Can blood flow in the bone be measured using coloured labelled Microspheres?

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Abstract

This study was designed to determine whether coloured micro-spheres represent a realistic alternative to more conventional techniques for the assessment of bone blood flow (BBF). BBF is normally measured using radioactive labelled micro-spheres, a technique that is accurate, but becoming increasingly challenged for safety and environmental reasons. Coloured micro-spheres come in two varieties, dye-fixed and dye eluting, and both have been employed for the measurement of blood flow in soft tissue for some time. However, measuring blood flow in bone presents a different challenge to that of soft tissue, insofar as the bone has to be dissolved using harsh chemicals to enable the harvesting of the micro-spheres for counting. This study therefore was designed to determine whether these micro-spheres would survive the harvesting process, and whether they could be identified and counted afterwards.

INTRODUCTION

Measuring the blood flow in soft tissues using the colour labelled micro spheres is an established method and a standard technique. There are many established studies in this field, for example, Cerebral blood flow and cerebral blood flow velocity during angiotensin-induced arterial hypertension in dogs. (Werner C. et al).(1)

In general, this is for soft tissue blood flow measurement. To apply this technique successfully in bone requires an additional aggressive step. The aim of this study is to determine whether this additional step compromises the micro spheres to such a degree that they are rendered inappropriate for bone blood flow application

BACKGROUND

The following is an introduction to establish methods in blood flow measurement in bone. There are three procedures that are currently used for the measurement of the blood flow in the bone:

I- LASER DOPPLER FLOWMETRY (LDF)

LDF is a continuous real time measurement, which allows the user to visualise the blood flow. Applying the laser probe onto the desired area of question uses it. The frequency of the ultrasound changes as a function of the blood speed as well as the angel at which the probe is being positioned with respect to the blood vessel (1). This is very similar to saying that a fast car coming towards you has a higher pitch sound than when it's leaving.

The procedure is sensitive but not specific since it measures all kinds of flows in the area where the laser passes through. And so, to measure the blood flow of the bone, we have to invade the probe (2).

Its great advantage is the ability of being applied clinically and accurately but not in slow blood flow (1).

II- RADIOLABEL MICRO SPHERES

It is the gold standard in the bone blood flow measurements for its high sensitivity and precision. It is widely used in measuring blood flow in bone and other soft tissues (3). The method of animal preparation and micro sphere injection with the radioactive are the same as that followed with TRAC colored micro spheres, as it is the standard technique in measurement blood flow in all kind of tissues, micro spheres in blood flow studies.

It has a great advantage in measuring a slow and a little blood flow of any tissue in the body regardless the size or percentage of the blood supply.

There are disadvantages faced when using the radioactive material: It cannot be applied clinically, the standard measurement technique using radioactive micro spheres prevents their use at institutions not able to neither dispose

nor afford the cost of radioactive waste and the Radioactivity safety.

III- POSITRON EMISSION TOMOGRAPHY (PET)

The PET is not sensitive or specific and it has a low field of view (about 4-5 mm from the probe). To measure the blood flow, it has to pass through the soft tissues.

However, the three earlier mentioned methods for measuring the blood flow in bone are accompanied by undesirable side effects, such as safety, sensitivity and specificity of the values, time effect, coast effect, permission and registration to some of them, restrictions of using a procedure, invasive procedure which of course needs sterilisation.

Having mentioned that, these methods may not be adequate for the implications they offer.

MATERIALS AND METHODS

This project was undertaken in Cardio Thoracic Laboratory at the Hammersmith Hospital Campus and the Orthopaedic and Traumatology Laboratory at Charring Cross Hospital Campus, the school of medicine at University of London and Imperial College of Science, Technology and Medicine. All the animals used in this study were sacrificed in accordance with Home Office Regulations

Two counting techniques were employed, manual counting using a microscope and spectrophotometric counting, relating micro-sphere number to dye concentration. The study was carried out in two stages: 1.In-vitro studies where the chemical stability and dye leaching properties of the micro-spheres were assessed. 2. Ex-vivo studies where the microspheres were employed in a rat model in which the tissues, both soft and bony underwent the entire harvesting process including the new step which involves the application of the highly corrosive chemical Conc HCl.

With these studies we wanted to determine two main factors, using both in-vitro and ex-vivo modelling techniques:

IN-VITRO, to determine the chemical stability of the two micro spheres species upon exposure to highly corrosive chemicals used to digest bone.

EX-VIVO, to determine whether the micro spheres survive the harvesting process and

Deliver a measurable end product in an animal model.

