Original Article

MEASUREMENT OF PULPAL BLOOD FLOW IN DOGS WITH NONRADIOACTIVE COLORED MICROSPHERES

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Abstract

The viability of dental pulp depends largely on regional blood flow, as in other organs. Measurement of absolute pulpal blood flow (PBF) and comparisons with blood flow in other organs allow the prediction of microvascular regulation in dental pulp. In previous studies, PBF was measured in dogs mainly with radioactive microspheres. However, this established technique is inaccessible to many investigators due to concerns over radiation safety and radioactive waste. To overcome these limitations, a new method has been introduced that involves the use of nonradioactive colored microspheres for measuring regional blood flow in the myocardium and in other organs in animals. However, no previous studies have investigated the use of this method to measure PBF in dogs. We attempted to determine whether blood flow in dental pulp, which comprises a small amount of the total tissue in dogs, could be measured using this technique by comparing the measured values with those for regional myocardial blood flow. Mean blood flow values were between 0.148 and 0.182 ml/min/g for dental pulp at four different sites and about 1.0 ml/min/g in regional myocardium. These values are comparable to those previously reported using radioactive microspheres. As nonradioactive colored microspheres safely permitted measurement of absolute PBF in dogs, this technique appears to be useful for research into microvascular blood flow in dental pulp.

Key words: Colored microspheres—Pulpal blood flow—
Regional myocardial blood flow

INTRODUCTION

The function and viability of dental pulp depend largely on regional blood flow, as in any organ. As dental pulp tissue is a low compliance environment, changes in blood flow and hemodynamic factors greatly affect its function and viability¹⁾. Therefore, detailed

knowledge of pulpal hemodynamics is important for future development of dental drugs and restorative materials, for establishing optimal conditions for tooth preparation¹⁴⁾, for developing treatment modalities for conservation of dental pulp during inflammation, and for research in the mechanisms of the dentin/pulp complex.

Pulpal blood flow (PBF) has been measured using such means as radioactively labeled microspheres (RMSs)^{15,16,18}, fluorescent microspheres (FMSs)^{20,29}, H₂ gas distribution techniques³⁸, laser Doppler flowmetry (LDF)²⁵, and the xenon washout method¹⁷. LDF is non-invasive, provides continuous data, and can be applied in human subjects; thus, it is the most common technique in current use^{1,24}. However, its major disadvantages are that LDF allows for only relative values in small areas, has limited reliability, poor reproducibility, and its accuracy is highly dependent upon the exact placement of the probe. Therefore, the RMS method has been accepted as a reliable standard method for yielding absolute values of blood flow and is employed for measurement of regional myocardial blood flow (RMBF)^{3,5} and regional flow in other organs. The use of RMSs has become the method of choice for determining PBF, and the absolute values obtained using this technique have become accepted²¹. However, because of the precautionary measures needed to minimize radiation exposure, the use of RMSs is not accessible to many investigators. The disposal of radioactive waste also raises environmental issues.

To eliminate some of the limitations inherent in the RMS method, a non-radioactive fluorescent microsphere (FMS) method¹⁰ and a colored microsphere (CMS)¹⁹ method have been developed for the measurement of RMBF. With the FMS method, the blood flow value is calculated principally from the number of FMSs measured on serial sections of the target tissue under a fluorescent microscope. The FMS method has already been adopted for the determination of rat PBF^{20,29}. Values for PBF measured by the RMS method are expressed with the unit (ml/min/100 g) based on wet weight^{13,15,16,18}, while PBF semi-quantified and visualized by the FMS method are calculated with the unit (ml/min/cm³ or ml/min/mm³) based on volume^{20,29}. The FMS method is appropriate only in small animals¹⁰; however, PBF values have been measured by the RMS method only in dogs. Therefore, PBF values measured by the FMS method cannot

be directly compared with previous PBF values measured by the RMS method.

