Near infrared spectroscopy (NIRS) to attain reduction-refinement–respect, the three Rs towards ANIMAL WELFARE in preclinical research

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Abstract

In order to support the effectiveness of Near Infrared Spectroscopy (NIRS) to enhance the WELFARE of laboratory animals comparative studies have been performed. In particular NIRS has been coupled with MRI as these are two major in vivo non invasive methodologies more and more applied in research.

Furthermore NIRS has been coupled with Standard Liquid chromatography-mass spectrometry (LCMSMS) analysis that allow determination of pharmacokinetic-pharmacodynamic (PK-PD) values of chemicals i.e. in brain. This in order to verify the possibility that changes of hemoglobin oxygenation in rat brain as measured by NIRS might be a useful index of brain penetration of chemical entities.

It appeared that

1. The data obtained in parallel NIRS – MRI experiments strength the proposition to consider NIRS a complementary and confirmatory measurement of MRI studies.

2. NIRS might contribute to assess in vivo real time brain penetration of chemical entities, i.e. significant changes in NIRS signals could be related to brain exposure, conversely the lack of significant changes in relevant NIRS parameters could be indicative of low brain exposure.

Moreover, a major achievement of non invasive-NIRS is that no surgery is needed, resulting in no pain for the animals with highest standards of hygiene. This is a substantial positive feature when confronting with current analytical approaches for tissue and/or in vivo studies requiring various preparative steps that cause stress, death (tissue studies) or surgery with possible infections, pain (in vivo studies, including MRI analysis).

Keywords: NIRS; MRI; PK-PD; Blood brain barrier; Cocaine; Animal welfare

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These results support NIRS as one if not the best technological tool to attain Reduction of number of animals/study-Refinement on the method of analysis—Respect towards ANIMAL WELFARE in preclinical research. This will therefore further support this non invasive methodology for studies on neurobiological processes and psychiatric diseases in preclinical but also as translational strategy from preclinical to clinical investigations.

Introduction

In general, the analytical approach for in vitro, tissue, ex vivo and/or in vivo studies, that are mostly invasive, requires various preparative steps that in most cases result unavoidably in stress, death (tissue studies) or surgery (pain: in vivo invasive studies).

The feasibility of in vivo non-invasive analysis of biological activities is a major attempt to overcome post mortem studies and primarily to avoid the major limitation of most part of in vivo methodologies that is invasiveness. One of the main objectives being the possibility of using the same analysing methodology for preclinical and clinical studies to allow direct translational strategy from preclinical to clinical investigations.

In particular, NON invasive near infrared spectroscopy (NIRS) was first used as a tool for the in vivo monitoring of tissue oxygenation in the early eighties. Indeed, NIRS provides a non-invasive, non-ionizing means to monitor 2 main forms of haemoglobin: oxy-haemoglobin (HbO2) and deoxy-haemoglobin (HHb). These are chromophores present in biological tissues and are markers of the degree of tissue oxygenation. In addition, total haemoglobin concentration (HbO2 + HHb) is considered as total blood volume (HbT) [1] thus providing an index of blood level. All together, these three NIRS parameters (HbO2, HHb, HbT) are indicative of the state of vascular activity, oxygen saturation and therefore the state of the metabolism in the tissue analysed.

Then NIRS has been rapidly improved at the level of theory and instrumentation for accurate qualitative and quantitative in vivo non invasive NIRS analysis [2-6].

Generally, main applications of NIRS are in the study of the transport of the oxygen to the muscles, the tissue-oxygenation index, the cellular metabolism and the cerebral haemodynamics.

In clinical studies NIRS is currently employed to monitor fetal hypoxemia [7,8] and in newborn infants to detect birth asphyxia and/or apnoea and hypoxia [9-12]. In addition, changes in oxygenation during occlusion of the carotid artery, or during cardiopulmonary bypass, circulatory alterations and/or arrest are also analyzed using NIRS [13-15].

