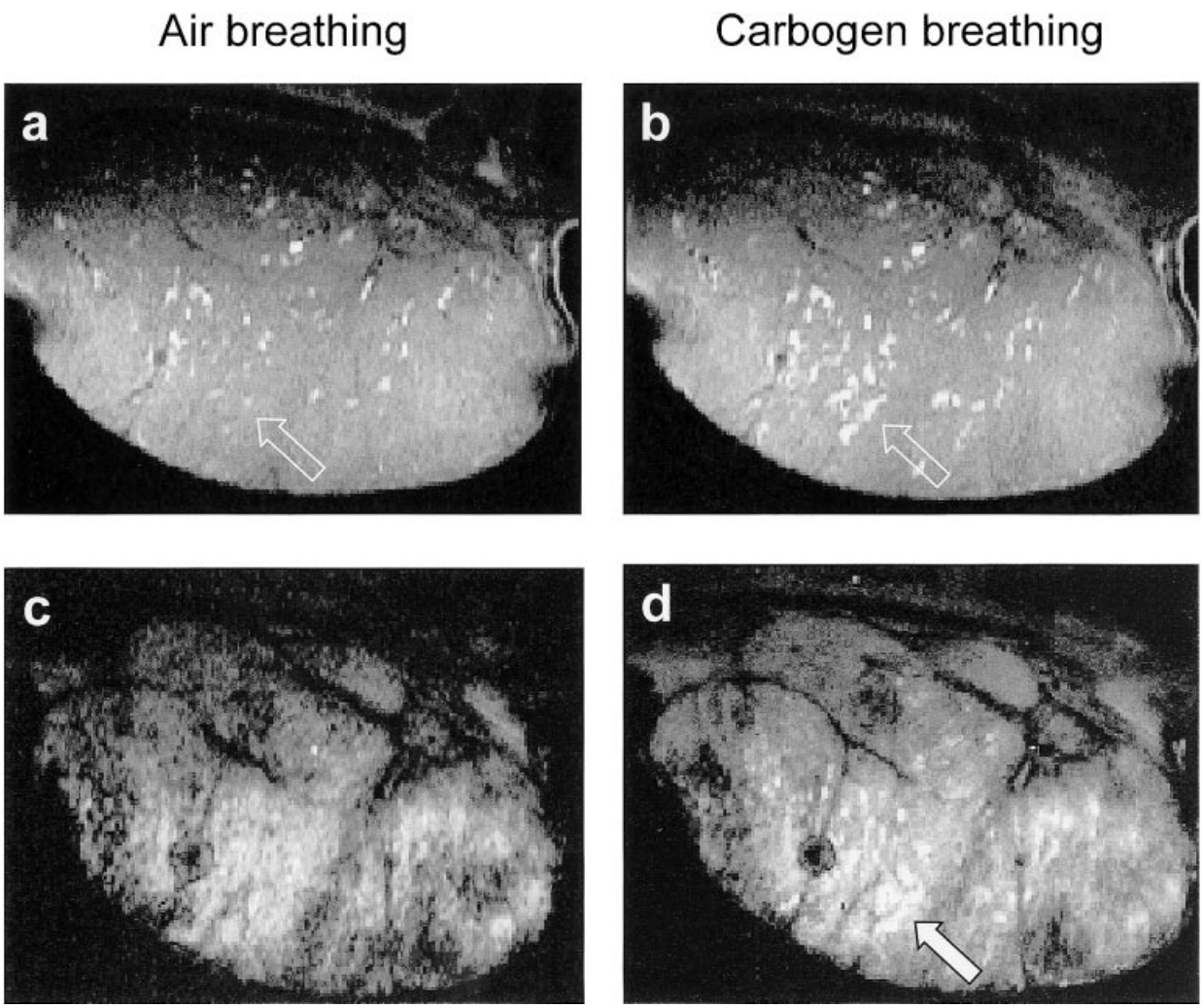

Figure 1. Demonstration of flow effects and vasodilation caused by carbogen breathing in a GH3 prolactinoma. (a) and (b) are SE (TR/*TE* = 300/20 ms) images which are sensitive to in-flow effects and the hyperintensities are blood vessels in cross-section. The open arrow indicates blood vessels that became dilated and showed increased in-flow signal enhancement during carbogen breathing. (c) and (d) are two-dimensional GRE (TR/*TE*/flip = 80/20/45°) images sensitive to both T_2* and in-flow signal enhancement. In (d) the filled arrow indicates hyperintense signals from blood vessels due to in-flow enhancement during carbogen breathing that dominate the image intensity increase due to elevated T_2*

