

Determination of Ocular Blood Flows with the Microsphere Method

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Core Messages

- The advantages with the microsphere method are: (1) it measures blood flow directly, (2) it is suitable for measuring blood flow in small pieces of tissue and in inaccessible tissues, and (3) it does not disturb the normal circulation, if the experiments are properly designed.
- Radioactive, colored, and fluorescent microspheres have been used for determination of ocular blood flow. A promising new development is the use of neutron-activated microspheres.
- For reliable measurements with the microsphere method, the size and number of microspheres should be optimized for the tissue under investigation.
- If few microspheres are trapped in a tissue, due to low blood flow and/or small sample size (e.g., retina and anterior uvea), the precision of the determinations can be increased by more experiments.
- Biological variation contributes more to the error in the measurements than paucity of microspheres.
- Differences in arterial blood pressure, anesthesia, and arterial blood gases contribute to the variation of reported values on ocular blood flows. Other factors such as diseases, age, gender, and hormonal and seasonal variations could contribute to the variation as well.

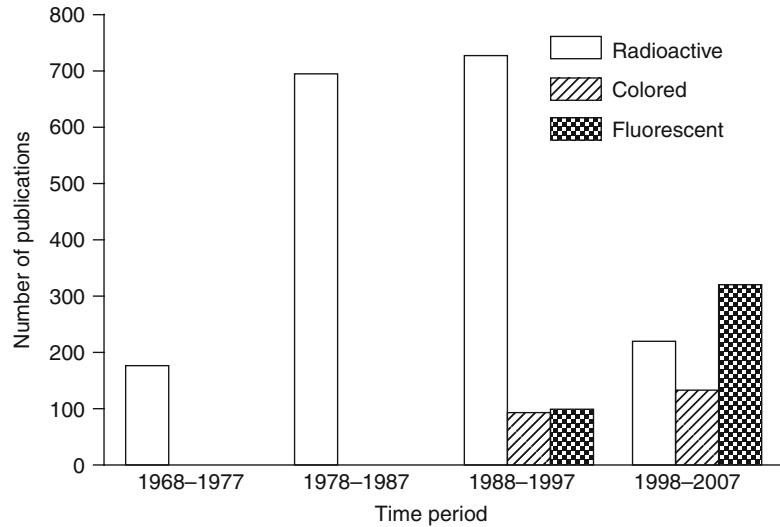
2.1 Introduction

The basic principle of the microsphere method is simple; microspheres injected into the systemic circulation are distributed and entrapped in tissues in proportion to the blood flow through the tissues. Although various types of solid particles had been used to study the microcirculation previously (see [36]), it was the introduction of plastic radioactive microspheres (RM) that boosted interest in the method. Rudolph and Heymann [77] were the first to use RM to study the blood flow distribution in the fetus of sheep and goat. Makowski [49] later included the collection of a reference blood sample

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Fig. 2.1 Number of publications with different types of microspheres published between 1968 and 2007. Data is based on a PubMed search on February 11, 2008, using the search terms “radioactive microspheres blood flow,” “colored microspheres blood flow,” and “fluorescent microspheres blood flow,” respectively. Numbers are approximate, as the publications were not checked for their relevance to blood flow measurement with microspheres or original articles versus reviews



from an artery, which made it possible to calculate actual blood flow values as

$$\frac{Q_R}{N_R} = \frac{Q_T}{N_T}$$

or

$$Q_T = \frac{Q_R \times N_T}{N_R}$$

where N_R and N_T are the number of microspheres in the reference sample and tissue, respectively, Q_R is reference flow, and Q_T is blood flow through the tissue. The method soon became a gold standard for measuring regional blood flow in various tissues and was adapted to measure ocular blood flows [4, 67, 93].

During the last decade, the number of studies using radioactive microspheres for blood flow determinations has declined, however (Fig. 2.1). There are several reasons for this; RM are relatively expensive, radiation exposure of personnel, decay during storage, difficulties and high costs associated with the disposal of the radioactive waste, and possible environmental hazards [71]. This has encouraged the search for alternatives, such as colored (CM) [10, 34, 40, 47] and fluorescent microspheres (FM) [1, 24, 32, 35]. Both types of spheres have been used to quantify ocular blood flows

[2, 25, 38, 65, 68, 92, 94]. The most recent addition is the use of neutron-activated microspheres (NAM), that is, microspheres containing stable nuclides that emit γ -irradiation when activated by neutron irradiation [41, 73]. In studies of myocardial blood flow, it has been shown that colored [34, 47], fluorescent [1, 24, 32], and neutron-activated [41, 73] microspheres give blood flow values that show a good correlation with those obtained by simultaneous injection of radioactive microspheres.

The potential sources of errors in connection with the use of radioactive and colored/fluorescent microspheres for determination of local blood flows have been extensively discussed elsewhere [11, 36, 71] and are similar regardless of the tissues under investigation. Thus, the present review will focus more on those issues that are of particular interest in relation to the measurement of ocular blood flows and our experience with the method. The terms microspheres and spheres will be used interchangeably in the remainder of the chapter.

2.2 Advantages and Disadvantages with the Method and Different Microspheres

The major advantages with the microsphere method, regardless the type of label, are that it (1) measures blood flow directly, (2) can measure

blood flow in small pieces of tissues, and (3) can be used for studies on not easily accessible tissues, without disturbing normal blood flow.

The ability to measure ocular blood flows without any eye surgery is very valuable. This is particularly true in species like the rabbit, which has eyes that are very sensitive to trauma. Measurement of uveal blood flow from a cannulated vortex vein in rabbits gave higher flow values than microspheres and made it difficult to study the effect of vasodilating agents, unless the animals were pretreated with indomethacin [20]. Extracapsular lens extraction and the corresponding sham operation increased blood flow in the posterior as well as the anterior uvea, an effect that could be abolished by aspirin [39].

The disadvantages with the microsphere method are that blood flow cannot be followed continuously and that relatively few measurements can be made in each animal. In addition, there is the risk of disturbing the normal microcirculation by occluding too many vessels with repeated injections, and separation of the different nuclides/colors becomes increasingly difficult with several different spheres (see below).

Colored and fluorescent microspheres can be counted in histological preparations [2, 10, 35, 65, 92] and in aqueous aliquots after digestion of the tissue [25, 34, 38, 40], or the absorbance/fluorescence can be measured after tissue digestion and extraction of dye from the microspheres [1, 24, 32, 47, 89, 94]. Thus, although CM and FM have longer shelf lives and are cheaper than RM, their use is more labor intensive, and hence the total cost may be equal. The waste disposal is simpler with CM and FM, but there are other health hazards for the personnel, as the sample processing requires the use of sodium or potassium hydroxide for digestion of the tissues and organic solvents for extraction of the dye. However, much of the sample processing can be automated [9, 87] to reduce the health risks as well as the costs. The advantages with FM over CM are that the measurement of fluorescence is more sensitive and can be done without spillover correction [32, 71], provided a limited number of differently labeled microspheres are used.

The NAM are cheaper than CM and FM, and no sample processing except drying is necessary. However, the samples have to be collected in special vials and sent to a central laboratory for analysis. To our knowledge, there is presently only one manufacturer of NAM, who also provides the analysis service. The analysis cost per sample is relatively high, but NAM are most likely cost effective for a laboratory that wants to do limited studies with few tissues. The major advantages with NAM are long shelf life, little tissue processing, and reduced health and environmental hazards. Furthermore, the samples may be archived and reanalyzed if necessary or used for other analyses [73].

2.3 Stochastic Error in the Entrapment of Microspheres

Provided that the microspheres are properly mixed with the blood, they will distribute according to the blood flow to different tissues. As the entrapments of microspheres are stochastic events, the number of microspheres in the reference and tissue samples will show a Poisson distribution around a mean value, if multiple samples are collected. Early theoretical and experimental data showed that for blood flow calculations to be made with 10% precision at the 95% confidence level, the reference and tissue sample should contain approximately 400 microspheres [23]. However, a later study showed that, providing the number of microspheres in the reference sample is ≥ 400 , the same precision can be achieved with considerably fewer microspheres in the tissue sample [66]. Still, the “400 microspheres/piece rule” has sometimes been considered a “must,” although it is only valid when one wants to determine the “true” blood flow through a tissue. If one merely wants to measure heterogeneity in blood flow or correlations, this can be achieved with considerably fewer microspheres [69], that is, if one wants to study differences in blood flow within a tissue, between tissues, or at different time points in the same animal.