EX-VIVO STUDIES

These studies were carried out using large (350 g) Sprague Dawley rats. Nine animals were anaesthetised using Midazolam and Fentanyl anaesthetic, they were placed supine on the operating table (fig.3).

Figure 1

Figure 1: Injecting the rat with the micro spheres on the operating table.

Micro spheres (1.000.000) were injected into the left ventricle via a needle inserted through the chest wall. The injections of micro spheres contained E-Z TRAC (Blue),

DYE TRAK (Yellow) or Both Micro Spheres.

After injection, the animals were allowed to stabilize for 15 min and were then sacrificed in accordance with Home Office Regulations.

All soft tissues were separated from the bones. Each limb was divided into two samples; the bone sample, which contained only the shafts of the long bones, and the muscle sample contained all the soft tissue that was removed from the long bones.

Prior to separation of the hard and soft tissues, the complete limbs were stored at -80□C in air evacuated polythene bags (fig.5). Once the tissues had been separated from the complete limbs, after thawing, they were placed in uniquely labelled sample containers and stored at –4 degrees until processed.

Figure 2

Figure 2: Limbs in reserved in air evacuated bags

The two micro spheres species (E-Z TRAC and DYE TRAK) employ essentially the same micro spheres harvesting protocol. However, this protocol is designed for soft tissue, for bone an additional step required.

Each sample, muscle or bone, was placed in a 15 ml polypropylene centrifuge tube and pushed to the bottom, 4 ml of diluted Tissue Digest Reagent I is added to each sample.

Tubes were placed in oven at 70˚-80˚ C and allow tissue hydrolysis overnight. The following day, vortex mixing was done for 15-20 seconds. The tissue samples in each tube have been completely homogenized into suspension, with only small particles of debris visible. Diluted Tissue Digest Reagent II was added to the sample suspension to bring the total liquid volume to 14-15 ml. Tubes were centrifuged for 30 min and the supernatant was aspirated to a level slightly above each pellet.

Each sediment was re-suspended in 10 ml of diluted Micro spheres Counting Reagent by vortex mixing.

An additional aggressive digestive step for harvesting microspheres from bone, involving the immersion of the bone samples in concentrated HCl for tissue digestion.

The two micro spheres species have two different recommended counting procedures,

Manual counting using microscope, and Spectrophotometer counting after dye elution.

During the course of these studies, it became apparent that dye elution counting was the most convenient method of assessing the micro spheres population. Therefore, in addition to recommended manual counting technique we derived a dye elution technique.

IN-VITRO STUDIES

The use of both micro sphere types in bone blood flow studies requires an additional digestive step to those outlined in the manufactures micro spheres harvesting protocol. This additional step requires extremely aggressive and corrosive chemicals, in particular concentrated HCl. There are two main concerns associated with the use of concentrated HCl in conjunction with micro spheres:

That concentrated HCl may alter the micro spheres structure in such a way that they are unidentifiable under the microscope.

That the interaction between HCl and the micro spheres may elute dye or product subsequent elution.

A number of simple IN-VITRO experiments were designed to answer these concerns.

Effect of concentrated HCl on micro sphere morphology was tested by adding 0.5 million micro spheres, of both species to 5 ml concentrated HCl and the mixture allowed to sit for 10 min. The acid was removed using a glass pipette and the vessel filled with 10 ml distilled water.

The tube containing micro spheres and H2O was centrifuged at 2500 rpm for 10 min and the water removed down to the 0.5 ml mark. The mixture of H□O and micro spheres was vortex mixed and an appropriate amount of the mixture was placed on a Hemacytometer and the structure of the micro spheres observed and recorded.

Effect of concentrated HCl on dye elution was tested by adding 0.5 million micro spheres of both species to 5 ml concentrated HCl and the mixture allowed to stand. The mixture was transferred by pipette into a spectrophotometer cuvette and placed in the reading bay or spectrophotometer. The absorbance of light at the appropriate wavelength for the two micro spheres was measured and compared to a control cuvette containing only concentrated HCl, this was repeated at 10, 30, 60 min and at 72 hours.

After 72 hours, the acid was removed after centrifugation and the micro spheres exposed to DiMethyle Formamide (DMF) and the elution of dye observed by colour change and by Spectrophotometric measurement.

RESULTS

IN-VITRO STUDIES

The in-vitro studies were carried out to determine whether the additional chemically aggressive step of adding

concentrated HCl to the bone samples to facilitate digestion, has any effect on either the morphology or dye eluting qualities of the micro spheres.

On close microscopic examination (x 400), there were no morphological changes to the micro spheres. The micro spheres appeared to be intact, with no apparent micro sphere litter present. It was possible to discern the colour of the micro spheres in all cases.