shows the largest change in GRE and SE intensity, we have measured the T_2 for the GH3 prolactinoma[4] and found it increased from 37 ± 3 ms (air) to 41 ± 3 ms (carbogen). For SE images with $TE = 20$ ms this translates into a 5% increase in signal intensity, which is less than the ΔSE% of + 15% observed for carbogen. We would also expect some T_2 sensitivity in the ΔSE% found with hydralazine, but smaller than for carbogen since the GRE signal changes are less.] Arterial (pH_A) and intracellular pH (pH_I) were altered by several of the vascular challenges, particularly those associated with blood flow changes. One surprising result was the increase in blood glucose levels with carbogen and nicotinamide.[5]

The results of an anoxia experiment intended to measure the baseline T_2* of tumours with fully deoxygenated blood are shown in Figs 2 and 3.

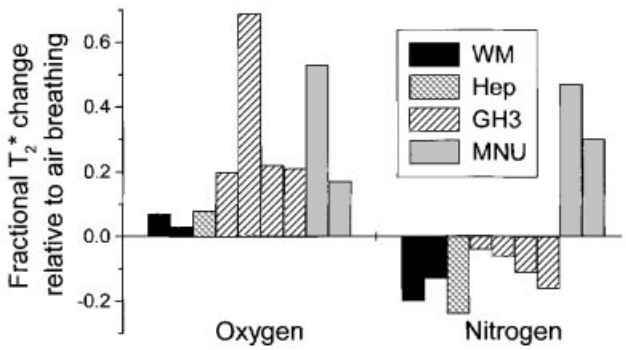

Figure 2. The fractional T_2* change, [T_2*(air) − T_2* (O_2 or N_2)]/T_2*(air), produced by different breathing gases. The change for N_2 breathing was calculated post mortem. WM, rat brain white matter; Hep, hepatoma; GH3, GH3 prolactinoma; MNU, mammary carcinoma

