Hexafluorobenzene (HFB) and perfluoro-15-crown-5-ether (15C5) were compared as fluorine reporter probes of tissue oxygenation using $^{19}$F MRI for dynamic assessment of muscle oxygenation, with special focus on muscle tissue toxicity of the probes, and consecutive alteration of animal behavior. The latter were also compared in terms of sensitivity to changes in oxygenation as well as of signal-to-noise ratio for accurate pO$_2$ measurements. For that purpose, mouse muscles were imaged at 11.7 T, at 2- and 36-h after intramuscular injection of HFB or 15C5. Histological analysis of the muscle tissue revealed a lack of toxicity for 15C5 from 2 up to 36-h postinjection, whereas HFB induced tissue necrosis, blood clots and thrombosis as soon as 24-h postinjection. This muscle toxicity led to a limitation in mice mobility 24-h after injection of HFB as evidenced by behavioral testing (open-field, grip strength, and catwalk tests), which was not the case after 15C5 intramuscular injection. Finally, pO$_2$ measurements assessed 2-h postinjection showed consistent values with both probes, evidencing cross-validation of the $^{19}$F MRI oxymetry technique for acute measurements. However, the measurement at 36-h was hampered for HFB, which showed significant lower values of muscle pO$_2$, whereas 15C5 was able to reliably assess muscle pO$_2$ at 36-h postinjection. Magn Reson Med 69:248–254, 2013. © 2012 Wiley Periodicals, Inc.

**Key words:** $^{19}$F-MRI; oxygenation; HFB; 15C5

Hypoxia (deprivation of adequate oxygen supply) can have serious consequences on vascular disease, specific pulmonary disease, kidney disease, cardiovascular disease, stroke, and cancer, among others (1). In each case, lack of oxygen is associated with poor prognosis, and efforts are made to improve tissue oxygenation. Regarding cancer therapy, the efficacy of many therapeutic approaches to tumor treatment is known to be modulated by oxygen tension (2). Moreover, hypoxia is also associated with local recurrence or distant metastases in a variety of tumors (2). There is therefore a critical need to develop dynamic methods for direct oxygen mapping (2). The ideal technique would assess hypoxia in vivo, be minimally invasive and take into consideration the temporal and spatial heterogeneity of oxygenation.

A variety of oximetry techniques are proposed to assess hypoxia, which can be broadly categorized into direct and indirect measurements according to different principles and the ability to quantify tissue oxygenation (2). Indirect methods include blood oxygen level-dependent imaging and recent $^3$H $T_1$-based magnetic resonance imaging (MRI) methods, positron emission tomography tracers retained in hypoxic regions, and near infrared spectroscopy (2). Methods to measure absolute pO$_2$ encompass invasive polarographic oxygen electrodes (Eppendorf system) and fluorescence quenching fiberoptic probes (OxyLite™), as well as electron paramagnetic resonance oximetry and $^{19}$F relaxometry (2).

Fluorine MR uses perfluorocarbon (PFC) compounds to evaluate interstitial oxygen tension. Indeed, molecular oxygen dissolves in PFC and proportionately increases the relaxation rates of PFC (3). The technique allows $^{19}$F MRI monitoring of the tissue oxygenation state and oxygen distribution in an organ (4). As there is a negligible endogenous $^{19}$F MRI signal from the body, the lack of background signal provides $^{19}$F MRI with a potentially extremely high contrast-to-noise ratio and specificity (5). Fluorine MRI, applied in the preclinical setting, is minimally invasive provided that PFC is not too toxic to surrounding tissues (6). Different PFCs have been used for in vivo oxygenation studies, including perfluoruron (7), hexafluorobenzene (HFB) (8,9), oxypherol (10,11), and perfluoro-15-crown-5-ether (15C5) (12,13), among others. PFCs can be administered intravenously or directly by intratumoral injection of PFC droplets or emulsions (3). The group led by R. Mason initially developed the FREDOM fluorocarbon relaxometry using echo planar imaging for dynamic oxygen mapping technique (9), which has been applied to examine the effects of vasoactive agents (14), vascular targeting agents (15), as well as hyperoxic gases (16), and correlated with invasive direct in vivo or ex vivo methods (3). Our group also used intratumoral HFB for short-term (<2-h) dynamic monitoring of tumor oxygenation following hyperoxic challenges or spontaneous fluctuations in...
tumor models, using a Look Locker approach in order to achieve a better temporal resolution than FREDOM (17,18).