Besides fewer NIRS studies have been performed at the Central Nervous System (CNS) level, mainly to monitor oxygen sufficiency and brain functions and/or brain mapping [16-18] as well as brain diseases [19-22] while only a limited amount of studies have used NIRS to analyse “vascular CNS” functions in animals. In order to implement such type of analysis, we have developed a Near Infrared Continuous Wave Spectroscopy instrument (NIR-CWS, based on the low extinction coefficient of tissue in the near infrared region [1]) that allows in vivo, real time non-invasive NIRS measurements in the rat brain [23,24]. The choice of developing NIRS in rodents comes also from the evidence of significant advantages of NIRS versus all the other non invasive techniques (fMRI, PET, MEG) that are: i) direct measurement of HbO2 and HHb; ii) needless of injection or inhalation of radioactivity; iii) movements are admitted during NIRS measurements; iv) lack of acoustic noise; v) robustness, small dimensions allowing portable device, minor cost.

On these basis, our attempt has been that of developing a methodology in order to perform in vivo NIRS measurements in the brain of rodents, therefore to implement NIRS as a putative translational strategy from preclinical to clinical investigations. Then, two studies have been addressed to validate that:

I) NIRS is complementary to fMRI as Magnetic Resonance Imaging (fMRI) and Near Infrared Spectroscopy (NIRS) are two major in vivo non invasive methodologies more and more applied in research. The first more than the second is largely used also in clinical domain.

II) NIRS is complementary to PK-PD studies so that it can be acting in the 3-Rs i.e. –

- Reduction in the number of animals required
- Refinement of the methodologies of analysis
- Respect of the animals i.e. reducing their suffering.

Methods & results

The Near Infrared Continuous Wave Spectroscopy instrument (NIRCWS, based on the low extinction coefficient of tissue in the near infrared region) that allows in vivo, real time non invasive NIRS measurements in rat brain as demonstrated earlier [1,23,24] has been used. In particular, it allows performing quantitative assessments of haemoglobin variations exploiting precise absorption measurements close to the absorption peak of the water, i.e. 975nm [1]. Under these conditions, the dominant absorbers are haemoglobin and water. They contribute to determine the value of absorption coefficient i.e. the log10 of the ratio of impinging and back reflected intensity per unit of the source-receiver distance (unit OD. cm-1) [25].

I) NIRS is complementary to MRI

NIRS and MRI experiments

Six adult male CD rats have been anaesthetised following the animal preparation requested for MRI studies and were individually positioned in a stereotaxic holder designed to allow the MRI receive coil, then set for MRI analysis in brain striatum as described [26].

Similarly, another 6 adult male CD rats anaesthetised and prepared as described above were individually positioned in a stereotaxic holder designed to allow the NIRS sources and receiver and prepared for NIRS analysis in the whole brain as described [24].

All animals were treated at first with vehicle (NaCl 0.9%, 200µl i.v.) and 60 min later they were treated with cocaine 0.5 mg/kg i.v. MRI as well as NIRS analysis were performed continuously during 30min each. Data are shown in Figure 1; note the significant, similar increase of both relative cerebral blood volume (rCBV) MRI and HbT NIRS signals after cocaine infusion to approximately 116 and 118% (MRI) or 115 and 113% (NIRS) 5min and 10 min later, respectively.
Data analysis

MRI data analysis were performed as described by Marota, et al., [27]. Briefly data were subjected to a paired t-test across the number of animals comparing pre-drug versus post-drug relative cerebral blood volume (rCBV) within each animal. It appears that acute intravenous cocaine infusion increased significantly rCBV contrast signal in the analyzed region of rat brain (*p, 0.0007 striatum).

NIRS row data were subjected to ANOVA, with comparison between “control” (vehicle) and “treatment” values performed using the Tukey test. Then, the results were presented as % of control values, mean ± S.D., *p < 0.05.