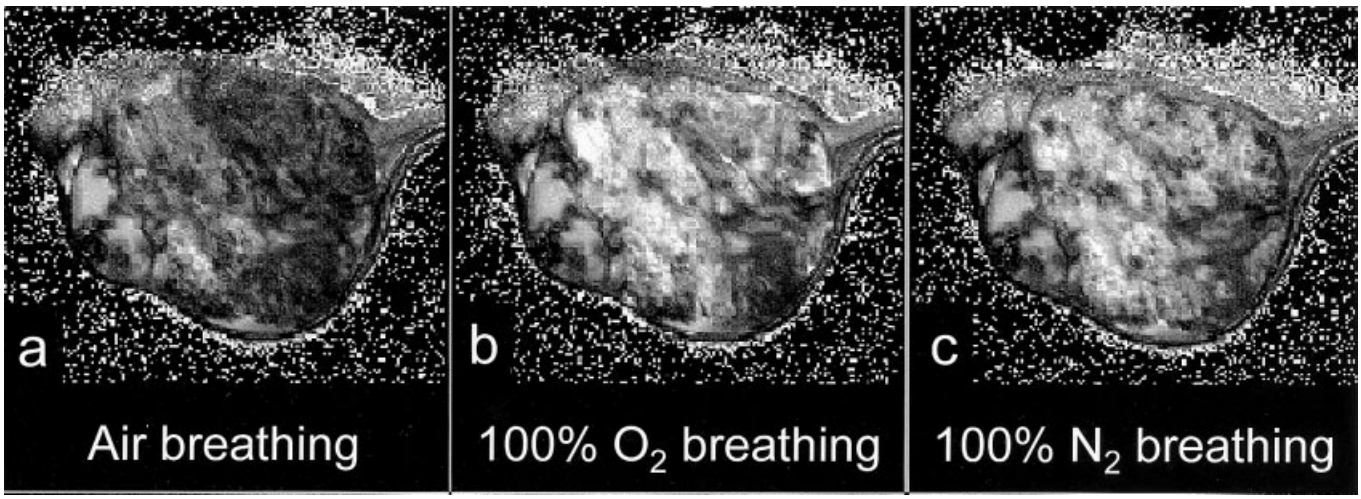

Figure 3. Calculated T_2* (grey scale 0–50 ms) images of a mammary tumour for different breathing gases. Note that the regions for which T_2* increase the greatest during 100% O_2 breathing are also those that show the greatest increase for 100% N_2 breathing. In (b) the 100% O_2 breathing reduces blood vessel deoxyhaemoglobin levels, in (c) we interpret the T_2* increase as being due to vascular collapse which reduces the overall tissue deoxyhaemoglobin concentration

Calculated T_2* images of normal rat brain and the three tumour types were determined during air breathing, 100% O_2 breathing and 100% N_2 breathing until post mortem. Figure 2 shows the fractional change in average T_2* for each tumour for the gas challenges relative to air breathing. With 100% oxygen breathing, there was an increase in T_2* for normal brain and all tumours, as would be expected due to increased arterial oxygenation. With 100% N_2 breathing, it was expected that T_2* should decrease as the blood became highly deoxygenated, and this was the case for brain, hepatoma and prolactinoma. However, unexpectedly, the MNU tumours showed an increase in T_2* by as much as that obtained for 100% O_2 breathing. Figure 3 shows the calculated T_2* images for one MNU tumour during each gas breathing episode. [*Note*: although we use R_2* in eqn (1), for discussion of changes in deoxyhaemoglobin levels we show calculated T_2* images which demonstrate the relaxation time changes more clearly than calculated R_2* images.]

Gd-enhanced images of a GH3 prolactinoma are shown in Fig. 4. These are inversion recovery images chosen at an inversion time for which the image intensity of tumour tissue was nulled prior to contrast administration. The signal intensity increases in Fig. 4(a) and (b) thus demonstrate the time course of Gd-DTPA uptake into the tumour. In Fig. 4(c), a difference map shows the