Even though PFCs are described as chemically inert and stable compounds (19), our group observed long-term tissue toxicity effects using HFB, which hampers its use for chronic studies. The molecule 15C5, with 20 fluorine atoms, presents a high signal-to-noise ratio and has been applied in chronic studies (12). The aim of the current study was therefore to compare HFB with 15C5 in terms of ability to assess physiological pO2 in muscle, as well as in terms of potential muscle tissue toxicity and alteration of mouse behavior.

METHODS

Animals and Products

Male NMRI mice (Elevage Janvier, France) were used for MRI experiments and PFC injections. Animals were anesthetized by inhalation of isoflurane (induction: 3%; maintenance 1.5%) delivered by a nose cone. HFB (Fluka, Sigma-Aldrich, United States) and 15C5 (Fluorochem, United Kingdom) were injected into the muscle of the right hind paw using a syringe with a 29G needle and deposited along three tracks (3 × 30 μL) encompassing both central and peripheral regions.

Histology

HFB or 15C5 was injected into the muscle of the right hind paw of 10 mice. After 0.5, 2, 4, 24, and 36 h, one mouse with HFB and one with 15C5 were sacrificed. The intact muscle tissue was dissected and stored in 4% formalin. The tissue was paraffin-embedded and sectioned into 5 μm sections encompassing the muscle injection site. Alternate sections were stained with hematoxylin and eosin and imaged using a microscope.

Behavioral Testing

All the behavioral tests were performed 24-h after intramuscular injection of 15C5 or HFB.

Open-Field Test [15C5 (n = 6) and HFB (n = 5)]

The open-field test was used to assess a nonforced ambulation as mice can move freely without any influence of the examiner. Briefly, mice were placed in a square arena (60 × 60 cm2) and video tracked (Ethovision 6.1, Noldus; Wageningen, The Netherlands) for 20 min (20). The total distance covered by the animals, the proportion of time during which each animal moved and the average speed of movement were measured.

Grip Strength Test [15C5 (n = 6) and HFB (n = 5)]

The grip strength test was used to measure the strength of combined fore limb-hindlimb muscles. Limb strength was recorded using a grid connected to a sensor (Panlab-Bioseb, Vitrolle, France). Mice were gently laid on the top of the grid so that both their front paws and hind paws could grip the grid. They were then pulled back steadily until the grip was released down the complete length of the grid (20). Two series of three tests were performed at an interval of 15 min. Results are presented as the mean of the two highest values of force recorded, related to body weight.

Catwalk test [15C5 (n = 6) and HFB (n = 4)]

The Catwalk test was used to assess the gait of voluntarily walking mice (Catwalk 7, Noldus; Wageningen, The Netherlands) (21). Paw print recording allowed analysis of various aspects of walking steps such as the basis of support, the pressure of the paws on the floor, the length of contact and the sequence regularity of steps (22). Five runs were performed per animal and analysis was performed on the fastest uninterrupted run.

The mice were weighed daily over a 4-day period after the PFC injections (day 1 corresponding to 24-h postinjection of the probes).

19F MRI Acquisitions

MRI was performed with an 11.7 T, 16 cm inner diameter bore system (Bruker Biospec, Ettlingen, Germany) equipped for respiration and temperature monitoring. Mouse temperature was maintained at 37°C ± 0.5°C by using a water blanket connected to a circulating water bath. A tunable 1H/19F surface coil was used for radiofrequency transmission and reception. Adjustments (shimming, geometry) were performed at the proton frequency (500 MHz), and then scans were acquired using the fluorine frequency (470 MHz).

MRI scout images were obtained for both 1H and 19F to reveal HFB or 15C5 distribution within the muscle. A polygonal region of interest (ROI) in muscle was defined and a nonlinear fit was used to determine the T1 relaxation in each pixel of the ROI. We then used a segmented FISP (Fast Imaging with Steady state Precession) sequence in FID mode to acquire 19F images of T1 relaxation times of HFB and 15C5 distributions. The acquisition parameters were TR/TE/FA/BW/NA/matrix = 4 ms/1.2 ms/5/100 kHz/2/64 × 64, four segments, and a total acquisition time of 1 min 36 s. In order to sample the recovery of the signal, a series of 100 images are acquired between 30 and 9930 ms (TR = 100 ms between segments) with a slice thickness of 10 mm. Images were then treated using a homemade program written in Matlab to determine the T1 relaxation (in ms) in regions of interest and convert it into pO2 values.

