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Original Article

An anesthetic method compatible with ^{18}F-FDG-PET studies in mice

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Abstract: The purpose of this study was to establish an experimental setting and an anesthetic method compatible with future sequential studies using ^{18}F-FDG-PET single scans, i.e. autoradiographic measurements, for the estimation of metabolic rate of glucose (MR_{glc}) in mice. In this study we had no access to a small animal PET scanner and therefore focus was on the anesthetic setting and optimization of the input function as a preparation for the future tumor metabolic studies. Initially, four combinations of intraperitoneal (ip) anesthesia were tested on tumor bearing mice. Fentanyl-fluanisone plus diazepam yielded low and stable blood glucose levels and kept the animals sedated for approximately 2 h. The anesthesia was also tested in a longitudinal ^{18}F-FDG study, where tumor bearing mice were anesthetized, injected with ^{18}F-FDG, and sampled for blood, before, one day after, and 8 days after treatment with cisplatin. The animals were in good condition during the entire study period. To validate the method, average MR_{glc} of whole brain and cerebellum in mice were calculated and compared with the literature. The average MR_{glc} in the whole brain and cerebellum were 46.2±4.4 and 39.0±3.1 μmol $100g^{-1}$ min^{-1}. In the present study, we have shown that an ip anesthesia with a combination of fentanyl-fluanisone and diazepam is feasible and provides stable and low blood glucose levels after a fasting period of 4 h in experiments in nude mice with xenografted human tumors. We have also verified that ^{18}F-FDG, intraperitoneally administrated, results in an expected plasma activity uptake and clearance. The method doesn't alter the uptake in brain which is an indirect indication that the anesthesia doesn't alter the uptake in other organs. In combination with meticulous animal handling this set-up is reliable and future sequential tumor studies of early metabolic effects with calculation of MR_{glc} following cytotoxic therapy are made possible.

Keywords: ^{18}F-FDG, metabolic rate of glucose (MR_{glc}), anesthesia, mice

Introduction

The most common radiopharmaceutical used within Positron Emission Tomography (PET) is 2-[^{18}F])fluoro-2deoxy-D-glucose (^{18}F-FDG) where the ^{18}F-FDG -molecule carries one radioactive [^{18}F] in position 2. Since ^{18}F-FDG is a glucose analogue it uses the same transportation into the cell as glucose. Inside the cell, ^{18}F-FDG is metabolized only in one step to 2-^{18}FDG-6-PO_4 that remains trapped inside the cell. Inside the cell, the concentration of radioactive metabolite grows with time in proportion to the cell's uptake of glucose.

PET-^{18}F-FDG is an established method for evaluation of metabolic response following cytotoxic therapy, and early metabolic response during chemotherapy in patients with Hodgkins lymphoma. The predictive value of early metabolic response in other tumors has yet to be clinically established in order to introduce sequential PET studies for monitoring cytotoxic treatment [1]. To accomplish this, it is important to gain basic knowledge of metabolic tumor effects of cytotoxic drugs in animal experiments. With well-designed experimental settings, such investigations can be conducted using human xenografts on nude mice.

Previous studies on mice have emphasized the importance of meticulous handling of the animals to achieve optimal conditions for ^{18}F-FDG

measurements. It has been shown that fasting before the PET scan and warming of mice before and after injection of ^{18}F-FDG is crucial [2]. Factors such as dietary state, temperature, and type of anesthesia have been studied and were found to interfere with blood glucose levels and ^{18}F-FDG kinetics [2-5]. Elevated blood glucose levels may interfere with the ^{18}F-FDG uptake in tumor cells, in humans as well as in mice, and may, therefore, considerably impair PET image quality and analysis outcome [6, 7].