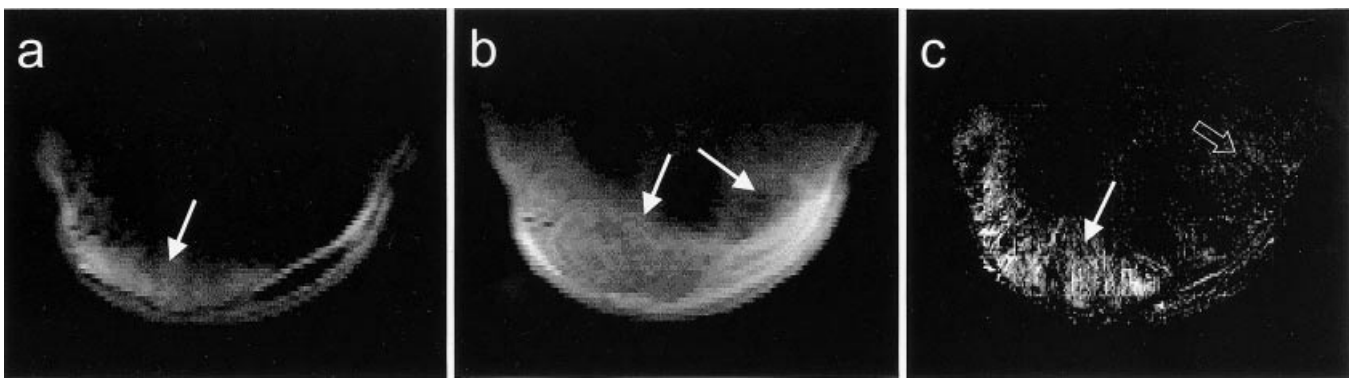

Figure 4. Comparison of Gd-DTPA uptake with carbogen induced T_2* changes for a prolactinoma. (a) and (b) are inversion recovery T_1 weighted images with the inversion time chosen to null pre-contrast tumor tissue and taken respectively at 0.3 and 8 min after administration of a Gd-DTPA bolus. (c) Difference image, T_2* (carbogen breathing) − T_2* (air breathing) displayed with 0–50 ms grey-scale. The magnitude of the T_2* increase is most likely related to vascular density, being lower for the open arrow than the solid arrow, with the central non-responding core being necrotic. The initial (at 0.3 min) rapid contrast enhancement correlates with the largest T_2* changes [solid arrows in (a) and (c)] and so indicates well perfused tissue. Late (at 8 min) enhancement could be either low perfusion or permeability [solid arrows in (b)]

T_2* increase observed with carbogen breathing for the same tumour. The regions which showed the most rapid enhancement by Gd also showed the largest T_2* increase.

DISCUSSION

The following discussion aims to highlight the importance of changes in tumour physiological parameters in the interpretation of FLOOD images following a vascular challenge aimed at altering tumour oxygenation.

In GRE images there can be large contributions to signal intensity from in-flow effects.[18] This is particularly pertinent to our tumour studies because we are investigating agents specifically designed to produce large tumour blood flow changes. For example carbogen and hydralazine both produce changes in the signal intensity of flow-sensitive SE images (Table 2). As demonstrated in Fig. 1, CO_2 can be a powerful vasodilator of tumour blood vessels, thereby increasing the effects of in-flow signal enhancement. In this example, there are large localized signal intensity increases (visible in both SE and GRE images) due to increased blood flow; however the flow component averaged over the whole tumour may be small.[4] To investigate a vasoactive challenge to blood oxygenation via eqn (1), R_2* should be measured using a multi-gradient echo imaging sequence[6,19] which is independent of flow effects. However, it would generally be expected that increased blood flow would also improve tissue oxygenation. So when a vasomodulator like carbogen is used that improves both blood oxygenation and flow, the effects on GRE signal intensity are additive. This effectively increases the sensitivity of FLOOD as an empirical method for detecting regions of tissue where oxygenation may have been improved. For the discussion that follows we use the average changes in tumour ΔR_2*, independent of flow, that we have measured in a number of studies.

By partial differentiation of eqn (1a) using the product rule and assuming k_i is constant, the change in tissue R_2* caused by changes in blood oxygenation and volume following a vasoactive challenge can be written as:

$$\Delta R^*_{2b} = \sum_{i=a,c,v} k_i \cdot [\Delta V_i(1 - Y_i) - V_i \Delta Y_i] \qquad (2)$$

In normal brain, large blood flow increases (~60%) occur which outweigh increases in oxygen utilization (~5% in fMRI) or blood volume (~30% in hypercapnia) and so give the positive BOLD response.[8] The situation in tumours, in contrast, is much less well defined. Ideally we would like the effects of a vasoactive challenge which is intended to improve tumour oxygenation to correlate with a decrease in R_2* (i.e. decreased deoxyhaemoglobin and increased Y_i) and the converse for agents designed to increase hypoxia. From eqn (2) we see that an increase in blood oxygenation in any compartment will decrease R_2* provided there are no volume changes. However, a vasoactive challenge that improves blood flow and so decreases the blood desaturation, is likely to be associated with a blood volume increase. Whether this produces a decrease in R_2* depends on the balance between changes in flow and volume with any change in oxygen utilization, and this balance is generally unknown for tumours.

We first consider the effects of breathing 100% O_2. The arteriovenous oxygenation difference, $Y_a - Y_v$ is related to blood flow (F) and oxygen extraction (oe) via Ficks law

$$oe = F[(Y_a - Y_b) + A(p_aO_2 - p_vO_2)] \qquad (3)$$

For completeness, we have included a term for the change in dissolved oxygen, which is a linear function of the difference in oxygen partial pressure in the arterioles (p_aO_2) and venules (p_vO_2) with a constant factor A. Assuming that oe is also constant, and ignoring the contribution of dissolved oxygen, then partial differentiation of eqn (3) implies that $\Delta Y_a = \Delta Y_v$, which must also equal ΔY_c within the capillaries, and from simple conservation considerations is also equal to the change in arterial blood saturation (ΔY_A). It has been shown in several tumour types that 100% O_2 breathing does not change blood perfusion[20] or cause vasoconstriction,[21] hence we shall start by assuming that ΔF and ΔV are zero for this challenge. With these assumptions the change in R_{2b}* from eqn (2) is simplified to:

$$\Delta R^*_{2b} = -\sum_{i=a,c,v} k_i \cdot V_i \cdot \Delta Y_A \qquad (4)$$

We can go no further with eqn (4) without making assumptions about the relative proportions of the compartments and the vessel sizes, which determine V_i and k_i, respectively. For a first approximation we shall simply assume all vessels are equivalent with a value of 600 for k, which is that calculated by Ogawa[22] for capillaries and corrected for use at a field strength of 4.7 T.[23] Equation (4) then simplifies to

$$\Delta R^*_{2b} = -k \cdot V_b \cdot \Delta Y_A \qquad (4a)$$

where V_b is the total blood volume fraction. We have previously measured the vascular volume to be 67 μl/g in GH3 prolactinomas (in a different cohort of tumours to which the MR data was acquired) using ^{51}Cr-radiolabelled blood cells.[4] Assuming that rat blood is 92% O_2 saturated[24] so that with 100% oxygen breathing ΔY_A is 0.08, and with $V_b \approx 0.07$ we would expect ΔR_{2b}* to be $-3.4\ s^{-1}$, which is within the range of ΔR_2* that we actually measure for 100% O_2 breathing (Table 2). Within the constraints of our assumptions, this suggests that hyperoxia could be used to produce a calculated ΔR_{2b}* image via eqn (4a) that relates qualitatively to the

gross heterogeneity of tumor vascular volume, which can range from zero to greater than 10% of tumour tissue.

We now consider how the ΔR_{2b}* in eqn (4a) is affected by the assumptions we have had to make in its derivation, and how this affects how we interpret the actual ΔR_{2b}* as a change in [dHb], where Δ[dHb] is proportional to $V_b \times \Delta Y$, or as an estimate of V_b. First, the value that we have used for k was originally derived for the average small vessel size in normal brain tissue. Ideally k should be determined for tumour tissue, which because of the structural heterogeneity of tumour vasculature may show both spatial variations and dependence on tumour type. An MRI study of vascular morphology using exogenous contrast agents has shown that the average micro-vessel diameter in a rat brain tumour is about a factor of two larger than for grey matter.[25] For vessels greater than about 10 μm the R_2* contribution of deoxygenated blood is fairly independent of vessel size[1] (for constant volume fraction), but greater than for smaller capillaries, hence ΔR_{2b}* will be increased (and Δ[dHb] or V_b over-estimated) in voxels with substantial contributions from arterioles and venules. The value of k in eqn (1) is also proportional to the fractional haematocrit, which is known to decrease with vessel size in both normal brain[26] and tumours.[27] We have also assumed that *oe* is constant; if this were not so and the extra oxygen were totally utilised then ΔY_v would be zero and ΔR_{2b}* will be reduced by approximately 50% if all vascular compartments had equal volumes. Hence, our V_b is underestimated and despite improved oxygen delivery to the tissue, our estimate for the apparent Δ[dHb] is low. Conversely, if *oe* is constant, but we now take into account the extra dissolved oxygen, the venular blood will be less desaturated than we have assumed. (*Note*: for rat blood which has 12.7 g/100 ml of haemoglobin, hyperoxia that produces a p_aO_2 increase from 100 to 400 mmHg, provides an extra 1.4 ml/100 ml of oxygen bound to haemoglobin and an extra 1.2 ml/100 ml of dissolved oxygen.) Hence if we were to use eqn (4a) to determine V_b on the assumption that $\Delta Y_i = \Delta Y_A$ then V_b would be underestimated.