II) in vivo NIRS is complementary to in vivo PK-PD studies

In these further experiments NIRS measurements have been performed in parallel with Standard Liquid chromatography-mass spectrometry (LCMSMS) analysis that allow determination of PK-PD values as described earlier [28]. Briefly, NIRS and PK-PD measurements have been performed in vivo in the same animals treated with 3 different doses of G18106, a glycine receptor antagonist (doses used: 0.1, 0.5 or 1mg/kg i.v. n=4 each dose). During the continuous in vivo NIRS analysis, approximately 100µl of blood was collected from the tail of the anaesthetised animals at 5, 15, 30, 60 min after treatment and submitted to LCMSMS analysis to monitor blood levels of the compound. The PK-PD analysis have been performed as described [28] and data about brain exposure [ng/g] as well as brain penetration (B/B) ratio of the compounds tested are present in figure 2 together with NIRS results, in particular NIRS-HbO2 values are shown. Briefly, The concomitant PK-PD and NIRS experiments performed when using the glycine receptor antagonist G18106 indicated parallel in time evolution of NIRS-HbO2 data and blood levels of the compound. This was evident for the doses 0.5mg/kg and 1mg/kg resulting in significant modification in time of NIRS-HbO2 levels in brain and blood levels of G18106. In particular 1mg/kg dose was followed by a progressive decrease of HbO2 to approximately half of its control value that appears paralleled by PK-PD analysis that was also showing a progressive decrease from approximately 23 μmoles/l down to 10 μmoles/l of G18106 in the blood samples. Both NIRS and PK-PD values decrease was significant (*p < 0.05 Tukey test).

The dose 0.1mg/kg was not modifying significantly HbO2 levels, and indeed related LCMSMS blood levels of G18106 were not detectable in vivo as well as in brain post mortem analysis (Table 1) indicating that there was no brain penetration when injecting such a low dose sistematically. This data is further supporting the direct relationship between the compound G18106 when injected at doses entering the brain and modified levels of HbO2.

Table 1

<table>
<thead>
<tr>
<th>G18106</th>
<th>1mg/kg</th>
<th>0.5mg/Kg</th>
<th>0.1mg/Kg</th>
<th>20 ± 6</th>
<th>6 ± 4</th>
<th>not detectable</th>
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Discussion

Today, thanks to its penetration depth, high temporal resolution and biochemical specificity NIRS is becoming a widely used research instrument to measure tissue oxygen (O₂) status non-invasively so that it can be applied to biomedical research and clinical environments to measure oxygenation in a variety of tissues including muscle, brain and connective tissue. More recently it has been used in the clinical setting to assess circulatory and metabolic abnormalities. Continuous-wave spectrometers are the most commonly used devices to apply NIRS measurements, they provide semi-quantitative changes in oxygenated and deoxygenated hemoglobin in small blood vessels (arterioles, capillaries and venules) [24,29].

In general, NIRS instruments are more and more used in clinical environments since they are now easy to use, sensitive, provide rapid analysis and could be complementary to other non invasive methodologies such as functional magnetic resonance imaging (fMRI), magneto-encephalography (MEG) and Positron Emission Tomography (PET). In particular, strong correlations were observed between the BOLD fMRI signal and the corresponding NIRS parameters in recent NIRS and fMRI parallel analysis of cerebral blood oxygenation changes during human brain activation [30,31].

Furthermore, “human” NIRS already has provided useful information involving monitoring of oxygen sufficiency and brain functions in the prevention and treatment of seizures and psychiatric concerns such as depression, Alzheimer’s disease and schizophrenia, as well as stroke rehabilitation [32].

The data obtained here in parallel NIRS – MRI experiments strength the proposition to consider NIRS a complementary and confirmatory measurement of fMRI studies. Furthermore, the relative easier and “cheaper” feasibility of the methodology designate NIRS as introductory approach, followed by fMRI when necessary as confirmatory analysis of CNS vascular activities. This will result in more rapid, less expensive preliminary experiments and in the present contest with a significative reduction of the number of animals tested as indeed NIRS analysis can be performed in anaesthetized rodents successively allowed to recover to be tested again.