For determination of ocular blood flows, we have usually used $1\text{--}2 \times 10^6$ spheres per injection in rabbits, cats, and monkeys, which will yield

Table 2.1 Approximate number of microspheres (n)^a trapped in ocular tissues of rabbit, cat, and monkey after injection of 1×10^6 spheres and theoretical coefficient of variation (CV)^b

	Retina	Choroid	Iris	Ciliary body
Species	n (CV)	n (CV)	n (CV)	n (CV)
Rabbit	25 (20%)	2,000 (2%)	130 (9%)	165 (8%)
Cat	75 (12%)	2,000 (2%)	165 (8%)	1,100 (3%)
Monkey	45 (15%)	640 (4%)	15 (26%)	115 (9%)

^aThe number of microspheres has been calculated based on the mean of the blood flow values in the references cited in Table 2.2 and assuming cardiac output to be approximately 450 g/min in rabbits [46, 53, 55, 82], 250 g/min in cats [56, 60], and 600 g/min in monkeys [8]

^bCV = $(\sqrt{n/n}) \cdot 100$

less than 400 microspheres in most ocular tissues, except in the choroid (Table 2.1). Table 2.2 shows values on ocular blood flows obtained in different studies published from the Department of Physiology at Uppsala University during more than 25 years. The experiments were made by several different researchers during a long time period, but the methodology has not changed very much. As expected from the low number of microspheres entrapped in the retina and anterior uvea, the coefficient of variation (CV) for these tissues is high in most studies. Furthermore, the observed CVs in the different studies (Table 2.2) are higher than theoretically expected, based solely on the number of microspheres (Table 2.1) for all tissues, including the choroid. Thus, one can assume that factors other than too few microspheres, most likely biological variability (see below), contribute to the error. Using the rat cochlea, another tissue with low blood flow, as a model, Hillerdal et al. showed that the biological variation contributed more to the error than the paucity of microspheres and that the precision could be improved by conducting more experiments [37].

Figure 2.2 is based on data from two studies in rabbits in which there was no difference in treatment between the left and the right eye during three blood flow determinations [54, 58]. The data shows that there is a good correlation between blood flow in the right and the left eye even in tissues with few microspheres, like the

rabbit retina (Table 2.3). Regression analysis (Fig. 2.2) shows that the best fitted line has a slope that is not significantly different from 1.0 for the retina and ciliary body, but for the choroid and iris, the slope is significantly less than 1.0, that is, 0.92 ± 0.04 ($P \leq 0.001$) and 0.91 ± 0.08 ($P \leq 0.001$), respectively. The reason for the slightly lower blood flow on the right side is not clear. It could be due to anatomical differences or the catheter used for the injection of microspheres could have interfered with normal hemodynamics, since in all experiments it was advanced into the left ventricle from the left brachial artery (see below). The difference between the right and left eye blood flow is too small ($\sim 10\%$) to be detected in the separate experimental series [54, 58], however.

Figure 2.3 is based on data from experiments with intracameral injection of test substances in rabbits [54], and shows the relationship between the first, second, and third blood flow determination, for the retina and choroid. The correlation between the three determinations was very good for the choroid and moderate for the retina (Table 2.4). Regression analysis of the retinal data showed that the slope of the regression line was not significantly different from 1.0, when blood flow was compared between the first and second or third determination, but the confidence interval is large (Fig. 2.3). For the choroid, the slopes of the regression lines were significantly lower than 1.0, when the second and third determinations were compared with the first determination (Fig. 2.3). This is more likely to be due to a decrease in the arterial blood pressure (81 ± 8 , 78 ± 8 , and 76 ± 8 mm during the first, second, and third determinations, respectively) than to disturbances of local blood flow in the choroid (see below).

Thus, despite the low number of microspheres in some ocular tissues, there is a fairly good correlation between eyes and between repeated determinations. Even with relatively few microspheres in the tissue and a limited number of experiments, it has been possible to detect blood flow changes of about 50% in the retina [55, 83] and of about 20% in anterior uvea [53, 85].

Table 2.2 Data on normal ocular blood flows determined with radioactive microspheres^a in rabbits, cats, and monkeys in papers published from the Department of Physiology, Uppsala University, from 1973 to 2001

Species/anesthesia	MABP (mmHg)	N ^c	Blood flow (mg/min) ^b				Reference
			Retina	Choroid	Iris	Ciliary body	
Rabbit	n.d.	13	8 ± 1	899 ± 77	75 ± 7	50 ± 6	[17]
Conscious	85 ± 2	10	15 ± 2	1,000 ± 63	68 ± 8	72 ± 5	[18]
	71 ± 2	10	10 ± 2	1,063 ± 58	39 ± 4	105 ± 10	[45]
	69 ± 2	10	11 ± 2	1,138 ± 68	44 ± 4	89 ± 13	[45]
	90 ± 3	14	n.d.	1,096 ± 163	84 ± 12	93 ± 15	[19]
	n.d.	5	11 ± 3	779 ± 97	65 ± 14	62 ± 12	[88]
	n.d.	7	n.d.	1,014 ± 101	58 ± 6	100 ± 17	[44]
	<i>Range of CV^d</i>		42–63	17–56	27–53	22–60	
Rabbit	71 ± 4	10	18 ± 3	1,020 ± 115	94 ± 24	82 ± 7	[57]
Urethane i.v.	75 ± 4	8	n.d.	1,044 ± 117	62 ± 15	97 ± 16	[46]
	n.d.	8	8 ± 2	678 ± 115	45 ± 8	35 ± 6	[59]
	n.d.	8	n.d.	1,131 ± 284	95 ± 23	116 ± 22	[43]
	n.d.	7	n.d.	842 ± 120	58 ± 10	95 ± 11	[43]
	n.d.	6	n.d.	585 ± 126	39 ± 12	52 ± 10	[44]
	n.d.	6	n.d.	609 ± 113	22 ± 7	47 ± 19	[44]
	n.d.	6	n.d.	578 ± 114	35 ± 10	59 ± 18	[44]
	n.d.	5	12 ± 6	567 ± 140	37 ± 13	65 ± 17	[88]
	63 ± 3	10	20 ± 5	700 ± 63	60 ± 10	65 ± 9	[58]
	77 ± 4	12	11 ± 2	805 ± 56	59 ± 10	82 ± 8	[53]
	74 ± 8	6	10 ± 4	811 ± 230	38 ± 16	68 ± 25	[82]
	84 ± 5	11	n.d.	1,101 ± 257	39 ± 10	99 ± 23	[83]
	65 ± 5	8	10 ± 2	729 ± 140	56 ± 14	50 ± 11	[55]
	60 ± 5	8	10 ± 1	633 ± 105	72 ± 20	59 ± 11	[55]
	60 ± 4	9	11 ± 2	552 ± 80	40 ± 10	30 ± 6	[55]
	<i>Range of CV</i>		28–112	24–77	46–103	27–99	
Cat	113 ± 5	12	15 ± 2	1,070 ± 122	60 ± 11	262 ± 30	[4]
Chloralose i.v. ^e	160 ± 5	11	17 ± 2	1,110 ± 120	23 ± 4	275 ± 13	[59]
	134 ± 9	7	25 ± 3	1,037 ± 104	63 ± 30	318 ± 43	[58]
	67 ± 6	6	28 ± 4	444 ± 94	33 ± 13	242 ± 45	[33]
	111 ± 7	6	17 ± 4	874 ± 123	58 ± 12	306 ± 25	[56]
	97 ± 5	7	19 ± 2	916 ± 144	37 ± 9	306 ± 50	[56]
	98 ± 4	8	23 ± 4	798 ± 92	39 ± 9	263 ± 34	[56]
	115 ± 7	7	18 ± 2	916 ± 170	47 ± 15	272 ± 35	[56]
	104 ± 4	9	17 ± 2	671 ± 56	21 ± 3	201 ± 14	[60]
	<i>Range of CV</i>		28–58	25–52	43–158	13–46	
Monkey	85 ± 5	5	30 ± 2	360 ± 48	5 ± 1	47 ± 4	[84]
Pentobarbital ^f	100 ± 6	12	32 ± 3	505 ± 75	8 ± 1	73 ± 7	[3]
	91 ± 8	7	16 ± 3	348 ± 70	5 ± 1	28 ± 7	[59]
	85 ± 6	5	32 ± 6	391 ± 36	13 ± 3	68 ± 9	[86]
	73 ± 6	6	25 ± 3	327 ± 51	15 ± 2	132 ± 27	[8]
	<i>Range of CV</i>		15–50	21–53	33–53	19–66	

^aThe number of microspheres given varied between 0.5 and 3×10^6 but was usually $1\text{--}2 \times 10^6$ ^bValues are the mean ± SEM for groups presented in the cited references. If data on blood pressure or flow values for different groups were not presented in the paper, the data were retrieved from original data whenever possible^cNumber of animals or eyes^dFor each study/group, CV was calculated as $(\text{SEM} \times \sqrt{n} \times 100) / \text{mean}$ ^eInduction of anesthesia was achieved by inhalation of chloroform [4, 58, 59], i.m. injection of alphaxalone and alphadolone acetate [33] or ketamine and xylazine [33, 56, 60]^fInduction of anesthesia was achieved with i.m. injection of methohexital [3, 59, 84, 86] or ketamine [8]