The main problem with using hyperoxia to determine vascularity, is of course its low sensitivity, which is proportional to the vascular volume and haematocrit, all of which can be inherently low in tumours. In humans $Y_a = 0.98$, and the maximum blood saturation change is $\Delta Y_a = 0.02$, hence the sensitivity is a factor of 4 lower than for rats. Nevertheless, the changes observed in Fig. 3, show that for some tumour models the T_2* changes are large enough to enable calculation of a ΔR_{2b}* map that may provide useful information on vascular heterogeneity. Furthermore, this technique uses endogenous deoxyhaemoglobin as a contrast agent, hence it gives a map of the density of actively perfused vasculature, in which non-responding regions can be interpreted as being those with the lowest vascular density or necrotic.

In laboratory experiments CGRP and hydralazine can be used to reduce mean arterial blood pressure (MABP) by typically 50%, hence reducing tumour blood-flow by the 'steal effect'.[13] The factor of 2 decrease in βNTP/Pi ratio (Table 2) confirms that there is reduced substrate delivery and the increase in R_{2b}* suggests increased blood desaturation (i.e. increased deoxyhaemoglobin levels) as a consequence of reduced blood flow. If we use our average V_b and ΔR_{2b}* for these tumours, and assume k as before, the average decrease in tumour blood saturation determined from eqn (4a) is 0.27 for CGRP. From this we can deduce no more than that during air breathing the average tumour Y was greater than 0.27, and this is not an unreasonable value compared to actual peri-arteriolar pO_2 measurements (typically 50 mmHg which is equivalent to a saturation of 0.45) in tumour models.[21] However, since we generally do not know the tumor blood volume *a priori*, any quantitative measure of a change in R_{2b}* can only be related to the change in total tissue deoxyhaemoglobin concentration ($V_b \times \Delta Y$). If tumour blood volume was constant following administration of a hypotensive agent, eqn (2) indicates that tumours with the largest blood volumes would most likely show the largest R_{2b}* changes. However, with changes in systemic MABP such as that induced by hypotensive agents like CGRP and hydralazine comes the possibility that, in addition to reduced blood flow, tumour blood volume may also decrease. Studies on experimental tumours have shown that following hydralazine administration, which reduces MABP, there can be a reduction in tumor blood volume.[28] It was proposed that because many tumor blood vessels have weak walls,[27] they collapse if the MABP is reduced, because of high interstitial fluid pressure in the tumor tissue.[28] We believe that we see an extreme example of the effects of blood volume changes due to vascular collapse in the R_{2b}* maps of our MNU-induced mammary tumour. R_{2b}* maps are shown in Fig. 3 for this tumour type following 100% O_2 breathing and then for 100% N_2 breathing by the host to post mortem. Subsequent to anoxia, these mammary tumours showed a heterogeneous increase in T_2* (decreased R_2*) post-mortem which corresponded, spatially, to the T_2* increase observed during hyperoxia. Since post-mortem, blood must become fully deoxygenated, we interpret the image changes as follows. The regions with high vascular density which are delineated by a large T_2* increase during hyperoxia because of increased blood oxygenation, are those that exhibit a large T_2* increase post mortem due to a greatly reduced tissue deoxyhaemoglobin concentration because of vascular collapse. It would be expected that the extent of vascular collapse will depend on the patency of the tumour blood vessels and the interstitial fluid pressure and so will be tumour dependent. Hence similar, but less pronounced, effects might well be occuring in the anoxia experiments on our other tumour types. This suggests caution in deducing the extent of tumour oxygenation

changes from R_2* changes following administration of hypotensive drugs. Although tumour hypoxia may be increased by the steal effect, reduced MABP may produce vascular collapse and reduce blood volume, thereby masking the effects of increased blood deoxygenation