Temporal resolution of NIRS is another crucial consideration in light of NIRS proposed scope. Since electrophysiologic study is often used to complement MRI, which is several orders of magnitude slower than the real time brain activity events being monitored, whereas electrophysiology measures as events occur, preliminary work has been performed in rats prepared for NIRS and electrophysiologic recording in the Raphe dorsalis Nucleus (RDN) and then supplied with pure oxygen. Data presented in figure 3 show parallel changes of NIRS and electrophysiologic signals recorded in RDN, proposing NIRS as well as electrophysiology as methodologies apt to real time analysis of brain events in vivo (for a review see ref 24). In particular, the increase of HbO2 is approximately 15 µmoles/L up to baseline. This value is in accord with previous work coupling both methodologies and showing linear relationship between changes in the BOLD MRI signal and NIRS parameters when giving pure oxygen to anaesthetized animals [33]. Therefore, these authors proposed such linear correlation for calibrating BOLD MRI, thus supporting NIRS as qualitative as well as quantitative tool for analysis of brain metabolism [33].

MRI’s claim to fame, notably, is its relatively high spatial res-
olution, down to about 1 mm, which helps in the pinpointing of brain nuclei. In contrast NIRS's spatial resolution is actually larger as shown in figure 4. However, this can be addressed via improvement of the hardware and software of the NIRS prototype used here i.e. via increasing the number of measurement points with a larger amount of optical fibers sources and receivers. This will allow reaching a 2-dimension imaging of the brain haemodynamics as proposed in figure 5.

Additionally, in this work we investigated the possibility that changes in brain metabolism as measured by NIRS might be a useful index of brain penetration of chemical entities. To test this hypothesis, the compound glycyne antagonist G18106 known to have good B/B penetration has been injected i.v. and the doses influencing brain NIRS values i.e. 0.5 and 1mg/Kg were also detected with PK-PD brain analysis both in vivo in blood samples as well as in post-mortem brain tissue. In contrast, the dose of 0.1mg/Kg of G18106 having no influence on NIRS values was also not detected in post-mortem PK-PD brain analysis, supporting the suggestion that significant changes in NIRS signals were related to brain exposure of such compound, or vice versa the lack of significant changes in NIRS parameters were indicative of low brain exposure of the glycyne antagonist G18106.

It is indeed possible that the low concentrations may be below the detection limits of the assay. This possibility may be verified via spectroscopic analysis with NIRS vis a vis PET analysis, that is, whether NIRS may eventually be used beyond hemoglobin oxygenation alone and so to monitor the presence of other reagents. And of course NIRS does not determine the other possibility the precise chemical form of the compound that induces brain metabolic activity, as it may be subject to metabolic processing once provided to the subject studied.

Nevertheless, NIRS analysis can therefore be an in vivo real time complementary and confirmatory measurement to post mortem Pharmacokinetic (PK) analysis of brain activity levels of chemicals. Indeed, it detects real time dose-related central influences of compounds with an evident advantage upon classic PK-PD measurements where rodents are generally sacrificed for brain analysis at each time selected for measurements. The advantage of in vivo NIRS is therefore evident because of the reduction of the number of animals needed, with the further advantage of a more refined PK analysis performed uninteruptedly in vivo on the same animal without the harmful condition of the animal sacrifice at each time of measurement.

Moreover, a major achievement of non invasive-NIRS is that no surgery is needed, resulting in no pain for the animals. Under these conditions the highest standards of hygiene are easily maintained. Indeed no infections were observed in any animal utilized in the experiments mentioned above. In addition, no one animal died during the experiments. This is in contrast to current analytical approaches for tissue and/or in vivo studies requiring various preparative steps that in most cases result unavoidably in stress, death (tissue studies) or surgery (pain, in vivo studies, including MRI analysis).