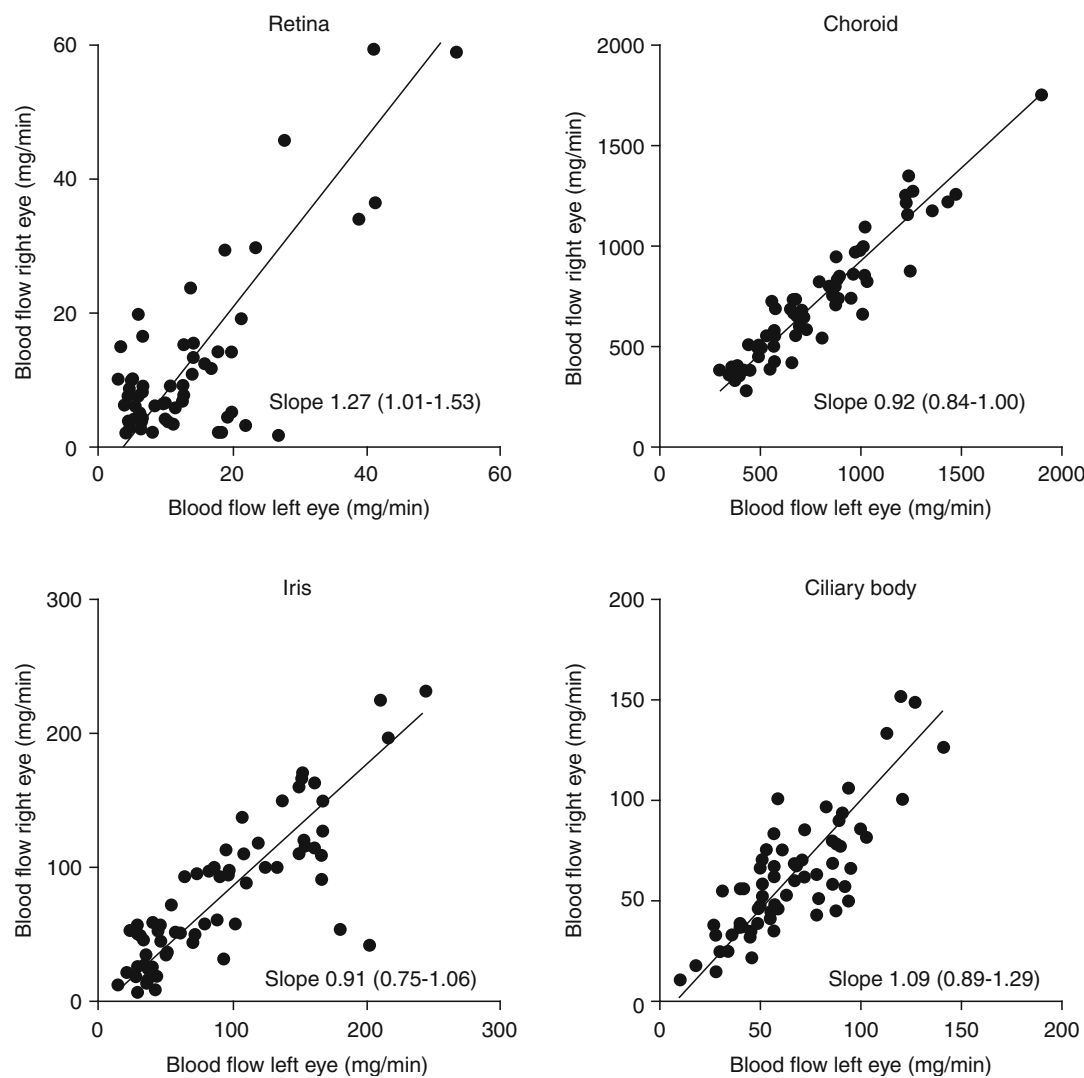


Fig. 2.2 Relationship between blood flow in the right and left eye in rabbits. Data are from 22 animals, with 3 blood flow determinations in each (Plotted from original data

[54, 58]). Regression line (Deming regression) with slope and 95% confidence intervals are given

Table 2.3 Correlation coefficients for comparison between ocular blood flows in the right and the left eye in rabbits^a

Tissue	<i>r</i> (CI) ^b	<i>P</i> value
Retina	0.77 (0.65–0.86)	<i>P</i> ≤ 0.0001
Choroid	0.95 (0.91–0.97)	<i>P</i> ≤ 0.0001
Iris	0.83 (0.73–0.89)	<i>P</i> ≤ 0.0001
Ciliary body	0.81 (0.71–0.88)	<i>P</i> ≤ 0.0001

^aBased on data from experiments in references [54, 58]
^bCorrelation coefficient with the 95% confidence interval

2.4 Methodological Errors and Practical Advice

2.4.1 Size of the Microspheres

Ideally, the number and size of microspheres should be optimized for each tissue and species. As discussed above, a large number of injected microspheres are desirable to get a high level of

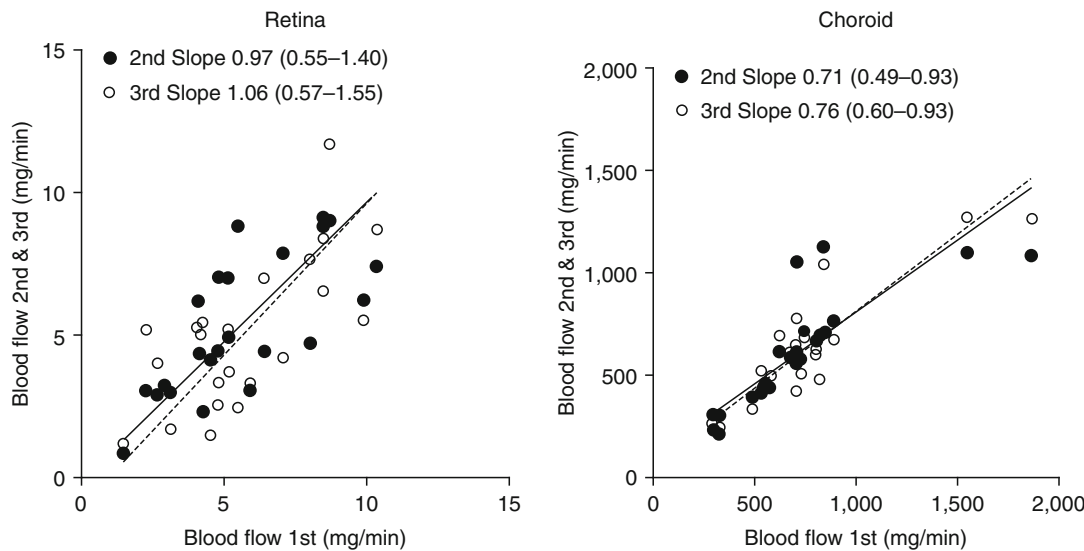


Fig. 2.3 Relationship between first and second blood flow determination (*filled circles, unbroken line*) and between first and third blood flow determination (*open circles, dotted line*) in rabbits. Data are from 12 animals,

each contributing 2 eyes each (Plotted from original data [54]). Regression line (Deming regression) with slope and 95% confidence intervals are given

Table 2.4 Correlation coefficients for comparison between the first, second, and third determination of retinal and choroidal blood flow in rabbits^a

Comparison	Retina		Choroid	
	<i>r</i> (CI) ^b	<i>P</i> value	<i>r</i> (CI) ^b	<i>P</i> value
1st and 2nd	0.72 (0.44–0.87)	<i>P</i> ≤ 0.0001	0.82 (0.62–0.92)	<i>P</i> ≤ 0.0001
1st and 3rd	0.70 (0.41–0.86)	<i>P</i> ≤ 0.0002	0.90 (0.78–0.96)	<i>P</i> ≤ 0.0001
2nd and 3rd	0.55 (0.16–0.78)	<i>P</i> ≤ 0.0084	0.92 (0.83–0.97)	<i>P</i> ≤ 0.0001

^aBased on data from experiments in references [54, 58]

^bCorrelation coefficient with the 95% confidence interval

precision in the measurements, but the risk of disturbing central and local hemodynamics will increase with the number of microspheres. The likelihood that disturbances of normal hemodynamics will occur is dependent on a combination of the size and number of microspheres and the species under investigation. Smaller animals have a smaller cross-sectional area of their vascular tree, and therefore the risk of disturbing their normal circulation is higher.