Different ways of assessing metabolic tumor activity with ^{18}F-FDG-PET have been proposed and the most commonly used are different types of standardized uptake value (SUV). SUV is a semi-quantitative analysis in which the tumor ^{18}F-FDG concentration is normalized to the amount of the injected activity and body-weight or -area. It is simple to perform but the drawback is the assumption that the shape of the arterial input function of ^{18}F-FDG is universal and only depends on the amount of ^{18}F-FDG injected. Also not all SUV-formulas take blood glucose levels into account. The metabolic rate of glucose (MR_{glc}) is a parameter providing quantitative information about tumor metabolism and, in contrast to SUV, calculation of MR_{glc}, either with nonlinear regression [8] or Patlak analysis [9] are based on measurements of the rate of glucose uptake over time. In small animal imaging, these approaches are extra demanding because of the rapid and frequent blood sampling required; nevertheless, attaining an accurate measurement of the tumor metabolism is a prerequisite for conducting reliable experiments. In this work we aimed to utilize an intraperitoneal injection (ip) of ^{18}F-FDG which results in a slower rise of the input function compared to intravenous injection (iv). This was used in combination with a modified autoradiographic formula, previously described by Rhodes [10] for the estimation of MR_{glc}.

$$MR_{glc} = \frac{C_{gl} \cdot C_i(T)}{LC \cdot \int_0^T C_p(t)\,dt} \quad \textbf{(Equation 1)}$$

The formula is based on a 3-compartment model [11] where the lumped constant (LC) represents the difference in transport and phosphorylation between glucose and ^{18}F-FDG, Cgl is the blood glucose value, Ci is activity in tissue, T is the time point post injection and Cp(t) is the plasma ^{18}F-FDG concentration as a function of time.

Equation 1 only requires one PET-scan at approximately 60 min after the injection of ^{18}F-FDG. It assumes that all radioactivity in the tissue is composed solely of 2-^{18}FDG-6-PO_4, no non-phosphorylated ^{18}F-FDG and that the dephosphorylation of 2-^{18}FDG-6-PO_4 is negligible. A similar equation, which corrects for the free non-phosphorylated ^{18}F-FDG has also been published by Brooks [12]. A prerequisite for using both calculations is stable blood glucose levels during the acquisition time and therefore we aimed to find an ip anesthesia which kept the animals sedated with a stable and a low blood glucose level.

The purpose of this study was to: 1). establish an easy and robust ip anesthesia resulting in low and stable blood glucose levels useful for ^{18}F-FDG-PET single scans, i.e. autoradiographic measurements, for the estimation of metabolic rate of glucose (MR_{glc}) in mice. 2). optimize the experimental settings for future sequential ^{18}F-FDG studies by looking at blood sampling frequency and the well-being of animals exposed to the chosen anesthesia. 3). validate the method by determining cerebral MR_{glc} values in mice exposed to the experimental setting and compare them to previously reported measurements.

In this study we had no access to a small animal PET scanner and therefore focus was on the anesthetic setting and optimization of the input function as a preparation for future tumor metabolic studies.

Material and methods

Animal handling was approved by, and performed in accordance with, the recommendations of the regional ethics committee. The animals were kept in a calm environment and handled by an experienced animal care taker. In-house bred, athymic nude (BALB/c nu/nu) mice of both genders were used in the study. Prior to the experiment, the mice were given food and water *ad libitum*. On experimental days, the lights were turned on 4 hours earlier than normal to end the active dark period (12 h), and a 4-hour period of fasting started. The mice were weighed and put in new boxes without food, but with free access to water. After the fasting period and prior to anesthesia, the weight was measured again and baseline values for rectal temperature and blood glucose

were recorded (Glucose meter™ from Bayer Contour, Leverkusen, Germany).

Anesthesia and blood glucose levels

Six to eight weeks old mice, weighing 21.7-31.5 g, were inoculated subcutaneously in one flank as previously described [13]. To find an anesthesia yielding a stable and a low blood glucose level while keeping the animals sedated for a sufficient period of time, four different anesthetic combinations were tested with ip administration on tumor bearing animals: a). Fentanyl, 0.5 μg/g body weight, and midazolam, 10 μg/g body weight, n=10. b). Fentanyl, 0.5 μg/g body weight, and diazepam, 50 μg/g body weight, n=9. c). Haloperidol, 10 μg/g body weight, and diazepam, 50 μg/g body weight, n=10. d). Fentanylcitrate-fluanisone, 0.25 and 0.8 μg/g body weight respectively, and diazepam, 50 μg/g body weight, n=10.