Calibration of HFB (R1 with respect to pO2) was performed at 11.7 T and at 37°C ± 0.5°C by measuring R1 in sealed tubes (n = 2) containing 300 μL HFB consecutively bubbled with nitrogen (0% O2 = 0 mmHg), air (21% O2 = 160 mmHg), and carbogen (95% O2 = 722 mmHg) for 20 min in a 37°C ± 0.5°C water bath before measurement.

For 15C5, a 10% w/v PFC emulsion with a pH of 7.22 and an isotonicity of 300 mOsm/kg was used for calibration (23). The composition of the emulsion was 10% PFC, 3% w/v purified egg lecithin, 1.8% glycerin and 18.7 mM phosphate buffer. Calibration measurements (n = 4) were then performed similarly to the procedure described for HFB.
A multiple linear least-squares regression was used to find the dependence of $R_1$ on dissolved oxygen for both HFB and 15C5. The correlation between $T_1$ (s) and $pO_2$ (mmHg) was used to obtain a map of the partial oxygen pressure for each voxel. The physiologically relevant data from 0 to 160 mmHg were used in our analysis.

**Statistical Data Analysis**

Results are presented as means ± standard deviation and paired $t$-tests were conducted on these results, with $P < 0.05$ being considered as significant. Two-way ANOVA with Bonferroni post hoc test was used to compare the weight loss over time between HFB and 15C5 mice.

**RESULTS**

**Effect of HFB and 15C5 on Muscle Tissue Histology**

Microscopic effects of HFB and 15C5 were compared at 4-, 24-, and 36-h after intramuscular injection in the right hind paw (Fig. 1). At 2-h after injection of 15C5 or HFB, the muscle tissue was not modified, with only small inflammatory infiltrates near the injection sites. We observed rapid cellular regeneration in muscle as soon as 4-h after HFB injection, which was not the case for 15C5 (Fig. 1a,d). At 24- and 36-h after HFB injection, toxic necrosis in muscular tissue was present, along with blood clots in vessels leading to thrombosis (Fig. 1e,f).

After 15C5 injection, the muscular tissue had no modified morphology at any time point, with only some inflammatory infiltrates (Fig. 1b,c).

**Effect of HFB and 15C5 on Mice Behavior**

General activity, measured in the open-field test, was systematically higher for mice injected with 15C5 than for mice injected with HFB. The total distance covered by mice injected with 15C5 was longer (9634 ± 1492 cm) than for mice injected with HFB (2356 ± 1592 cm; $P < 0.0001$). The 15C5 mice were also more often in movement (71 ± 5 % vs. 21 ± 16% of the total time, $P < 0.0001$; Fig. 2a) and moved faster (8 ± 1 cm/s) than mice injected with HFB (2 ± 1 cm/s, $P < 0.0001$; Fig. 2a).

For the grip test, a sensitive gauge recorded the peak force applied by each animal. The force developed by mice injected with 15C5 was higher with 15C5 (7.5 ± 0.6) than for mice injected with HFB (6 ± 0.9, $P < 0.01$; Fig. 2b).

On the catwalk test, we observed a higher speed of locomotion crossing the runway for mice injected with 15C5 (34 ± 4 cm/s) than for mice injected with HFB (20 ± 2 cm/s, $P < 0.0001$; Fig. 2c). The duration of contact of the injected paw with the ground was also significantly higher with 15C5 (72.5 ± 3.9 % vs. 26.4 ± 11.6 %, $P < 0.0001$; Fig. 2c) and the area of contact with the ground was larger with this product (31.3 ± 7.6 mm² versus 1.1 ± 0.8 mm², $P < 0.0001$; Fig. 2c).
Finally, mice's weight was recorded daily over a 4-day period after injection, all mice having the same origin and age. With HFB, we observed a decrease in the mice's weight, whereas the weight of 15C5 mice was stable after injections (Fig. 2d; $P < 0.001$ at day 2, 3, 4 while comparing HFB and 15C5 groups; two-way ANOVA).