Obviously, if the microspheres are too small, they can pass through the tissue and hence cause an underestimation of blood flow. However, it is desirable that the microspheres do not get trapped until they reach the capillary bed of the tissue under investigation. Injection of microspheres that are too large can underestimate flow

and disturb local hemodynamics. Ideally, the microspheres should be injected as a bolus injection, but this is not possible as it will increase blood pressure. Thus, a large sphere that gets trapped early in an arteriole could prevent other spheres from entering the capillaries supplied by the arteriole, and hence cause an underestimation of blood flow. Furthermore, as the total cross-sectional area of the arterioles is much smaller than the total cross-sectional area of the capillaries, blood flow to a larger area will become occluded. By occluding many arterioles in many tissues, larger spheres may disturb central hemodynamics by increasing total peripheral resistance and hence blood pressure or the large spheres may impair cardiac blood flow and therefore decrease cardiac performance.

The most commonly used size of microspheres for determination of ocular blood flows is 15 μm , which has been validated in rabbits, cats, dogs, pigs, and monkeys [3, 4, 7, 76, 78, 85]. In cats and monkeys, there is a good correlation between uveal blood flows measured with 15- and 35- μm spheres injected simultaneously [3, 4, 7]. Injection of 8–10- μm and 15- μm spheres in rabbits indicated that the smaller spheres may pass the capillaries in the iris and ciliary body, as the larger spheres gave higher flow values [7, 85]. In dogs, uveal blood flows were not significantly different when measured by 15- and 25- μm spheres, but a small percentage (3%) of the injected 15- μm microspheres was found in the venous effluent from the eye [76]. Total ocular blood flow measured with four different sizes of microspheres (10, 15, 25, and 35 μm) in pigs showed that 10- μm spheres gave significantly lower flow values than 15- μm spheres. The values obtained with the larger spheres were higher but not significantly different from those obtained with the 15- μm spheres [78]. Collection of venous blood from a cannulated vortex vein indicated that more than 99% of 15- μm spheres were trapped in the pig eye [90]. Retinal capillaries are generally smaller in diameter than choroidal capillaries [15, 16, 26, 61–64], which indicate that smaller spheres are to be preferred when the main goal is to study circulation in the retina and optic nerve [31, 42]. Using smaller spheres also makes it possible to inject a larger number of spheres, which will simultaneously improve the reliability of the measurements.

A recent study showed that 10- μm spheres are more suitable than 15- μm spheres for measuring choroidal blood flow in the rat, as many 15- μm spheres were trapped before the choriocapillaries and injection of 10- μm spheres caused a larger number of spheres to be trapped in the choroid [92]. Similarly, as in larger species, smaller spheres (8 μm) were found to be best suited for measurement of blood flow to the retina and optic nerve [92]. Thus, for determination of retinal and choroidal blood flow at the same time in the same animal, one should, if practically possible, simultaneously inject two sizes of

microspheres using a higher number of the small-sized spheres [91].

Care regarding the size of the microspheres may be warranted also when one wants to study regional differences within an ocular tissue. In a recent histological study on cat eyes, it was observed that 15- μm spheres did not reach into the tapetum lucidum, whereas in nontapetal choroid, the microspheres were observed in precapillary arterioles just before the choriocapillaries [50]. Furthermore, the diameter of the precapillary arterioles was smaller in the tapetal region (4.7 ± 0.8 μm) than in the nontapetal region (6.2 ± 0.9 μm). Due to these findings, the authors suggested that 15 μm may not be suitable for measuring choroidal blood flow [50]. Although their findings may be of importance when studying regional differences within the choroid, it is most likely not true for determinations of total choroidal blood flow. Larger spheres show axial streaming, that is they move in the center of the vessel. The concentration of microspheres will therefore be lower in the periphery than in the middle of the vessel. This causes disproportionately fewer spheres to enter small branch arteries, which will underestimate flow in the area supplied by the small arteries. Too large spheres may therefore incorrectly show unevenness in the distribution of blood flow within a tissue [36]. Thus, the findings by May and Narfström [50] could be of importance when studying differences in blood flow between tapetal and nontapetal regions of the choroid. However, axial streaming is not likely to contribute to the error when measuring total choroidal blood flow with 15- μm spheres in cats. First, there is a good correlation between choroidal blood flow values obtained with 15- and 35- μm spheres [6, 7], even though axial streaming is more pronounced with larger spheres [36]. Second, it has been shown that flow biasing due to axial streaming is significant only when the feeding vessel to the tissue is just a few times larger than the diameter of microspheres [13]. As the choroidal arteries are much larger than 15 μm , axial streaming ought not to affect the results when total choroidal blood flow is measured.

2.4.2 Physical Characteristics of Microspheres

All microspheres are made of polystyrene, but their physical characteristics differ somewhat, depending on the type of label. RM [36] and NAM [41] are much heavier than blood (density ~1.5 g/ml compared to 1.05 g/ml), whereas the CM and FM have a density close to that of blood [89]. Due to their density and hydrophobic nature, the microspheres will sediment on the bottom of the storage vial and form aggregates. To reduce this problem, the microspheres are suspended in saline with a small amount of surfactant (e.g., Tween) added. Saline with dextran has also been used as a suspending medium to slow the sedimentation of microspheres. Still, the microspheres need to be sonicated and vigorously shaken before being removed from the storage vial, and prevented from sedimenting again before injection. One should be aware that the additives can influence hemodynamics in some species; Tween [51] and dextran [30] have been reported to cause hypotension in dogs and rats, respectively.

In early studies with RM, the variability in the diameter of the microspheres was quite large and not always in agreement with the manufacturers' specifications [36, 52]. Although the variability in size has diminished [71] (an acceptable SD of 0.1 μm for 10- and 15- μm spheres is now common), it is wise to check the size of new batches.

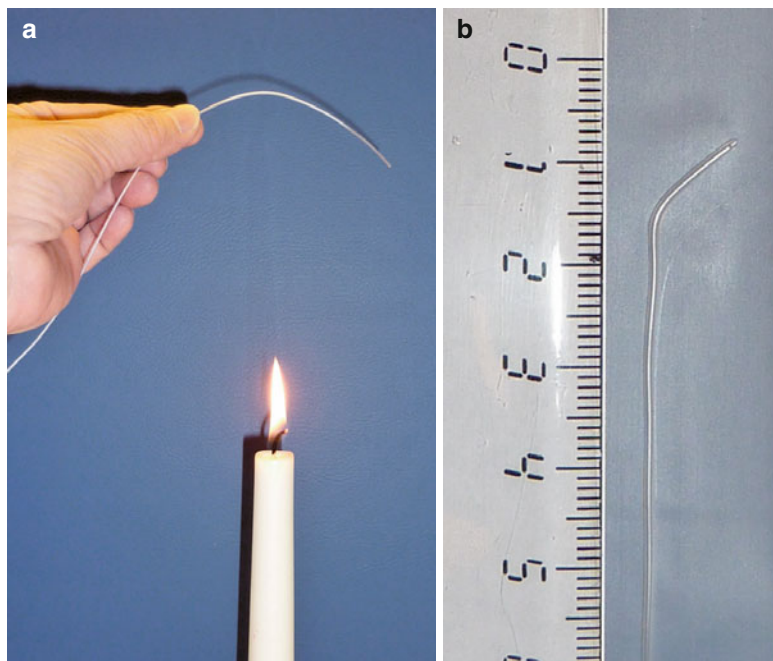
2.4.3 Injection of Microspheres and Collection of Reference Sample

The microspheres should be thoroughly mixed with the blood when they are injected, which is best achieved by injection into the left atrium or ventricle. In earlier studies, we injected the microspheres directly into the left ventricle via a steel cannula connected to a syringe with polyethylene tubing. In larger animals, this procedure demands a thoracotomy and artificial ventilation, whereas in rats, the microspheres can be injected into the left ventricle through the thoracic wall. To be able to measure ocular blood flows in conscious

rabbits, we started to do a heart catheterization by introducing a polyethylene tubing from a brachial artery. As this procedure caused less damage to the heart and minimized the risk of injecting the microspheres into the right ventricle, we subsequently used it in anesthetized animals as well. Empirically, we found that a slight bend (approximately 120°) at the tip of the catheter (OD approximately 1 mm) and using the left brachial artery in rabbits and the right brachial artery in monkeys and cats was the easiest way to succeed. To create the bend of the catheter, the tubing was briefly held above the flame of a match or candle (Fig. 2.4). As the catheter was advanced toward the left ventricle, it was gently rotated, and the pulse pressure wave was continuously recorded to register its entry into the ventricle. Before fixing the catheter in place, care was taken to check that an excessive length of catheter had not been advanced into the ventricle, that it did not disturb normal heart rhythm, and that blood could be easily aspirated. After the experiments, the proper location of the catheter was checked by visual inspection. Furthermore, we usually analyzed a piece of lung tissue to reveal possible injection of microspheres into the right ventricle due to damage by the catheter or possible septum defects.