Fentanyl: (Fentanyl®, B Braun, Melsungen, Germany); Midazolam: (Midazolam®, Actavis, Hafnafjörður, Iceland); Diazepam: (Stesolid novum®, Actavis, Hafnafjörður, Iceland); Haloperidol: (Haldol®, Janssen-Cilag, Sollentuna, Sweden); Fentanylcitrate-fluanisone: (Hypnorm®, Vetapharma Ltd, Leeds, UK).

During anesthesia, the mice were kept on a heat pad at 38°C. Blood glucose levels were measured 10, 30, 40 and 60 min after induction of anesthesia. Blood samples were collected in 10-μL capillary tubes from the medial canthus of the eye. To determine which combination of anesthetics that resulted in the most stable glucose level (mmol/L), the blood glucose values for every animal were normalized by division with the averaged value from the four measurements (10, 30, 40 and 60 min) and the standard deviation of the blood glucose levels was calculated from the animals within the four groups.

The Fentanylcitrate-fluanisone + diazepam (d) anesthetic yielded a low blood glucose level with the smallest standard deviation and was therefore chosen for further studies.

Blood sampling and sequential ^{18}F-FDG experiment

The ^{18}F-FDG was supplied by the in-house cyclotron unit in Lund which serves the clinical PET facilities in the southern part of Sweden [14]. Animals were injected with 1 MBq of ^{18}F-FDG. Blood glucose and blood radioactivity samples were collected in 10-μL capillary tubes from the medial canthus of the eye. Whole blood ^{18}F-FDG concentration was measured in an automated gamma-well counter (1480 Perkin Elmer, Wizard 3). For the well-being of the animals a minimum of blood was collected for radioactivity measurements. To optimize the number of blood samples the integral of the input function was calculated based on 10 blood samples drawn every 5 min from 10 animals. Due to the slow rise of the input function, related to the ip injection, the number of blood samples could be decreased to only five samples taken every 10 min with the first sample taken 5 min post injection. This still preserved the shape of the activity curve, with only a maximum ± 5% integral change compared to the integral values from 10 samples.

In order to avoid encapsulation of the ^{18}F-FDG in the anesthetic agents, the injection of ^{18}F-FDG was made on the opposite side compared to the anesthetics. The final, optimal, injection site and time protocol is given in (**Figure 1**).

The experimental setting was tested in a sequential ^{18}F-FDG experiment, without PET-scans, where the well-being of nine tumor bearing mice was studied during 10 days. An established cisplatin-sensitive, in-house cell line, LU-HNSCC-7, originating from a human primary, untreated, head-and-neck squamous-cell carcinoma was used as xenograft [15]. Mice, six to eight weeks old, were inoculated subcutaneously in one flank as previously described [13]. The inoculated tumor developed a size of 10-12 mm in approximately 4 weeks. The sequential experiment followed the protocol from **Figure 1** and was repeated three times over 10 days.

Day 1: 30 min after induction with fentanyl-fluanisione and diazepam, ^{18}F-FDG (0.5-1 MBq) was injected ip in tumor bearing mice (n=9). After recovery from anesthesia all mice were given a subcutaneous rehydration dose of 0.5 mL physiological NaCl solution.

Day 2: cisplatin, 5 mg/kg body weight, corresponding to maximal tolerated dose, was injected ip.

Day 3: the procedure from day 1 was repeated.