The CMS method has been successfully employed in the measurement of RMBF^{6,7,12,19}, making this method desirable, because it employs dye-labeled microspheres instead of radiolabeled tracers and eliminates the need for precautions against exposure to radiation. The dye is recovered from the microspheres by elution with an organic solvent, and blood flow data are obtained through determination of the photometric absorption of the dye solution with a spectrophotometer. This enables the comparison between the PBF values obtained by the CMS method and the previous PBF values obtained by the RMS method. However, no studies have used the CMS method to determine hemodynamics in the oral cavity of dogs. It is not known whether this technique would allow measurement of PBF through an adequate number of trapped CMSs for spectrophotometry in the very small amount of tissue that comprises dental pulp. The purpose of this study was to examine the validity of the CMS method by determination of PBF and comparing the results with those using RMBF.

MATERIALS AND METHODS

Experiments were conducted in accordance with the Guideline Principles for the Care and Use of Animals as approved by the Council of the Physiological Society of Japan.

1. Surgical procedures

Experiments were performed on seven conditioned, healthy Labrador-Retriever and Beagle hybrid dogs (NRB; Nihon Nosan Kohgyo, Yokohama, Japan) ranging in weight from 15.2 to 18.4 kg (mean $\pm$ SD, 16.3 $\pm$ 1.0 kg) and in age from 7 to 8 months. Anesthesia was induced by intravenous bolus injection of sodium thiopental 10 mg/kg and vecuronium bromide 0.5 mg/kg. After tracheal intubation, an adequate depth of anesthesia was maintained by inhalation of a nitrogen-oxygen-isoflurane gas mixture under artificial ventilation (Model

613, Harvard Apparatus, South Natick, MA, USA), which was adjusted to provide an end-expiratory $p\text{CO}_2$ of 30–40 Torr.

The heart was exposed through a left thoracotomy in the fourth intercostal space, and the pericardium was excised and sutured to cradle the heart. A catheter for microsphere injection was inserted into the left atrium through the left atrial appendage and fixed by ligature when the tip of the catheter was placed in the atrium under pressure monitoring. Another catheter was introduced through the right femoral artery and advanced toward the abdominal aorta for collection of reference blood samples and for continuous monitoring of arterial pressure by a pressure transducer (P23ID, Gould, Oxnard, CA, USA). Blood pressure was continuously recorded (Polygraph series 360; NEC San-ei, Tokyo, Japan). A 5-Fr Swan-Ganz catheter (TC-504, Nihon Kohden, Tokyo, Japan) was inserted through the right jugular vein and advanced with the tip placed in the pulmonary artery. A cold water outlet placed in the right ventricle for determination of cardiac output by the thermodilution technique. The cardiac index (CI) was calculated by a hemodynamic profile computer (SP1145, Viggo-Spectramed, Oxnard, CA, USA). The open chest was covered with a sheet of vinyl chloride to minimize changes in the surface temperature of the heart. The total peripheral resistance index was calculated according to the equation: $(\text{mean arterial pressure}) \times 80 / \text{CI}$ ($\text{dyne} \cdot \text{sec} / \text{cm}^5 / \text{m}^2$).

Prior to the experiments, periapical radiographs of both maxillary and mandibular canine teeth were taken to assure that apical formation had been completed.

2. Injection and recovery of colored microspheres

For each measurement, 8 ml of CMS suspension (from a 20 ml vial containing approximately 3×10^6 spheres/ml; CMS diameter, mean $\pm$ SD, $15 \pm 0.1 \mu\text{m}$; density, 1.09 g/ml; Dye-Trak, Tritontechnology, San Diego, CA, USA) was injected into the left atrium. Before the CMS injection, the vial was shaken vigorously with a vortex mixer. Each bolus injection

of approximately 24×10^6 CMSs was followed immediately by a flush of 10 ml saline solution to wash the inside of the catheter and stir the CMS suspension in the atrium. The two injections were completed within 5–10 sec. Arterial reference blood samples were drawn from the abdominal aorta at a rate of 7.1 ml/min, beginning 10 sec prior to microsphere injection and continuing for 150 sec, by means of an infusion withdrawal pump (FP-W-100, Toyosangyo, Tokyo, Japan). After about 20 min, injection of different colored CMSs was completed, followed by blood sample collection (Fig. 1). Before each injection of microspheres, arterial blood pressure and CI were measured.