**Ability of HFB and 15C5 to Assess Muscle Oxygenation with $^{19}$F-MRI**

**PFC Calibrations**

The effective $p_O^2$ was determined by measuring the $^{19}$F $T_1$ of 15C5 emulsion or HFB solution versus dissolved oxygen concentration for three different oxygen tensions. Figure 3 shows the calibration curves measured at 11.7 T and 37°C ± 0.5°C for HFB and 15C5, respectively. Using linear regression analyses, we obtained the relationship between $R_1 (= 1/T_1)$ and $p_O^2$ given by: $[R_1 = 0.00303 (p_O^2) + 0.63639]$ for HFB and $[R_1 = 0.0063 (p_O^2) + 1.7447]$ for 15C5, where $R_1$ and $p_O^2$ have units of s$^{-1}$ and mmHg.

**Sensitivity Index and Signal-to-Noise Ratio**

The calculated sensitivity indexes (representing the sensitivity of each probe to changes in oxygenation), defined as $b/a (=\text{slope/intercept})$ in an $R = ax + b$ calibration curve (3), are 0.00476 for HFB and 0.00361 for 15C5, at 11.7 T, 15C5 (25% less sensitive). However, thanks to its higher number of fluorine atoms and its higher density (15C5 vs. HFB: 20 vs. six fluorine atoms and 1.78 vs. 1.61 g/mL), 15C5 presented a S/N ratio of ~40 for 15C5 (at 2- and 36-h postinjection), whereas HFB showed a S/N ratio of ~20 at 2-h postinjection and of ~6 at 36-h postinjection, illustrating the loss of signal over time (Fig. 4). Overall, taking into account the slightly lower sensitivity of 15C5 and its higher (double) S/N ratio, our analysis resulted in a mean of 40 pixels analyzed (being correctly fitted with a $T_1$ error/$T_1 < 30\%$) in muscles at 2-h postinjection of 15C5 ($n = 4\text{/group}$) vs. 27 pixels for HFB ($n = 4\text{/group}$). At 36-h, the number of pixels analyzed was significantly reduced for HFB but not for 15C5 (91 pixels analyzed for 15C5 and 13 pixels for HFB at 36-h).

**Measurement of Muscle Oxygenation Using HFB and 15C5**

HFB or 15C5 were injected in mouse muscle ($n = 4$ for HFB and 15C5). Muscles were imaged using $^{19}$F MRI at 2- and 36-h postinjection. Two hours after injection of PFC, $p_O^2$ was similar in both groups of mice (Fig. 5). At 36-h postinjection, the HFB mice showed a decrease in muscular $p_O^2$ ($P < 0.05$; Fig. 5), whereas the 15C5 mice were stable and did not show any significant change in $p_O^2$ between 2- and 36-h. The apparent decrease in muscle $p_O^2$ using HFB could be due to the modification of muscle tissue histology as well as to the selective retention of the probe in less well perfused regions (which are therefore also more hypoxic), while being washed out from well perfused regions.
DISCUSSION

A method for directly monitoring the heterogeneous distribution of partial oxygen pressure is the linear increase of the longitudinal relaxation rate ($1/T_1$) of PFCs with increasing $pO_2$ (3). Vascular delivery of PFC has been shown to favor delivery to well perfused and hence well oxygenated regions (10). Thus the most effective approach that has been suggested to achieve representative interrogation of a tumor is direct intratumoral delivery of the reporter molecules (3). The aim of the current study was to compare the ability of two PFCs, namely HFB and 15C5, to assess muscle tissue oxygenation and to compare their effects in terms of tissue toxicity, sensitivity, and signal-to-noise ratio.

The two PFCs used in this study are highly sensitive to $pO_2$, present a high signal-to-noise ratio, a single narrow spectral peak, and a linear relationship across the entire range of oxygenation. Since 15C5 is more sensitive to temperature (3.0 mmHg/°C) (3) than HFB (0.1 mmHg/°C) (3), it is critical to closely monitor animal temperature as well as in vitro samples to generate calibration data. In addition, the sensitivity of 15C5 is 25% lower than HFB at 11.7 T. However, this effect was more important at lower magnetic fields, as described by others: at 4.7 T, HFB presented a sensitivity index of ~0.02 vs. ~0.005 for 15C5 (four times less sensitive; Table 1). An important advantage of 15C5 over HFB, however, is its longer half-life and higher number of fluorine atoms, resulting in a higher signal-to-noise ratio that allows daily measurements without additional injection of the probe, contrary to HFB, which provides less signal 24- or 48-h after injection (as illustrated in Fig. 4). Zhao et al. showed in their study (15) that additional doses of HFB are required for the 24-h follow-up study of $pO_2$ for the control tumor group. For these control tumors, no significant differences in $pO_2$ were found with HFB in any of the measurements (immediately after HFB injection or 24-h after) (15).