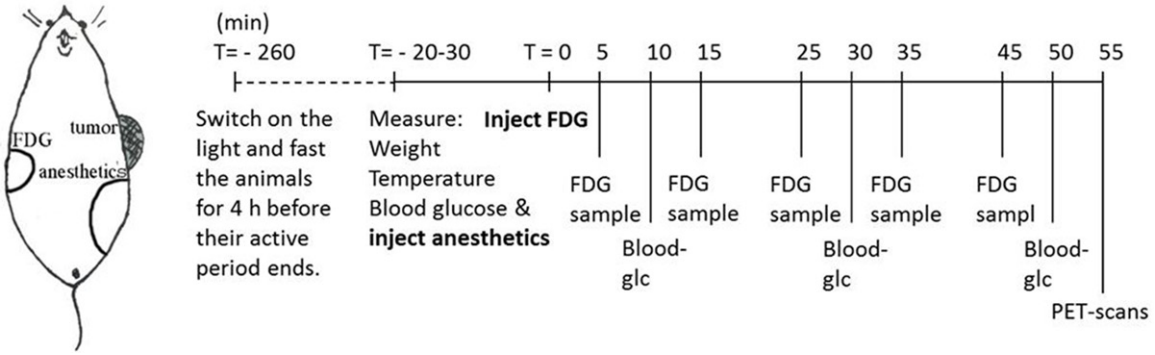

Figure 1. The protocol including time points for start of fasting, injection of anesthetics, and injection of ^{18}F-FDG. Blood glucose and blood activity sampling time points during the experiments are also shown, Glc=glucose.

Day 10: the procedure from day 1 was repeated, after which the mice were sacrificed by cervical dislocation.

Validation of the method with cerebral MR_{glc} estimations

Four mice, 17 weeks old, without tumors, weighing 20-26 g were anesthetized with fentanyl-fluanisone combined with diazepam and injected ip with 5 MBq of ^{18}F-FDG according to the protocol in **Figure 1**. Blood glucose and blood radioactivity samples were collected in 10-µL capillary tubes from the medial canthus of the eye. Whole blood ^{18}F-FDG concentration was measured as described earlier. Evaluation of iv ^{18}F-FDG ratios of plasma to whole blood in mice have been published earlier [16] where **Equation 2** describes the time dependent part of the erythrocytes that are not available for free diffusion of ^{18}F-FDG:

$$A_{plasma} = A_{whole\ blood} \cdot (0.39 \cdot e^{-0.19t} + 1.17)$$ (**Equation 2**).

Due to the ip injections, which results in a slow diffusion of ^{18}F-FDG into the blood stream, the radioactivity blood samples were compensated for erythrocyte uptake and converted to plasma radioactivity by applying only the steady state correction factor of 1.17 from the above exponential equation. At 55 min the animals were sacrificed by cervical dislocation and the brains were removed. Cerebellum was removed from the whole brain and the two were then washed and weighed before being measured in the gamma-well counter. The average from the three blood glucose samples was used for calculation of the average MR_{glc} in the whole brain and cerebellum according to **Equation 1** with LC=0.625 [17]. The input functions were integrated between times of ^{18}F-FDG injection to cervical dislocation. All values were decay corrected to injection time. An earlier report has demonstrated MR_{glc} estimated in occipital and parietal cortex for mice [18]. Due to the difficulties in removing cerebral cortex tissue from the small mouse brains in this experiment the average whole brain MR_{glc} in the mice was translated to occipital and parietal cortex values in mice by using ratios, 68/107 and 68/112 respectively, between average whole brain and occipital and parietal cortex MR_{glc} determined in conscious albino rats [19].

Results

Anesthesia and blood glucose levels

The normalized glucose levels from the anesthetics had standard deviations of: a (σ=0.27), b (σ=0.19), c (σ=0.22) and d (σ=0.12). The absolute average blood glucose levels were: a=5.9±1.6, b=5.4±1.3, c=8.1±2.1 and d= 5.6±0.9 mmol/L. The fentanyl-fluanisone plus diazepam combination (d) resulted in the most stable blood glucose levels with a standard deviation of 0.12 and in an anesthesia adequate for proper sedation with no need for any extra doses of anesthetics during the sedation period (approximately 2 h).