At the end of the experiment, the heart was arrested under general anesthesia by an intravenous injection of an adequate amount of potassium chloride (1 M/liter). About ten min after death, myocardial tissue samples were taken from several sites (10 mm or less in depth) in the perfusion field of the left anterior descending coronary artery. The maxillary and mandibular canine teeth were then extracted carefully using a bone chisel and mallet. The extracted teeth were fissured using a diamond disk and cracked with a bone chisel and mallet. The hard tissue surrounding the pulp was removed, and the dental pulp was separated from the canine teeth. Dental pulp and myocardial tissue samples were weighed, and wet weights were recorded. Each tissue sample was placed in a 50 ml screw-cap glass tube to which a 4 M potassium hydroxide solution containing 2% Tween 80 (surfactant, polyoxyethylene sorbitan mono-oleate) was added. The glass tubes were closed and placed in a heated bath shaker at 72°C for 24 more hrs to secure adequate tissue digestion with mixing. The digested tissue solutions were filtered to recover the microspheres under vacuum suction using a vacuum filtration chamber (Millipore, Bedford, MA, USA) fitted to a removable polyester membrane filter with a pore size of $10 \mu\text{m}$ and a diameter of 25 mm (Dye-Trak, Tritontechnology, San Diego, CA, USA). To eliminate any impurities adsorbed by the microspheres, the filters were rinsed twice with saline containing 2% Tween 80 and

Fig. 1 Schematic protocol of colored microsphere (CMS) injection. Arterial reference blood samples were drawn from the abdominal aorta at a rate of 7.1 ml/min, starting 10 sec before microsphere injection and continuing for 150 sec. After about 20 min, CMS injection was repeated using microspheres of a different color.

then twice with 70% ethanol. The procedure was repeated with about 18 ml of the reference blood samples placed in 50 ml capped glass tubes to which a 16 M potassium hydroxide solution containing 20% Tween 80 had been added. The reference samples were processed in the same way as the tissue samples.

3. Calculation of regional blood flow

The filter that trapped the spheres was removed from the filtration chamber, folded, and placed in a 1.5 ml micro tube. A total of 100–800 μ l of N, N-dimethyl-formamide (DMF) was added to each tube for elution of the dye. The tube was vortex-mixed and centrifuged at $2,000 \times g$ for 5 min. A 50 μ l volume of the dye solution was transferred by a micropipette into a cuvette for absorbance analysis with a spectrophotometer (U2001 Hitachi, Tokyo, Japan) (wave length range, 300–820 nm). The composite spectrum of each dye solution was

resolved into spectra of individual constituents by a matrix inversion technique²⁷⁾. The absorption spectrum of each dye for the two types of reference blood samples was measured separately, and this served as a reference for the matrix inversion in determining the contribution of each color to the measured composite spectra at two fixed wavelengths. Thus, the regional blood flow (Q_m) in ml/min/g was calculated for each tissue sample from the following equation: $Q_m = (A_m \times Q_r) / A_r$ where Q_r is the recovery rate for the reference blood sample (ml/min), A_m is the absorbance by the CMSs trapped in a 1 g sample (AU/g), and A_r is the absorbance by the total CMSs in the reference blood sample (AU).