At the moment, the animals tested with NIRS are anaesthetized for the only reason that it is the way to preserve the optic fibers from the rodents. The stress related to anaesthesia will be solved in the future improvement of the method (i.e. optic fibers protected from the reach of the animal). However, the anaesthesia can be reversible, so that at the end of each experiment the animal can be allowed to recover and to be re-employed for further analysis: this resulting in reduced number of animals needed for experiments.

For instance, different types of treatments (i.e. different chemicals) can be performed successively within the same group of animals, resulting in more consistent comparison of data obtained in significant less number of animals.

These results support NIRS as one if not the best technological tool to attain:
- **Reduction** of the number of animals/study
- **Refinement** on the method of analysis
- **Respect** towards ANIMAL WELFARE in preclinical research.

This will therefore further support this non-invasive methodology for studies on neurobiological processes and psychiatric diseases in preclinical but also as a translational strategy from preclinical to clinical investigations.

**Figures**

Figure 1: shows the results obtained following all treatments i.e. response after vehicle (white square for MRI or white circle for NIRS experiments, respectively) or cocaine infusion (black square for MRI or black circle for NIRS experiments, respectively).

Data are presented as means ± S.D. for the percentage increase of the MRI as well as NIRS signals detected: i.e. relative cerebral blood volume (rCBV) (26) and total blood volume HbT (1), respectively. They are relative to a 5 min baseline (control) obtained immediately before vehicle or cocaine i.v. infusion.

Data obtained following vehicle are presented without S.D. values for clarity.

Note the significant, similar increase of both MRI and NIRS signals after cocaine infusion to approximately 116 and 118% (MRI) or 115 and 113% (NIRS) 5min and 10 min later, respectively (stars: see Data analysis).
Figure 2: SQUARES: in vivo real time laser-NIRS values of HbO2 expressed as μmoles/L ± S.D. Values of HbO2 at time 0 are control levels, i.e. mean of continuous NIRS measurements within 5 min before treatment. Then treatments with G18106 were performed (arrow). Doses used: 0.1, 0.5 or 1mg/kg i.v., n=4 each dose.

DOTS: in vivo blood levels of G18106 are shown using the same scale values as μmoles/L (i.e. from 0 up to 25 absolute value). The levels are measured in the blood collected from the tail of the anesthetized animals at 5, 15, 30 and 60 min after treatment and submitted to LCMSMS for PK-PD analysis and are expressed as ng x10 / ml ± S.D.

Figure 3: Left: electrophysiological data gathered in the RDN before (0-5min, control period) and after application of exogenous oxygen (1 bar/2min, arrow) directly into the mouth of an anesthetized rat. Right: concomitant NIRS recordings showing simultaneous to electrophysiology changes of NIRS parameters. In particular pure oxygen supply is increasing HbO2 levels from steady state baseline [considered as zero] up to approx. 15 μmoles/L and significantly decreasing HHb to a similar negative extent. This effect is reversible as soon as the influx of oxygen is stopped. Similar data have been observed in other 5 animals (for further details see ref. 24). V= total volume.

Altogether these results indicate concomitant activation of RDN neurones and brain metabolism further to oxygen supply, therefore supporting NIRS as methodology capable of real time measurement of biological events.

Figure 4: Theoretical brain areas monitored in the rat brain with the NIRS instrumentation used here i.e. computer simulation of photon paths based on photon migration theory (for a review see ref 24).

S1 – S4 the four laser sources used, R = receiver. (For further details see refs 23, 24).

Figure 5: TOP: actual NIRS measurement of the effect of cocaine 1mg/kg i.v. (n=9) or vehicle (saline, NaCl 0.9% 1.4ml, n=8) upon HbO2 levels in rat brain. Note the significant increase of HbO2 levels after cocaine injection (*p < 0.05, Tukey test) from steady state baseline considered as zero, versus no significant changes following saline. For further details on this experiment see ref. 35.

BOTTOM: FUTURE measurement: 2-dimension imaging of rat brain haemodynamics will be obtained when larger number of optical fibers sources and receivers will be applied. This will allow reaching a 2-dimension imaging of the brain haemodynamics and higher spatial resolution.
References