Sequential ^{18}F-FDG experiment

The compatibility of the fentanyl-fluanisone plus diazepam sedation with sequential metabolic tumor studies was tested in a longitudinal study over ten days including three anesthetic

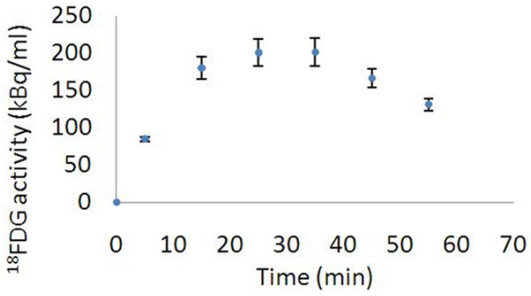

Figure 2. Typical representative time-activity curve from an ip injection of ^{18}F-FDG. Errors from gamma counting statistics (< 3%) were taken as reported by the software. Error in the blood volumes in the capillary tubes was conservatively estimated at 10%. The total uncertainty in the data points in **Figure 2** was calculated by combining these contributing errors in quadrature. The last data point, at 55 min post injection, was extrapolated linearly from the two previous.

cycles with full blood sampling for ^{18}F-FDG measurements and blood glucose levels. A typical time-activity curve is depicted in (**Figure 2**).

During the whole experimental period the mice had good performance status, showing normal behavior and only minor changes in weight. The baseline weight varied between 21.7 and 31.5 g, and there was an average increase of 2.3 g (-1.5 g to 2.8 g) during the study period. Body temperature declined in all animals during anesthesia, by an average of 1.5°C. After the first and second anesthesia, the mean temperatures were 34.9°C and 35.5°C, respectively. Blood glucose levels during the ten days remained within the range 3.3-8.4 mmol/L.

Validation of the method with cerebral MR_{glc} estimations

The mice mean temperature was 36.7°C after 5-10 min after injection of anesthetics and remained stable during the experiment. The average MR_{glc} in whole brain and cerebellum were calculated and whole brain MR_{glc} was transformed to occipital and parietal cortex by using ratios from average whole brain to occipital and parietal cortex in rats [19] (**Table 1**).

Discussion

In the present study, we established a simple and robust experimental set-up based on ip injection of both anesthesia and ^{18}F-FDG for estimation of MR_{glc} in mice. We tested the method's potential for future sequential metabolic tumor studies in xenografted nude mice during cytotoxic therapy. The method was validated by measurements of cerebral MR_{glc} in normal mice.

Based on previous experience, we designed a standard protocol regarding fasting time, light cycles, temperature control, rehydration, anesthetic administration and general animal handling. In this setting, we tested four different types of anesthesia aiming for one that kept the animals sedated for approximately 2 h with minimal interference with the blood glucose levels, the ^{18}F-FDG-uptake, and the well-being of the animals. In this set-up, we starved the mice for no more than 4 h prior to ^{18}F-FDG injection in order to avoid the risk of weakening the animals. In previous studies, different fasting times have been employed. Woo et al [6] kept mice fasting for 20 h and Fueger et al [4] used 8-12 h. The 4-hour period of fasting in this study was, however, sufficient to achieve stable blood glucose levels, while retaining good physical vigour. To avoid hypothermia during the anesthesia, we put the mice on a heat pad set at 38°C, which is above the zone of thermoneutrality described for mice [20]. This resulted in only small fluctuations in body temperature. It has been shown that by warming the fasting animal, a decreased uptake of ^{18}F-FDG in brown fat can be achieved compared to fasting alone [4], but this was not further explored in the study.

Regarding general anesthesia for small animal imaging, several agents and methods may be used [21]. If inhalants are considered, sevoflurane is preferred in order to obtain stable blood glucose levels [5]. Inhalants require complex and expensive equipment and in this study we preferred the feasibility of injectable agents. Intravenous as opposed to ip injection is a matter for discussion. There is a risk of misplacement of the ip injection, even though the animal keeper is experienced, and occasional failures of injections should be taken into account if a predicted effect is missing. The incidence of miss injections are low, and the simplicity of the ip injection makes it preferable to intravenous injection in mouse experiments [22]. However a state-of-the-art MR_{glc} estimation technique requires arterial blood sampling in combination with iv injections [16]. Wong et al [23] have previously shown that an intravenous injection of ^{18}F-FDG gives a sharp peak in plasma activity

Table 1. Comparison of MR_{glc} with literature data

	MR_{glc} (μmol×(100 g)$^{-1}$×min^{-1})		
	This study	[18]	
Region	Fentanyl-fluanisone/diazepam (n=4)	Awake mice (n=6)	Ketamine/xylazine (n=6)
Cerebellum	39.0±3.1	-	-
Whole brain	46.2±4.4	-	-
Occipital cortex	72.7±7.0[1]	73.0±12.4	42.1±10.2
Parietal cortex	76.1±7.3[2]	74.0±13.5	49.3±7.2