Upon adding DMF to the previously acid exposed micro spheres, there was a clear leaching of yellow dye from the micro spheres into the solution. Blue dye was not apparent upon visual inspection. Analysis using the spectrophotometer confirmed that the yellow dye has leached, and that the process has no effect on the blue E-Z TRAC micro spheres

Long term exposure to DMF demonstrates that it is possible to elute dye over a long period of time from the supposedly dye-stable E-Z TRAC micro spheres. This may be useful in applications where chemical processes affect the elution in conventional DYE TRAK micro spheres, but where manual counting is confounded by low micro sphere numbers. It was clear from these studies that both micro sphere species were impervious to exposure to strong acid, insofar as in neither case was there any significant dye present at either wavelength in the acid after exposure. Furthermore, the DYE TRAK micro spheres appeared to give up most of the dye present in a short time (around 15 minutes) in the presence of DMF, with no significant further elution beyond this time.

The E-Z TRAC micro spheres did not appear to release dye over the short time scale, but prolonged exposure to DMF did result in the release of significant amounts of dye. This suggests that the E-Z TRAC micro sphere can be employed as a dye-eluting device if exposed for a long period of time. However, with no real calibration data from the manufacturer, it is difficult to quantify the number of micro spheres present as a function of the dye concentration at this stage for E-Z TRAC, but this action may be useful as a method for verifying manual counting of this micro sphere species.

EX-VIVO STUDIES

Manual counts were carried out on both soft tissue and bone samples. The counts are shown in table 1 for bone and table 2 for muscle. These values are expressed as the number of micro spheres per gram of tissue in each sample.

Figure 3

Table 1: The manual counting of micro spheres in bone, group 1 was the yellow injected rats, group 2 was the blue injected rats and group 3 was the both micro spheres injected rats (NO= None Observed).

GROUP	Blue	Yellow	MS/g Yellow	MS/g Blue
1		✓	219.2 ➨ 121.4	NO
2	✓		NO	227.3 ➨ 199.4
3	✓	✓	287 ➨ 49.4	198.9 ➨ 71.3

Figure 4

Table 2: The manual counting of micro spheres in muscle, group 1 was the yellow injected rats, group 2 was the blue injected rats and group 3 was the both micro spheres injected rats (NO= None Observed).

GROUP	Blue	Yellow	MS/g Yellow	MS/g Blue
1		✓	1806 ➨ 782	NO
2	✓		NO	942 ➨ 490
3	✓	✓	2305 ➨ 448.4	968 ➨ 497.3

These data show quite clearly that it is possible to identify both micro spheres types in both bone and muscle, by simple microscopy. However, the numbers of micro spheres seen were fairly low in both bone and muscle and the difference between the two tissues types in terms of the number of micro spheres distributed to each tissue does not appear to confirm the expressed blood flow distribution patterns . This may be due a combination of counting errors and poor injection technique.

In all samples, there was a difference in adsorption after exposure to DMF in the yellow dye wavelength (448 nm), but no change in the blue (672 nm), when compared to control DMF, tables 3 and 4.

Figure 5

Table 3: Adsorption (ADS) of yellow (Y) and blue (B) dye in bone.

GROUP	ADS Yellow (AU)	ADS Blue (AU)
1	0.005 ➨ 0.02	0.00046 ➨ 0.003
2	0.003 ➨ 0.0014	0.0009 ➨ 0.002
3	0.0091 ➨ 0.017	0.007 ➨ 0.005

Figure 6

Table 4: Adsorption (ADS) of yellow (Y) and blue (B) dye in muscle.

GROUP	ADS Yellow (AU)	ADS Blue (AU)
1	0.076 ➨ 0.04	0.016 ➨ 0.02
2	0.0125 ➨ 0.005	0.0034 ➨ 0.004
3	0.133 ➨ 0.14	0.015 ➨ 0.025

It is possible to compute the number of micro spheres in the sample volume by using manufactures control sample adsorption data. In the case of yellow DYE TRAK micro spheres, 1000 micro spheres in 100 micro l DMF typically are associated with an adsorption at 448 nm of 0.068, which increases in proportion to the number of micro spheres present. Using these data, and the known weight of tissue in each sample, it is possible to express these adsorption values as number of micro spheres per gram of tissue.

For the blue E-Z TRAC micro spheres, the adsorption is 0.045 for 1000 micro spheres in 100 micro-litres of DMF. This estimation is based upon values employed for evaluation blue DYE TRAK micro sphere populations, and cannot be deemed accurate at this stage. However, the wavelength of 672, which is recommended for DYE TRAK blue, is precisely where one observes light adsorption with the blue dye associated with the E-Z TRAC species.