4. Statistical analysis

All values in the text and figures are presented as mean $\pm$ standard deviation (SD),

Table 1 Hemodynamic parameters before microsphere injection

	Blood pressure					
	Heart rate (beats/min)	Systolic (Rorr)	Diastolic (Rorr)	Mean (Rorr)	Cardiac index (liter/min/m ²)	Total peripheral resistance index (dyne-sec/cm ⁵ /m ²)
First injection*	124 ± 21	127 ± 18	90 ± 18	103 ± 18	4.07 ± 1.83	2,288 ± 772
Second injection*	122 ± 15	120 ± 13	85 ± 16	99 ± 14	3.31 ± 1.12	2,573 ± 753

Values are mean ± SD. Values were obtained from seven dogs.

Data were obtained before microsphere injection.

Two measurements were made in each dog.

*: No significant differences between first and second measurements. $p < 0.05$

Table 2 Wet weight and blood flow for each site of dental pulp

	Maxillary canine teeth		Mandibular canine teeth	
	Left	Right	Left	Right
Wet weight (g)	0.266 ± 0.099	0.269 ± 0.105	0.232 ± 0.098	0.223 ± 0.081
Pulpal blood flow (ml/min/g)	0.153 ± 0.052	0.172 ± 0.057	0.148 ± 0.058	0.182 ± 0.070
Number of measurements	13	14	8	10

Values are mean ± SD.

Wet weight of dental pulp was measured after hard tissue surrounding dental pulp was removed.

Pulpal blood flow (ml/min/g) was calculated from wet weight.

with the number of observations shown as (n). Statistical analysis was performed using one way analysis of variance followed by the Student-Newman-Kleus test for multiple comparisons. A p value of 0.05 or less was considered to be statistically significant.

RESULTS

1. Pulpal and myocardial blood flow

Hemodynamic parameters before each CMS injection are shown in Table 1. Values represent means of 14 measurements in seven dogs. There were no significant differences between the first and second measurements, indicating that CMS injections did not cause significant changes in hemodynamics. Wet weight for each dental pulp site is shown in Table 2. Values for PBF (mean ± SD) were: left maxillary canine teeth, 0.153 ± 0.052 ml/min/g (n = 13);

Fig. 2 Comparison of blood flow in regional myocardium and dental pulp in seven dogs. n = number of measurements. Values are given as mean ± SD. Statistical analysis was performed using one-way analysis of variance followed by the Student-Newman-Kleus test for multiple comparison.

*: No significance among different canine teeth $p < 0.05$
 **: Significance between myocardium and canine dental pulp $p < 0.05$

Fig. 3 Relationship between regional myocardial blood flow and right maxillary pulpal blood flow. Fourteen measurements were made ($n=14$) among 7 dogs. There was a significant correlation ($r=0.62$) between regional myocardial blood flow and right maxillary pulpal blood flow.

Fig. 4 Relationship between regional left pulpal blood flow and upper right pulpal blood flow in maxillary teeth. Thirteen measurements were made ($n=13$) among 7 dogs. There was a significant correlation ($r=0.70$) between regional left pulpal blood flow and upper right pulpal blood flow in maxillary teeth.

right maxillary canine teeth, 0.172 ± 0.057 ml/min/g ($n=14$); left mandibular canine teeth, 0.148 ± 0.058 ml/min/g ($n=8$); and right mandibular canine teeth, 0.182 ± 0.070 ml/min/g ($n=10$). No significant differences were found in PBF in relation to the site of canine teeth. RMBF per unit wet weight was 1.07 ± 0.59 ml/min/g ($n=14$), and was significantly higher than PBF at all four sites (Fig. 2).

2. Relationship between pulpal blood flow and regional myocardial blood flow

To evaluate the accuracy of measurements of PBF using CMS methodology, we investigated the relationship between PBF in right maxillary canine teeth and RMBF (Fig. 3). There was a significant correlation ($r=0.62$) between right maxillary PBF and RMBF. We examined the difference in PBF between two sites in maxillary canine teeth from seven dogs (Fig. 4). There was a significant correlation ($r=0.70$) between regional left PBF and regional right PBF in maxillary canine teeth. PBF in mandibular canine teeth was measured in four dogs, and a significant correlation ($r=0.63$) between regional left PBF and regional right PBF in these teeth was shown.