Challenges that aim to improve tumour blood flow are carbogen breathing and nicotinamide. The CO_2 component of carbogen is a potent vasodilator and increases blood volume by as much as 50% in GH3 prolactinomas.[14] Suggested actions of nicotinamide on tumour blood vessels have been inhibition of arterial vasoconstriction[29] or decreased tumour interstitial fluid pressure,[30] both of which could cause improved blood flow. Without knowing the actual blood flow change for these challenges, or the resulting changes in the venous and capillary blood oxygen saturation, it is not possible to quantitatively relate the R_2* changes directly to blood saturation by eqn (2). Nevertheless, for the GH3 prolactinoma we know from other measurements that carbogen breathing increases blood volume, blood flow and arterial blood saturation (Table 2). Hence, where we observe a decrease in R_2* we can infer from eqn (2) that this must be associated with increased blood oxygenation with the size of the R_2* increase being modulated by the blood volume (i.e. highly vascularised regions showing the greatest ΔR_{2b}*). If there are regions of tumour for which we observe no change, or even an increase, in R_2*, we cannot make an unambiguous interpretation. Unlike brain, where arteriolar blood is assumed fully saturated with oxygen (i.e. $Y_a \sim 1$), tumour vasculature is often derived from host vessels which already contain partially desaturated blood so $Y_a < 1$. In addition, tumours are frequently hypoxic. An increase in blood flow may not decrease the blood desaturation if the fraction of oxygen extraction increases. Also, if vasodilation of arterioles containing partially desaturated blood occurs the tissue deoxyhaemoglobin concentration can increase. Under both these circumstances there is improved oxygen (and glucose) delivery and elevated tumour oxygenation, but with an R_2* increase, which is the opposite of the correlation we would ideally like. Unlike in the GH3 prolactinoma tumour model, there are other tumour types in which carbogen breathing produces vasoconstriction and reduced tumor blood flow.[31] The response to hypercapnia appears to be very dependent on tumour type and site of implantation, and is likely to be a balance between the local effects of CO_2 on the vascular endothelium and that mediated by the sympathetic nervous system.[32] When vasoconstriction occurs, interpretation of R_2* changes is again confounded, because reduced blood volume masks the effect of increased blood deoxygenation.

Another possible confounding factor in correlating the ΔR_{2b}* due to changes in blood oxygen saturation with tissue oxygenation is a change in blood pH. We have observed changes in pH_A of -0.15 during carbogen breathing (Table 2). This acidification causes a right shift of the oxygen saturation curve due to the Bohr effect, and so results in more O_2 being released from haemoglobin. Using the Hill equation ($Y = P^n/[P_{50}{}^n + P^n]$) and literature values for the blood P_{50}[24,33] we can calculate the change in deoxyhaemoglobin levels. For a pH shift of -0.15, and at a blood partial pressure of 30 mmHg, the deoxyhaemoglobin level will increase from 67 to 79% for rat ($P_{50} = 38$ mmHg and pH 7.4) and from 38 to 52% for man ($P_{50} = 25$ mmHg at pH 7.4). These fractional increases in deoxyhaemoglobin could, in principle, produce an R_{2b}* increase as a consequence of increased delivery of oxygen to the tissue. However, extracellular pH values of tumours are generally acidic,[34] lying between pH 6 and 7; hence, despite arterial blood pH changes, capillary and venular blood pH may change very little.

A consequence of carbogen breathing and nicotinamide administration, was the two-fold increase in blood glucose (Table 2). Tumours have mixed oxidative and glycolytic metabolism, and may preferentially favour one or other of these according to substrate supply. Both carbogen and nicotinamide increase the βNTP/P_i ratio by a similar factor, indicative of improved substrate delivery and metabolic status. If hyperglycaemia causes a switch to a more glycolytic metabolism (the Crabtree effect) then there is the possibility of an R_{2b}* decrease due to sparing of tissue O_2.[35] Conversely, the additional glucose may stimulate oxidative metabolism.[36] Metabolic changes such as these are an unpredictable influence on blood saturation and R_{2b}* [eqns (2) and (3)], but nevertheless need to be assessed.