After dispensing from the storage vial, the microspheres were diluted with saline to a volume of approximately 1 ml (for experiments in rabbits, cats, and monkeys) in a test tube. As the microspheres were aspirated into a plastic syringe, a small air bubble was allowed to remain in the syringe. By gently turning the syringe, the air bubble moved up and down and helped in preventing the microspheres from sedimenting in the syringe. After the syringe had been attached to the catheter, blood was withdrawn and mixed with the microspheres by the aid of the air bubble. This procedure is advantageous when one wants to do the blood flow determinations at fixed time points during or after an intervention, e.g., at the start of nerve stimulation or administration of a drug, as it only takes a few seconds before the microspheres start to sediment. Care was taken to hold the syringe in a tip-down position during injection to avoid injecting the air bubble, and after injection,

Fig. 2.4 Preparation of a catheter for heart catheterization. By holding the polyethylene tubing above a flame (a), a bend of about 120° was created (b)



blood was aspirated into the syringe to remove microspheres left in the catheter. If cardiac output was to be measured, triplicate samples (10 μ l) were collected from the test tube before aspirating the microspheres into the syringe, and the exact volume in the syringe was noted. After the injection, the content of the syringe was emptied into the same test tube, after the volume had been noted, and triplicate samples were collected. This made it possible to calculate the exact amount of radioactivity injected and hence cardiac output.

Usually, the microspheres were injected during 10–20 s, and the reference sample was collected during 1–2 min. Collection of the reference sample can be done by withdrawal with a syringe pump at a known speed or by free flow from a cannulated artery into preweighed vials. A high reference flow is desired to get a large number of spheres in the reference sample and to make sure that no microspheres remain in the tubing. On the other hand, the reference flow needs to be low enough to avoid disturbing normal hemodynamics. Pump withdrawal has the advantage of making it possible to calculate flows in volume/min, but it involves the transfer of blood from the syringe and rinsing of the syringe. We have been using free flow into preweighed counting vials,

collecting blood for 1 min with 10 s for each vial (or 2 min with 20 s for each vial). This procedure has the advantage that in every experiment we could confirm that no microspheres remained in the tubing and we avoided the transfer of the reference sample. Furthermore, with this sampling technique, it is possible to detect recirculation of microspheres. With appropriate-sized tubing, the free flow sample from a femoral artery (rabbits, cats, and monkeys) was usually 1–2 ml, only slightly more than the injected volume and less than 1% of the animal's total blood volume. Usually, the peak radioactivity was seen in tube 1 or 2, with little or no activity in tubes 3–4, and background activity in tubes 5–6.

2.4.4 Dissection

After the experiment, the eyes were enucleated and kept moist until dissection. During dissection, the eyeball was first cleaned of extraocular tissues and was then cut open along the ora serrata when dissecting cat and monkey eyes. The zonular fibers were cut with fine scissors to remove the lens. The iris could then be separated from the ciliary body by gently pulling with a pair of small

forceps or by cutting. The ciliary body was scraped off the sclera with a pair of forceps. The posterior part of the eye was divided into four quadrants by cutting from the ora serrata to the optic nerve head. After removal of the vitreous, the retina was gently removed from the choroid, which was subsequently scraped off the sclera. In rabbits, a slightly different technique was used due to anatomical differences. For example, the ciliary muscle is poorly developed, and the ciliary processes extend almost all the way to pupillary margins. Usually, the optic nerve head was removed, and the eyeball was cut open from the posterior pole to the ora serrata by four incisions. The lens is also not as firmly attached in rabbit as in monkeys and cats, which makes it possible to remove it by just a pair of forceps. The vitreous and retina were removed and the iris-ciliary body pulled off the sclera. The ciliary processes were then scraped off the back of the iris with a pair of forceps. In albino rabbits, the iris is almost white, whereas the ciliary processes are blood filled, which makes it relatively easy to see that the processes have been completely removed. The choroid was scraped off the sclera.

As we weighed our blood samples and it is sometimes difficult to completely remove the vitreous from the retina and ciliary body, we usually expressed ocular blood flows as total flow in mg/min, whereas many other investigators use ml/min/g tissue. The disadvantage with total flow is of course that the tissues have to be quantitatively sampled. However, this is not a big problem, and it may even be easier to do this if one lets some of the vitreous remain attached to the retina. After dissection, the tissue samples as well as the blood samples were centrifuged to make sure that the samples were in the bottom of the test tube before counting (see below).

2.4.5 Detection of RM and NAM

Although NAM have several advantages over RM, as concerns occupational and environmental hazards, the mode of their detection is the same, gamma irradiation, and hence the potential errors in their detection are similar. Radioactive disinte-

grations are stochastic events; that is, measuring the same sample repeatedly will result in a Poisson distribution around a mean value. The contribution of the stochastic nature of radioactivity to the total error is likely to be low, however [11].

Other possible sources of error are dependent on the gamma detector and include errors due to the separation of the energy spectrum of different radionuclides, coincidence, and geometry [11, 27, 36]. The energy spectrum for different radionuclides almost always overlap to some extent, which makes it necessary to correct for the spillover between different counting windows when using more than one nuclide in an experiment. Several different correction methods have been used to correct for the spillover between different radionuclides [12, 36, 80, 95]. With increasing number of radionuclides, the calculations become increasingly extensive. One can imagine that this was a large obstacle when the microsphere method was first introduced, but with modern gamma detectors, including software that automatically corrects for spillover, this ought not to be any major problem for the experimenter. If the radioactivity in a sample is very high, the true radioactivity of the sample may be underestimated due to coincidence; that is, if two disintegrations occur at the same time or very close in time, the detector will only register one event. Considering the low blood flow and/or small tissue pieces that can be obtained from ocular tissues, this is not likely to occur in conjunction with determination of ocular blood flows, unless the radioactivity in the blood sample is very high. Nor is the geometry likely to be of any great concern when determining ocular blood flows. As the radioactive microspheres have a higher density than blood, they will rapidly sediment on the bottom of the test tube that contains the reference blood sample. Thus, during counting, the microspheres will be close to the bottom of the detector. The microspheres in tissues samples will be dispersed through the tissue, however, meaning they will be at different heights in the detector. As most detectors are constructed as wells with a height of a few centimeters and the sensor at the bottom, disintegrations occurring higher up in the well have a lower probability to be detected. A difference in height of 2 cm

between blood sample and tissues sample has been shown to cause up to a 13% systematic error in blood flow calculations [27]. Considering the small size of the tissue samples, geometry should normally not be a significant source of error when measuring ocular blood flow, as long as one checks that the samples are at the bottom of the counting vial. Another prerequisite is of course that the automatic movement of samples in and out of the detector functions properly.

2.4.6 Detection of CM and FM

As there are several different ways to quantify the colored and fluorescent microspheres, the potential sources of error vary with the mode of detection. The spheres can be counted in histological preparations manually or by the aid of different imaging systems. After tissue digestion, the spheres can be counted in aqueous aliquots by a hemocytometer or the spheres can be dissolved to release the dye, which then is quantified by spectrophotometry or fluorospectrophotometry.

Ocular blood flows have been studied by counting of microspheres in histological sections of the optic nerve [31, 42, 81, 92] or flat mounts of the retina and choroid [2, 65, 92]. In these types of experiments, different counting errors may contribute to the variability. Spheres that are close together may be counted as one sphere or fail to be detected by the imaging system [65]. In histological sections, spheres may be counted twice if they are cut in half by the sectioning.

If CM or FM are counted in aqueous aliquots or determined by photometry (both methods have been used to quantify ocular blood flows [25, 38, 68]), the tissues must first be digested and the microspheres isolated. For photometry, the dye is subsequently extracted from the microspheres with an organic solvent. During this processing of the tissues, some microspheres may be lost, which could increase the variability in the measured blood flow values. The ease by which different tissues can be digested varies, depending on how dense the tissue is and the fat content of the tissue. In lung tissue, the organic solvent can directly dissolve the dye from the microspheres, without prior digestion

of the samples [32]. Other tissues have to be digested in sodium hydroxide [40, 68], potassium hydroxide [1, 9, 24, 32, 35, 47, 87, 94], or ethanolic KOH [70, 89]. Tissues with a high fat content are more difficult to digest, but the digestion can be improved by letting the samples autolyze for 1–2 weeks [70, 89]. Two different methods have been used for the separation of microspheres from the digested tissue: negative pressure filtration [24, 32, 47] and sedimentation [70, 89]. As the former method involves transfer of the sample from one vial to another, it is recommended to add an internal standard to the samples to calculate recovery [24]. The latter method is based on the difference in density between the FM and the ethanolic KOH (1.05 compared to 0.893 g/cm³). As the FM are heavier than the solvent, they will easily sediment by centrifugation [89]. The supernatant can then be aspirated and the FM dissolved in the same vial [70, 89]. Van Oosterhout et al. [89] achieved almost 100% recovery with the sedimentation method.