DISCUSSION

The chemical process required to harvest micro-spheres from bone in blood flow experiments is extremely harsh. In common with blood flow assessment in other, softer tissue, it is necessary to first dissolve the bone. This is normally achieved in soft tissue by utilising a hydrolysing agent, which over a period of time will dissolve the tissue itself, releasing the micro spheres into the resultant solution. Bone is unaffected by this process, and therefore will not release micro spheres contained within it readily for subsequent harvesting and counting. To achieve this release of micro spheres with bony tissue, it is necessary to employ a strong acid (Concentrated HCl) at the release stage. Concentrated HCl is highly corrosive, and at the outset of these experiments we feared that is may destroy, or at least damage the micro-spheres to such an extent that they may be rendered inappropriate for bone blood flow experiments. We therefore chose to study two discreet micro spheres species, DYE TRAK, dye eluting micro-spheres, and E-Z TRAC dye stable, manual counting micro spheres. The reasoning behind this choice was to give an option for this type of experiment in the event that the dye eluting micro spheres released dye upon acid exposure. We tested the stability of the micro spheres in two environments: IN-VITRO, where we studied the effects of the acid step on both the morphology of the micro spheres and their dye eluting properties, and EX-VIVO, where we studied the effects of the various chemical steps in an animal model which reflects the environment in which the micro spheres will ultimately be employed.

At no time did we attempt to actually measure blood flow with these micro spheres, rather the aim was to determine, using a simple experimental model, whether the micro spheres would survive the harvesting process, whether we could detect them in proportion to the number expected with regard to known relative blood flow distribution rates, and therefore, to determine whether they represent a valid and appropriate vehicle for assessing blood flow in animals where bone is being harvested.

The results of the in-vitro studies support both micro sphere species as being valid for bone blood flow studies. Both micro spheres survived exposure to concentrated hydrochloric acid without either significantly leaching dye, or undergoing apparent morphological changes. There was very low-level light adsorption at 448nm (0.00016 ± 0.00021 AU), indicating that there was some yellow dye leakage, but taking into account the large number of micro spheres in this preparation, this level is not significant and probably represents background adsorption. This is supported by the fact that this level did not increase over the 72 hours test period; rather it decreased to “none detectable” levels towards the end of the experiment. There was absolutely no blue dye detected at 672 nm throughout the 72 hours test period, confirming that the E-Z TRAC micro spheres were indeed dye-stable in the acid. These studies, combined with the fact that there were no morphological changes to the micro spheres upon acid exposure, support both species with regard to their suitability for use in bone preparations. Further studies, aimed at determining whether, after exposure to acid, the dye eluting micro-sphere retained a dye eluting property, and that the dye-stable micro sphere remained stable, were then performed to further demonstrate this suitability. These studies further support both species in this regard. It was apparent that the dye eluting micro spheres retained this property when exposed to DMF, a strong dye leaching agent, in all cases of exposure of yellow DYE TRAK micro spheres to DMF, significant light adsorption was detected at 448nm, and this was higher in muscle than in bone, suggesting that there were more micro spheres present in the muscle preparation than in the bone. These experiments therefore confirmed that yellow microspheres were detectable in both muscle and bone and that significantly different numbers of microspheres were present in both tissue types. Utilising this adsorbance data to calculate the numbers of microspheres present confirm this difference. Once again for Group 1 animals, receiving only yellow dye eluting micro-spheres, these numbers were 660.2 ± 600.96 MS/g for bone tissue and 1602.3 ± 843.34 MS/g for

corresponding muscle tissue. This ratio of around 2.5:1 for micro-spheres in the muscle to those in the bone, differs from that predicted by physiological principals of blood flow, which would suggest that the relationship should be in the region of 3:1. However, in the context of these pilot studies, this is a very close match and demonstrates the validity of this technique. Additional IN-VITRO studies showed that the blue E-Z TRAC micro spheres, thought to completely dye-stable were not susceptible to the strong acid solution, but that it was possible to elute the dye from the micro-spheres using DMF, but over a much longer exposure time. This finding suggests that this species of micro sphere may be suitable for dye eluting studies where conventional dye eluting micro spheres are not suited, for example where complex chemical processes such as histological fixing, is employed.

CONCLUSION

Overall, this study, though small in scale, suggests for the first time that there is a true alternative to radiolabelled micro spheres for the study of blood flow and blood flow distribution in bone. The technique is inexpensive, and devoid of the taxing safety considerations associated with traditional radio labelling techniques.

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