Although there are many issues to be resolved to relate ΔR_{2b}* first to blood oxygenation changes and thence to oxygenation of the tumor tissue, Fig. 4 illustrates a potential advantage of using deoxyhaemoglobin as an endogenous contrast agent in a tumour model, when the physiological effects of a vasoactive challenge are known. A carbogen-induced T_2* increase is shown to correlate with rapid image enhancement following a bolus of Gd-DTPA [Fig. 4(a) and (c)]. The large carbogen-induced change in T_2* [solid arrow Fig. 4(c)] occurs in well perfused tissue, since it arises from changes in deoxyhaemoglobin concentration, and a small T_2* increase most likely indicates a low capillary density [open arrow on Fig. 4(c)]. The interpretation of Gd-DTPA enhancement, however, can be ambiguous since it depends on several parameters: the product of blood vessel permeability and surface area, and on blood flow. Slow enhancement [arrows on Fig. 4(b)] can therefore result if blood vessels have low permeability, as opposed to being low in density or having poor flow. Rapid enhancement, however, requires high flow and reasonably dense and permeable vessels, and the correlation we observe implies that the carbogen-induced T_2* change is largest in regions satisfying these conditions. Hence contrast enhancement using endogenous deoxyhaemo-

Table 3. Possible physiological changes in experimental tumours after a vascular challenge and potential confounding effects on R_2* BOLD imaging

Challenge	Physiological change	Expected tumour ΔR_2*	Confounding physiological effect	Effect on BOLD imaging
Hypotensive agents (CGRP, hydralazine)	MABP reduction and reduced blood flow	Increase	Possibility of vascular collapse and reduced blood volume	ΔR_2* less than expected or decreases
Hyperoxia	Increased arterial blood saturation	Decrease	Vasoconstriction of some blood vessel types (e.g. skin derived)	ΔR_2* decreases from blood volume reduction rather than improved oxygenation
Hypercapnia	Increased blood flow	Decrease	Vasodilation of vessels carrying unsaturated blood	Increased ΔR_2* due to increase of total tissue deoxyhaemoglobin
			Blood pH reduced	Increased ΔR_2* from tissue deoxyhaemoglobin increase due to Bohr effect
Nicotinamide	Reduction of transient hypoxia	Decrease	Increased blood glucose	Stimulus of oxidative or glycolytic metabolism changes blood oxygen extraction fraction

globin may provide more accurate information on the spatial distribution of well-perfused tissue, without the need for exogenous blood pool agents.

CONCLUSIONS

A summary of the potential problems of interpretation of FLOOD experiments in tumors is given in Table 3. We conclude that in many instances ΔR_{2b}* in tumours correlates with the expected changes in blood oxygenation following a vascular challenge, but this is not a simple linear correlation and there is a 'grey region' where ΔR_{2b}* is smaller, or even the reverse, of what is expected. In the case of challenges that improve blood oxygenation this can occur when increased blood flow and oxygen delivery do not outweigh the effects of increased blood volume of partially deoxygenated blood, or of altered metabolism. In the case of agents designed to reduce blood flow by reducing MABP, there is the possibility of vascular collapse that could reduce blood volume. If we are to more precisely assign changes in R_2* to changes in blood (and hence tissue) oxygenation, our model must include knowledge of changes in physiological parameters such as blood pH, MABP, and blood flow and volume. We must also consider carefully how both glucose and oxygen are utilised by the tumor. Information obtained using ^{31}P MRS[2,5] and MR-compatible techniques such as near infra-red spectroscopy,[37] and tissue pO_2 and perfusion measurements[38] will thus be invaluable.

Despite these caveats, under specific circumstances where the tissue pathology and physiology are controlled and understood, BOLD contrast is capable of providing information on tumour vascularity and oxygenation non-invasively. The sensitivity of R_{2b}* to blood vessel density has been used to monitor angiogenic activity and blood vessel maturation.[39] Microelectrode measurements have shown correlations between changes in tissue pO_2 and [dHb]-sensitive spectroscopic imaging[40] and MR imaging has also been shown to correctly predict the relative effects of radiosensitisers on tumor hypoxic fraction.[41] We have recently observed a correlation between the carbogen-induced ΔR_{2b}* as measured pre-treatment, with the reduction in tumour size following radiation treatment.[42] In conclusion, although there are many issues still to be resolved before FLOOD can be a general tool to investigate tumor oxygenation, changes in tumour R_{2b}* following a vascular challenge may well provide both diagnostic and prognostic information for studies in both experimental and clinical settings

Acknowledgement

This work was supported by the Cancer Research Campaign grant no. SP1971/0402.

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