In analogy with the overlap of energy spectrum for different radionuclides, there is an overlap in the wavelength of different colors that has to be corrected for when using CM and FM. The spectral overlap is less with FM [32] than with CM [47], and the spillover only occurs between adjacent colors. FM labeled with up to six different colors can be separated without spillover correction [32]; with spillover correction, up to 13 colors have been separated [79]. Colors in the blue and violet regions of the spectrum should be avoided if possible, however [79]. Autofluorescence from the solvent and compounds released from the tissue increase background in the blue region, which increases variability in the calculated blood flow values [79, 89]. Scarlet and violet FM have minor secondary peaks that overlap with the blue spectra [79]. The spillover is also dependent on the intensity of the fluorescence; if two adjacent colors have a big difference in intensity, it can increase spillover [89]. Furthermore, one must establish that there is a linear relationship between the dye concentration (number of microspheres) and the fluorescence, as at higher dye concentrations the relationship becomes curvilinear due to quenching [32]. This must be done for each new batch of fluorescent microspheres, as the dye load

may vary from one batch to another, and hence, the relative fluorescence intensity of the different colors and the spillover may vary. The quenching is not merely dependent on the concentration of the dye that is being measured at a given wavelength; increasing the number of colors that are used increases the quenching and decreases the linear range [79]. Other potential sources of errors in conjunction with fluorescence measurements are dilution errors, unclean or unmatched cuvettes, and machine variability.

2.5 Biological Variation

2.5.1 Blood Pressure

Despite the same type of anesthesia, the mean arterial blood pressure (MABP) varies considerably between different studies. Although all data presented in Table 2.2 were determined under control conditions, there are several differences that could have influenced the blood pressure: (1) the extent of surgical intervention before the first blood flow determination varied, which may have influenced the depth of anesthesia needed, (2) in some studies, muscle relaxant drugs were used, and the type of muscle relaxant varied between studies, (3) differences in arterial blood gases, and (4) other factors such as age, gender, and subclinical diseases could have contributed.

The data from urethane-anesthetized rabbits show that there is a significant correlation between MABP and blood flow in the choroid and ciliary body, that is, correlation coefficients of 0.85 (95% confidence interval 0.42–0.97; $P \leq 0.01$) and 0.85 (95% confidence interval 0.43–0.97; $P \leq 0.01$), respectively. No correlation was found between MABP and retinal or iridal blood flow (Fig. 2.5). For the studies done in conscious rabbits, there was no significant correlation between arterial blood pressure and ocular blood flows (data not shown). The reason for this discrepancy between urethane-anesthetized and conscious rabbits is not clear, but it could be due to the relatively few studies in conscious rabbits for which blood pressure data could be retrieved. Another possible explana-

tion could be that in conscious animals, a high blood pressure (possibly due to a higher level of stress) could be a sign of high sympathetic activity, which will prevent choroidal blood flow from rising, whereas retinal blood flow is not expected to correlate with blood pressure within a normal range due to autoregulation [22].

The data from studies with cats show a significant correlation between MABP and choroidal blood flow with a correlation coefficient of 0.84 (95% confidence interval 0.41–0.97; $P \leq 0.01$) (Fig. 2.6). For monkeys, the relationship between MABP and choroidal blood flow was of borderline statistical significance only, with a correlation coefficient of 0.82 (95% confidence interval –0.24–0.99; $P = 0.09$), possibly due to the few studies (Fig. 2.6). There was no significant correlation between blood pressure and blood flow in the retina or in the anterior uvea, neither in data from studies in cats nor in data from studies in monkeys.

Thus, examining the combined data from several microsphere studies reveals the same pattern as had previously been observed in single studies [4, 5]; choroidal blood flow changed passively with the arterial blood pressure in anesthetized animals, whereas blood flow in the anterior uvea and retina was less pressure sensitive, indicating true autoregulation. Hence, some of the variability in choroidal blood flow, within as well as between studies (Table 2.2), may be due to differences in arterial blood pressure. Furthermore, these differences between the ocular tissues have to be considered when one evaluates blood flow data and there are differences in arterial blood pressure.

2.5.2 Influence of Anesthesia

General anesthesia may affect ocular blood flows in several ways: (1) depression of the cardiovascular center in the medulla oblongata or (2) general vasodilatation can decrease the arterial blood pressure and hence decrease blood flow in the choroid and anterior uvea. Some anesthesia may affect ocular blood flows (3) by direct effects on the vascular smooth muscles or (4) by changing

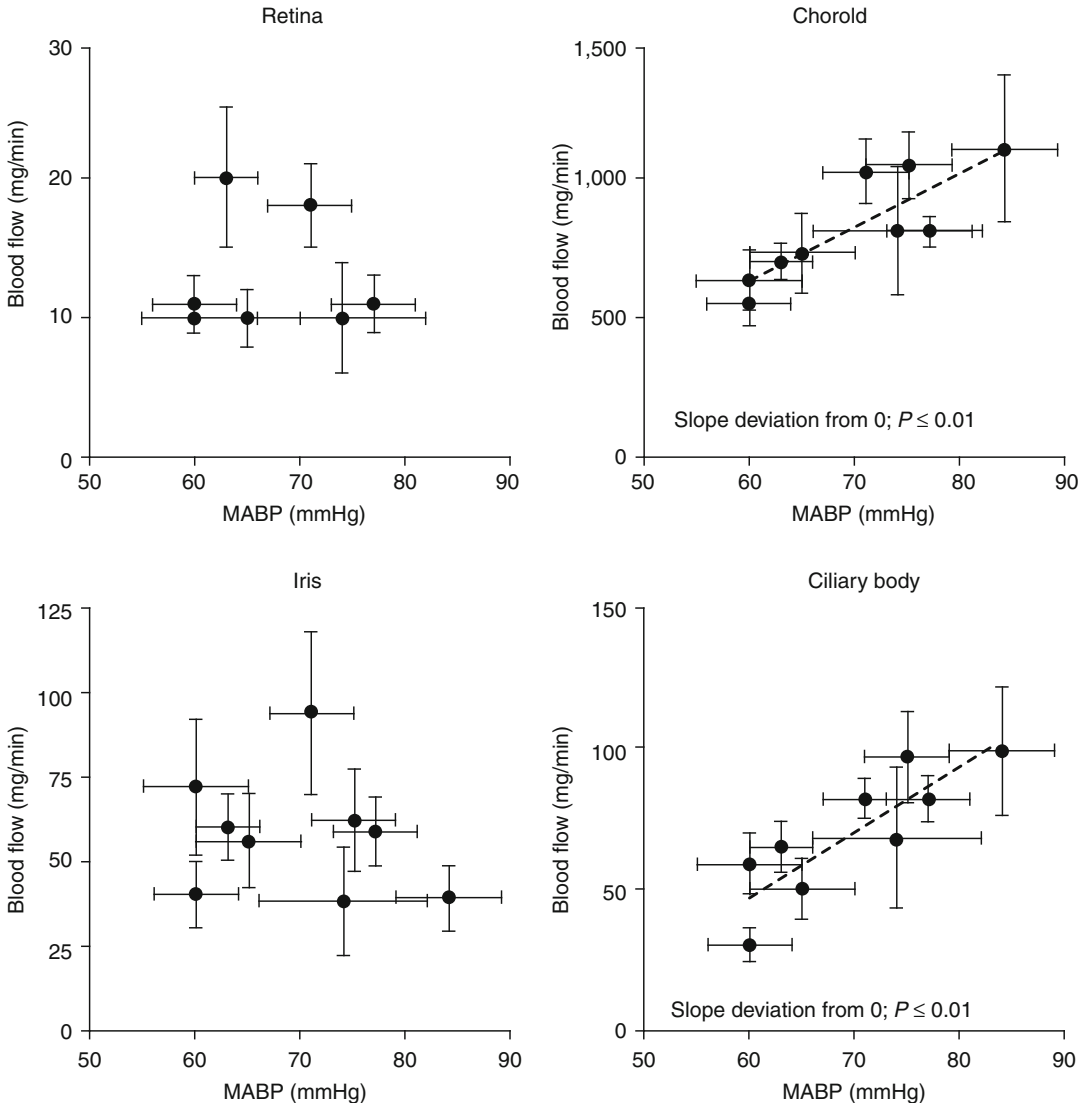


Fig. 2.5 Ocular blood flows in urethane-anesthetized rabbits, as a function of mean arterial blood pressure (MABP) (Plotted from data presented in Table 2.2). Dotted line is the linear regression line

neuronal or hormonal control of the vascular tone. For instance, pentobarbital anesthesia causes vasodilatation in the anterior uvea of the rabbit by abolishing a cholinergic vasoconstrictor tone [21]. Much of the rabbit ocular blood flow data that we have obtained are from rabbits anesthetized with urethane. Under this type of anesthesia, there is a slight sympathetic tone to the uveal blood vessels; unilateral sectioning of the cervical sympathetic nerve increases uveal blood flow on the sectioned side [46, 53]. This can be

advantageous when one wants to study vasodilatory agents or to determine if drugs or test substances exert their effects via the sympathetic nervous system. However, urethane is classified as carcinogenic, and its use demands special precautions (a special permit is needed in Sweden).