[1]Occipital cortex is calculated from whole brain divided with 68/107. [2]Parietal cortex is calculated from whole brain divided with 68/112. Blood plasma activity of ^{18}F-FDG during acquisition showed a slow rise and a broad peak with a similar profile in all mice.

within 10 s while an ip injection produces a lower and broader peak, the latter similar to our findings (**Figure 2**). A benefit with the ip injection is the slower rise of the input function which makes the blood sampling less stressful and less sensitive to the exact timing of the sampling. For iv injections, it is important to have a more frequent sampling interval in the beginning of the time activity curve to "catch" the rapid rise of the blood activity peak.

Irrespective of the use of iv or ip anesthesia, ketamine-xylazine is commonly used in experimental animal models but is known to induce hyperglycemia [24]. In an earlier study it was shown that reduction in uptake ratios and PET image contrast caused by both ketamine-xylazine and pentobarbital anesthesia were due to high blood activity rather than to differences in absolute tumor uptake levels [5]. In the same study it was also shown that the effect is reduced with prolonged fasting. In this work we have not investigated tumor uptake ratios but the result presented in **Table 1** shows that the method doesn't alter the uptake in brain which is an indirect indication that the anesthesia doesn't alter the uptake in other organs. In 1984, Flecknell and Mitchell [25] concluded that fentanyl-fluanisone in combination with midazolam or diazepam, given ip, was preferable if surgical anesthesia and muscle relaxation is desired in different laboratory animals. Complete surgical anesthesia prior to PET scan is not needed, but a sufficient depth of anesthesia to avoid disturbing muscle activity is important.

To validate the method, average whole-brain and -cerebellum MR_{glc} were estimated and translated to the occipital and parietal cortex (**Table 1**). Mouse-MR_{glc} in occipital and parietal cortex brain has previously been reported to approximately 70 μmol×(100 g)$^{-1}$×min^{-1} (in conscious mice) using the 2-^{14}C-DG autoradiographic method [11]. When ketamine/xylazine anesthetics were applied the MR_{glc} decreased to approximately 40 μmol×(100 g)$^{-1}$×min^{-1}. All these results are reasonably consistent with the ones obtained in the present study (**Table 1**). In the present study we used LC=0.62 which is valid for brain. For most tumors the LC-value is still unknown and therefore the correct MR_{glc} in the tumors cannot be determined. However, for a certain tumor-type the LC-value is assumed to be consistent irrespectable of the individual studied. This implies the possibility of conducting both intra- and interindividual comparisons without knowing the LC-value which therefore enables accurate sequential studies focusing on metabolic effects of cytotoxic treatment.

Fluctuation of blood glucose levels is a serious source of error in clinical and experimental ^{18}F-FDG PET studies. Initially we explored different types of ip anesthetic combinations. The combination fentanyl-fluanisone plus diazepam resulted in reliable sedation for approximately 2 h and satisfactory blood glucose levels with only minor intra-individual variations. When stable experimental conditions had been obtained, we carried out the sequential experiment, to test the well-being on mice, where tumor bearing mice was studied on three time points, one before chemotherapy and on days 2 and 8 after chemotherapy. The experimental setting was well tolerated. All mice could carry out three rounds of anesthesia (with full blood sampling) as well as the cytotoxic therapy.

Conclusion

In the present study, we have shown that an ip anesthesia with a combination of fentanyl-flua-

nisone and diazepam is feasible and provides stable blood glucose levels after a fasting period of 4 h in experiments in nude mice with xenografted human tumors. The anesthesia doesn't alter the uptake in brain and we have also verified that ^{18}F-FDG, intraperitoneally administrated, results in an expected plasma activity uptake and clearance. In combination with meticulous animal handling this set-up is reliable and future sequential tumor studies of early metabolic effects with calculation of MR_{glc} following cytotoxic therapy are made possible.

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Disclosure of conflict of interest

None.

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