DISCUSSION

The function and viability of dental pulp, as with other organs, depend largely on the supply of arterial blood. Comparison of blood flow in dental pulp with that in other organs can elucidate the properties of microvascular regulation in dental pulp. Therefore, accurate measurement of absolute PBF will be needed for future dental research. The CMS method is likely to be employed in dental research, because it is the only current method that estimates absolute PBF without a radiation hazard. This method is based on the principle that CMSs injected into the left atrium or into the left ventricle are distributed in proportion to regional blood flow. CMSs are trapped in the arterioles of various organs and tissues. Regional blood flow can be estimated by comparing the quantity of CMSs in reference blood samples with that of CMSs trapped in the target tissue. These principles and procedures are similar in those of the RMS method. However, differences exist between the CMS method and the RMS method in the tracers used to label the microspheres, the detection technique, and regional blood flow computation procedures.

The reliability of PBF determinations based on the CMS method should be verified, because dental pulp involves such a small amount of tissue. The evaluation of the CMS method should include (i) comparison of its accuracy with that of the established RMS technique, (ii) comparison of methodology for measurement of PBF between the two methods, and (iii) assessment of reproducibility of PBF measurements by the CMS method.

(i) A comparison of RMBF measurements by the CMS method with those by the established RMS technique demonstrated a high degree of correlation (dogs, $r=0.97$; swine, $r=0.98$), indicating that the utility of the CMS method is comparable to that of the RMS method¹⁹. In comparing reported measurements of RMBF using the CMS method^{6,7,12} and the RMS technique^{3,5}, we found that the yielded values were very similar, demonstrating the validity of data obtained by the CMS method¹⁹. The RMBF values at the level of 1.0 ml/min/g in this study (Fig. 2) are similar to those reported previously^{6,7,12}.

The presence of foreign materials on the CMS surface, such as incompletely digested blood or tissue fragments, will affect color and interfere with the accuracy of the spectrophotometric assay. Since separation of CMSs from foreign material is accomplished by filtration, all tissue and blood samples must be sufficiently digested to allow for passage through the filter. This digestion is generally achieved by placing the glass tubes containing samples in a heated shaker bath at 72°C for 4 hrs¹⁹. However, in this study, more than 24 hrs were required for this procedure. Following filtration of the tissue solution digested with a strong alkali, the filtration chamber and sample tubes were first rinsed with a saline solution containing 2% Tween 80 and then with a 70% ethanol solution, maximizing the recovery of filtered CMSs. In other studies, to ensure the adequate trapping and recovery of CMSs, several procedures have been developed, including performing all steps prior to spectroscopy without changing test tubes⁹ and dye extraction by high performance liquid chromatography (HPLC)²².

(ii) Since dental pulp involves a very small amount of tissue, accurate spectrophotometric quantification of dye eluted from spheres is an important element in the CMS method. Complete recovery of spheres and processing of spheres to ensure clean surfaces are required as well as securing an appropriate number of spheres in the target tissue. In attempting to trap sufficient CMSs in dental pulp, we could not use the large number of microspheres that have been used for regional blood flow measurement in other organs (i.e., 5×10^6 CMSs); thus, we did not achieve a measurement of PBF in our preliminary experiment. With the RMS method, isolated tissue samples reportedly must contain more than 384 trapped microspheres to secure a 95% or higher statistical reliable blood flow measurement³. This meant that, theoretically at least, approximately 1×10^6 RMSs are necessary for RMBF measurements in dogs²³. A greater number of RMSs appears to be necessary for PBF measurement than for myocardial measurement, because dental pulp is comprised of such a small amount of tissue. Kim *et al.* reported that PBF measurement in dogs required injection of about $6-8 \times 10^6$ RMSs/10 kg body weight^{15,18}. In contrast, RMBF measurement in dogs weighing about 20 kg required $3-11 \times 10^6$ CMSs^{6,7,12}. In this study, we injected 24×10^6 (3×10^6 /ml) CMSs for PBF measurement in dogs aged 7–8 months, which were selected to secure relatively large dental pulp samples. Selection of young dogs, which have greater amounts of dental pulp, is an important factor in measuring PBF with the microsphere method. Thus, the CMS method appears to require a larger number of microspheres than the RMS method, because blood flow is computed from the concentration of the recovered dye. The condition of the circulation is important in measuring regional blood flow. The fact that there were no significant differences between the first and second hemodynamic measurements (Table 1) indicates that the CMS injections in this study did not result in significant changes in hemodynamics.