In our search for a suitable replacement for urethane, we tested different inhalation anesthetics. In these experiments, anesthesia was induced by i.v. infusion of a mixture of ketamine (5 mg kg⁻¹) and xylazine (2 mg kg⁻¹). A tracheotomy was

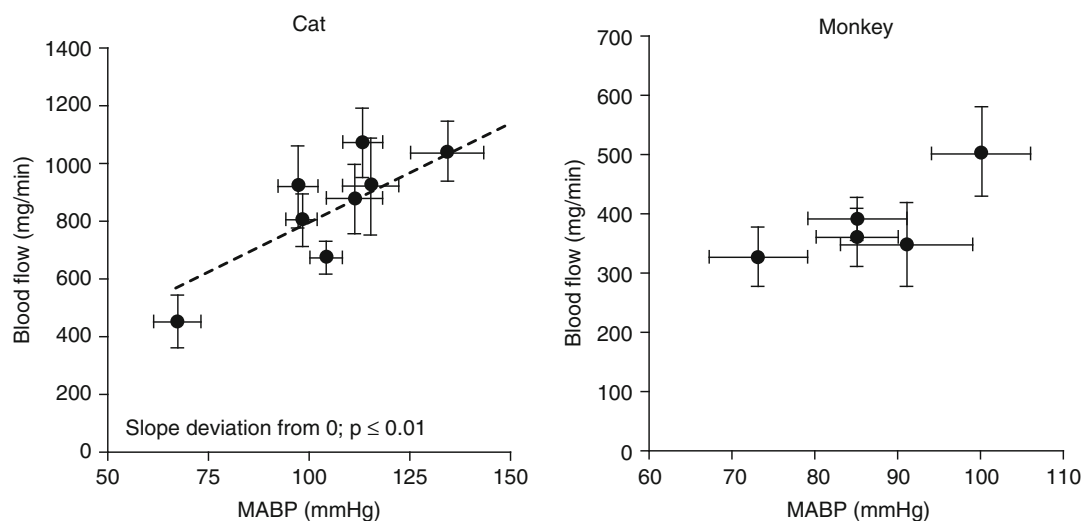


Fig. 2.6 Choroidal blood flows as a function of mean arterial blood pressure (MABP) in cats and monkeys (Plotted from data presented in Table 2.2). Dotted line is the linear regression line

made to insert a tracheal tube, and the cervical sympathetic nerve was cut on one side. The rabbit was then artificially ventilated with an Anesthesia WorkStation (AWS, Hallowell EMC, Pittsfield, MA) connected to a vaporizer for administration of the anesthetic. The AWS has a carbon dioxide absorber, as it is constructed as a closed system and intended for using oxygen as the carrier gas. As we wanted to use air as the carrier gas (to keep arterial PO_2 at a normal level), we had to remove the absorber and use a flow of air of 1.5–2.5 $l \cdot min^{-1}$ to maintain arterial blood gases at normal levels. The respiration frequency was 55–65 $breaths \cdot min^{-1}$ and the tidal volume 15–20 ml, adjusted to keep the positive airway pressure at about 10 cmH_2O . The concentration of anesthetic gas was then adjusted to maintain a surgical plane of anesthesia during the remaining part of the preparation: insertion of two arterial catheters for blood pressure registration and reference blood sampling, a venous catheter, and heart catheterization as described above. The depth of anesthesia was judged by checking the reaction to a pinch in the ear, the blinking reflex, and the corneal reflex. During surgery, it was necessary to increase the concentration of anesthetic until the corneal reflex disappeared to achieve surgical anesthesia.

Initial experiments with halothane and isoflurane revealed that when the effect of the initial dose of xylazine wore off, the rabbits tried to breathe spontaneously, making it difficult to adjust the acid–base balance. This could be prevented by a slow i.v. infusion of xylazine (a bolus dose of 0.2 mg/kg followed by continuous infusion of 0.05 $mg \cdot kg^{-1} \cdot min^{-1}$), which therefore was used subsequently. Xylazine is an adrenergic α_2 -agonist with sedative and muscle relaxant properties. Thus, in addition to causing the necessary muscle relaxant effect to allow artificial ventilation, xylazine caused a slight rise in MABP. Before the blood flow determinations, the concentration of anesthetic was reduced until a weak corneal reflex was observed, which also increased MABP slightly. Unlike halothane and isoflurane, enflurane could be used without the addition of xylazine and without causing difficulties with the ventilation. In the experiments with enflurane, ocular blood flows were therefore determined during enflurane only and at 20 and 50 min after the start of xylazine infusion. Sevoflurane could not be used without the addition of xylazine.

In the experiments with enflurane, the infusion of xylazine increased MABP slightly (47 ± 2 , 51 ± 5 , and 49 ± 5 mmHg during the three blood flow determinations, respectively), whereas uveal

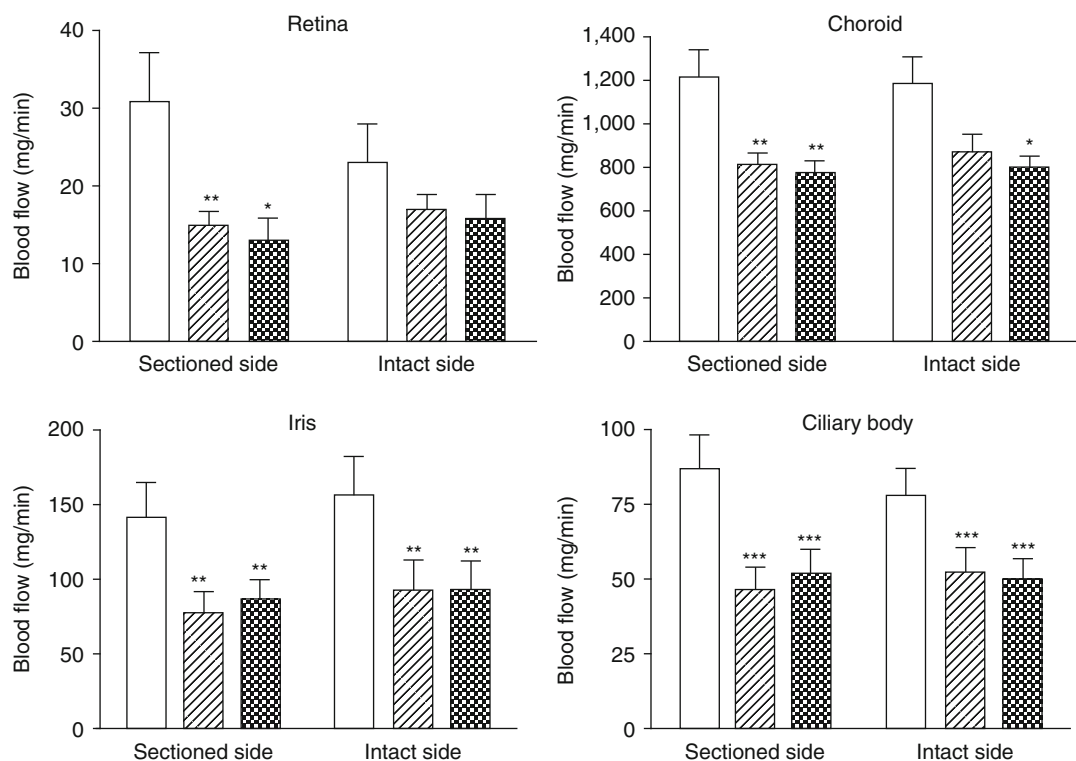


Fig. 2.7 Ocular blood flows during enflurane anesthesia alone and in combination with xylazine (0.2 mg/kg as a bolus dose followed by continuous infusion of 0.05 mg kg⁻¹ min⁻¹) in rabbits. Blood flow determinations were made during enflurane alone (open bars) and 20

(hatched bars) and 50 min (shaded bars) after the start of xylazine infusion. Mean values \pm SEM are shown ($n=7$). Statistical comparison was made with one-way ANOVA, with Tukey's test as post-ANOVA test. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$

blood flows decreased (Fig. 2.7). Retinal blood flow was also decreased by xylazine, but the effect was only statistically significant for the side with the sectioned sympathetic nerve (Fig. 2.7). Thus, xylazine appeared to cause vasoconstriction in the retina as well as in the uvea.