It has been reported that HFB exhibits no mutagenicity or teratogenicity (19) and clears from the body rapidly owing to its highly volatile nature (within 24 h). HFB has been used in many species including cats, dogs, rats and mice, among others (3). However, to our knowledge, no study has focused on the tissue toxicity of HFB for “chronic” monitoring of tissue oxygenation (>2-h). At 2-h postinjection of the probes, the muscle tissue was unaltered with 15C5 as well as with HFB. However, the histological analysis of the muscle tissue showed modifications as soon as 4-h after injection of HFB, contrary to 15C5. We show here that injection of HFB in muscle causes a toxic necrosis with thrombosis in vessels 24-h after injection, a toxicity that is not present with 15C5. For this reason, the use of HFB to assess physiological measurement of $pO_2$ in a tissue should be limited to a maximum of 2-h postinjection. Moreover, the half-life of HFB in poorly perfused organs is reported to be ~10-h or less in the more vascularized organs, which makes daily monitoring of $pO_2$ difficult without additional injection of the probe (28), thereby generating even more tissue toxicity and bias in $pO_2$ measurements. We show here that 15C5 is more adapted to chronic experiments since the probe does not induce tissue toxicity (as is the case for a physiologic liquid injection that shows only a small inflammatory reaction at the site of injection). $pO_2$ measurements assessed 2-h postinjection showed consistent values with both probes, evidencing cross-validation of the $^{19}$F MRI oximetry technique for acute measurements. However, the measurement at 36-h was hampered for HFB, which showed significantly lower values of muscle $pO_2$, whereas 15C5 was able to reliably assess muscle $pO_2$ at 36-h postinjection. The apparent decrease in muscle $pO_2$ using HFB is likely to be due to the modification of muscle tissue histology as well as to the selective

FIG. 3. Calibration curves of $R_1$ ($1/T_1$) versus $pO_2$ (mmHg) for HFB (a) and 15C5 (b), at 11.7 T (37°C).

FIG. 4. Evolution of signal-to-noise (S/N) ratio for 15C5 and HFB at 2- and 36-h postinjection of the probes.
retention of the probe in less well perfused regions. In addition, using multiple tests, we observed that the intramuscular injection of HFB was able to influence mice behavior as soon as 24-h after injection. Indeed, mice injected with HFB did not use the injected paw in an optimal way, whereas 15C5 mice presented normal displacement. The weight loss and decrease of exploration behavior of the mice demonstrate the toxicity of HFB 24-h postinjection.

Most of the previously published studies using HFB were ‘acute’ studies with either similar doses of HFB (17) or even lower doses of HFB (8), and showed consistent monitoring of tissue \( pO_2 \). Our \(^{19}\)F MRI oximetry values are in line with the recently published results of Liu et al. (4), who studied the level of oxygenation of different organs and tissues in rats using fluorine relaxometry along with HFB probe. However, these values are higher than those usually obtained with other direct techniques in skeletal muscle in rodents, including electron paramagnetic resonance (29–31), the Eppendorf system (32,33), or OxyLite probes (17). Nevertheless, the use of the technique for individual monitoring or comparison between groups has found broad applications in the preclinical field, and yet remains a unique minimally invasive technique for direct monitoring of tissue oxygenation.

**CONCLUSIONS**

In summary, even though \(^{19}\)F MRI oximetry technique provides a sensitive method for measuring regional organ tissue oxygen tension and dynamic changes, it is important
to use an appropriate probe for chronic measurements. Indeed, the use of HFB as a reporter probe is mostly applicable for acute measurements (<2-h). We did show that 15C5 is better suited to chronic measurements of pO2 with 19F MRI, since it presents a lack of tissue toxicity and impact on animal behavior, and because of its longer half life and signal-to-noise ratio that does not imply repeated injections of the probe. Acute pO2 measurements showed consistent values with both probes, evidencing cross-validation of the 19F MRI oximetry technique for that purpose. However, the measurement at 36-h was hampered for HFB, which showed significant lower values of muscle pO2, whereas 15C5 was able to reliably assess muscle pO2 at 36-h postinjection.

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