The mean values of PBF measurements in this study ranged from 0.148 to 0.182 ml/min/

g, while measurement with RMSs in canine teeth yielded PBF values of about 28 ml/min/100 g and 41 ml/min/100 g^{15,18)}. When these differences between the CMS method and the RMS method are taken into consideration, the PBF values of this study, being comparable to those (about 22 ml/min/100 g) measured by the RMS method¹⁶⁾, appear reasonable.

(iii) Determination of regional blood flow using a microsphere technique is based on the phenomenon that microspheres injected into the left atrium or left ventricle are trapped in the arterioles. The diameter of microspheres is important in determining blood flow, because microspheres of an inappropriate size will pass through the capillaries or areas of arteriovenous anastomoses. Microspheres 15 μ m in diameter are reported to be suitable for regional blood flow measurement in various organs, while 9 μ m spheres will yield a low rate of trapping in arterioles^{4,8)}. Comparison of PBF values measured in canine teeth with 8 μ m or 9 μ m and 15 μ m RMS have demonstrated that an appreciable number of 9 μ m RMSs passed through the capillaries and arteriovenous anastomoses, thus indicating the appropriateness of 15 μ m RMSs^{13,23)}. The density of our CMS is 1.09 g/ml, which closely approximates the density of erythrocytes, while its diameter is 15 μ m. Taking these factors into consideration, the CMS in this study appears reasonable.

The values of right maxillary PBF measurements in this study, for instance, ranged from 0.087 to 0.279 ml/min/g, while values of RMBF ranged from 0.55 to 2.38 ml/min/g in seven dogs. As with any organ or tissue, PBF and RMBF should be regulated by cardiac output and vascular resistance of regional arterioles. We investigated the relationship between right maxillary PBF and RMBF (Fig. 3) and found a good correlation. We also found a good correlation between regional left PBF and regional right PBF in maxillary teeth (Fig. 4). However, previously these relationships were not studied to elucidate the properties of dental pulp. The appreciable differences in PBF observed among different studies, even within the same species of experimental animals, may

be due to differences in such factors as hemodynamics at the time of the experiment, growth of teeth, development of pulpal vasculature, and the method of observation. A previous study with laser Doppler velocimetry in dogs demonstrated that drug-induced changes in PBF correlated well with those in femoral arterial pressure²⁶⁾. Since blood pressure is determined by two major factors, cardiac output and peripheral resistance, the relationship between systemic circulation and regional blood flow must be evaluated by these factors.

In conclusion, the CMS method offers distinct advantages. First, it provides a quantitative estimation of absolute blood flow in dental pulp. Second, it allows regional blood flow in several different target organs to be simultaneously determined in a single subject without radiation hazard. In this study, PBF and RMBF were simultaneously measured by the CMS method and the results compared. An injection of 24×10^6 microspheres in dogs aged 7–8 months allowed for blood flow measurement in the canine pulp, by producing adequate microsphere trapping in the tissue without hemodynamic disturbance. PBF and RMBF values in this study are comparable to those reported previously for the RMS technique.

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