Comparison between the different inhalation anesthetics (with the addition of xylazine) did not reveal any significant differences, except for a significantly higher blood flow in the anterior uvea during halothane anesthesia. There was no significant difference between the eye with the sectioned sympathetic nerve supply and the control eye. Nor was uveal blood flow much different from blood flow during urethane anesthesia when compared to a previous study [53] (Table 2.5). However, during inhalation anesthesia, the MABP was lower (Table 2.5) than in most of the studies with urethane (Table 2.2), and choroidal

blood flow was higher (Table 2.5) than expected from the MABP (Table 2.2 and Fig. 2.5). Calculations of uveal vascular resistances showed that the choroidal vascular resistance was significantly lower during halothane, enflurane, and sevoflurane anesthesia than during urethane anesthesia (Fig. 2.8). Vascular resistance in the iris was also lower during inhalation anesthesia, but only halothane was significantly different compared to urethane (Fig. 2.8). Vascular resistance in the ciliary body was not significantly different during inhalation anesthesia compared to urethane (Fig. 2.8). Retinal blood flow tended to be slightly higher (Table 2.5) and retinal vascular resistance lower (data not shown) with halothane, enflurane, and sevoflurane than with isoflurane and urethane.

At least two factors could have contributed to the lower vascular tone in the uvea with the inha-

Table 2.5 Systemic cardiovascular parameters, arterial blood gases, and ocular blood flows during different inhalation anesthesia combined with i.v. infusion of xylazine (0.2 mg/kg as a bolus dose followed by continuous infusion of 0.05 mg kg⁻¹ min⁻¹) in rabbits

	Halothane (1–1.5%) ^b <i>n</i> =6	Enflurane (2–3.5%) ^b <i>n</i> =7	Isoflurane (1–2%) ^b <i>n</i> =7	Sevoflurane (1.6–3.5%) ^b <i>n</i> =7	Urethane ^a <i>n</i> =12
<i>Cardiovascular parameters</i>					
MABP (mmHg) ^c	56±3**	51±5**	61±4*	64±3	77±4
Heart rate (min ⁻¹)	189±10**	182±4**	190±10**	161±7**	312±8
Cardiac output (g min ⁻¹)	457±53	341±26	267±20**	438±36	415±29
TPR (100 mmHg min g ⁻¹) ^d	13±2*	15±1	24±3	15±1	19±1
<i>Arterial blood gases</i>					
pH	7.42±0.01	7.43±0.01	7.48±0.01	7.48±0.02	7.47±0.01
PCO ₂ (kPa)	5.7±0.1**	5.5±0.1**	5.5±0.1**	5.5±0.1**	4.6±0.1
PO ₂ (kPa)	10.9±0.7	11.2±0.3	13.2±0.3	11.7±0.5	11.9±0.5
<i>Ocular blood flows^e</i>					
Retina I	14±1	17±2	11±2	15±3	11±2
Retina S	15±2	15±2	10±2	12±1	13±2
Choroid I	1,129±103	863±92	783±86	1,054±153	805±65
Choroid S	1,127±118	814±56	836±86	1,021±105	951±79 ^f
Iris I	136±12*	93±19	52±10	86±10	59±10
Iris S	152±19**	77±15	59±12	86±7	71±12
Ciliary body I	75±12	52±8*	42±2*	51±8*	82±8
Ciliary body S	82±16	47±7**	41±5**	55±9**	105±12 ^f

* $P \leq 0.05$, ** $P \leq 0.01$ ^aComparison was made with urethane anesthesia (one-way ANOVA, with Dunnett's test as post-ANOVA test); urethane data is from a previously published study [53]^bInhaled concentration that produced a weak corneal reflex^cMean arterial blood pressure^dTotal peripheral resistance^eI=intact sympathetic nerve supply, S=cervical sympathetic nerve sectioned^fDenote a significant difference ($P \leq 0.05$) between the two sides (two-tailed students' *t*-test; paired data)

lation anesthetics. A direct vasodilatory effect on the uveal blood vessels is one possibility, but decreased vascular tone due higher PCO₂ in the experiments with inhalation anesthetics (Table 2.5) could also have contributed (see below). Furthermore, the vasoconstrictive effect of xylazine in the enflurane experiments indicates that the vasodilatory effect of the inhalation anesthetics could be even larger.

In the cat, increased inhaled concentrations of enflurane (0.5, 1.0 and 1.5 MAC) caused a concentration-dependent decrease in MABP and choroidal vascular resistance and a significant increase in choroidal blood flow by the highest concentration. Retinal blood flow was not significantly affected, but retinal vascular resistance fell with increasing concentrations [75]. In similar

experiments with halothane, MABP and choroidal blood flow decreased with increasing concentration, whereas choroidal vascular resistance was not significantly affected. Retinal blood flow increased and retinal vascular resistance decreased with increased concentration [74]. These data indicate that the two inhalation anesthetics may have different effects on choroidal and retinal vasculature in the cat. Enflurane appears to have a direct vasodilatory effect on choroidal vessels but no or a much smaller effect on retinal vessels. With halothane, the effects seem to be reversed; that is, retinal vessels are dilated, but choroidal vessels are not significantly affected. In our experiments, choroidal vascular resistance was lower than with urethane for both enflurane and halothane, indicating that both anesthetics dilated

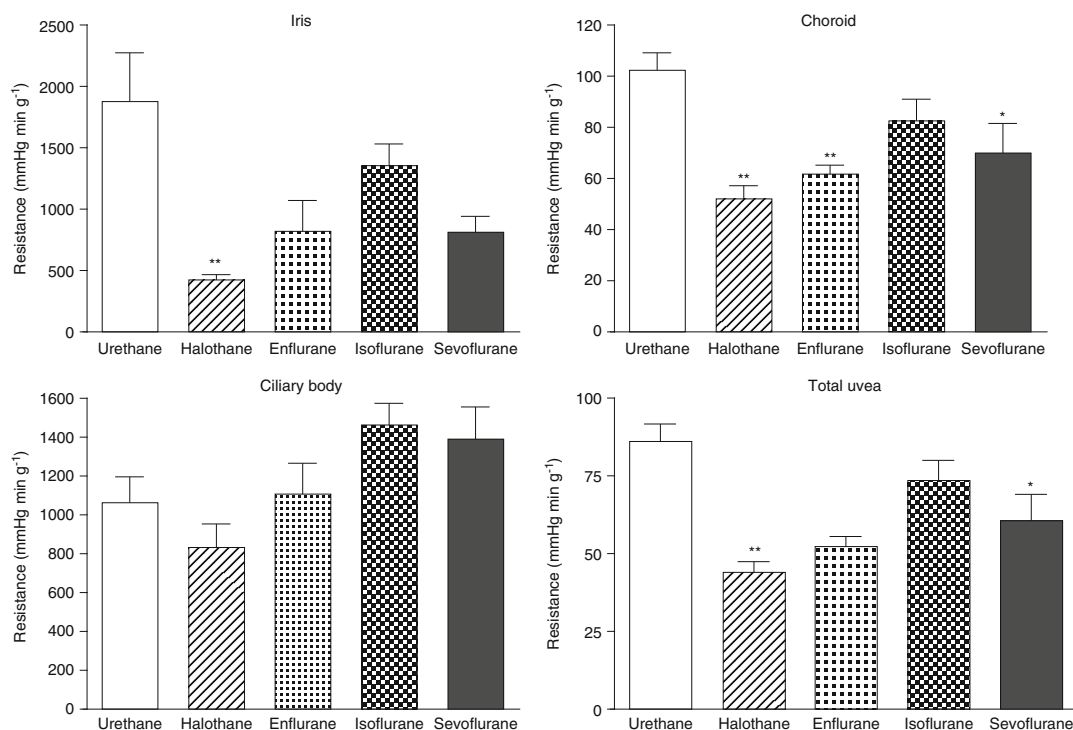


Fig. 2.8 Uveal vascular resistances under different types of inhalation anesthesia in combination with i.v. infusion of xylazine (0.2 mg/kg as a bolus dose followed by continuous infusion of 0.05 mg kg⁻¹ min⁻¹) in rabbits. Mean values \pm SEM are shown ($n=6$ for halothane; $n=7$ for

enflurane, isoflurane, and sevoflurane; and $n=12$ for urethane). Comparison was made with urethane anesthesia (one-way ANOVA with Dunnett's test as post-ANOVA test) (Data from a previously published study [53])

choroidal blood vessels. Thus, it seems the effect of enflurane on choroidal vasculature may differ between rabbits and cats. Furthermore, the high retinal blood flow during enflurane only and the decrease in blood flow caused by xylazine (Fig. 2.7.) indicate that enflurane has a vasodilatory effect on retinal vessels in the rabbit. The inhaled concentrations of enflurane and halothane in our experiments correspond to 0.7–1.2 MAC [28], which is in the same range as used by Roth in cats [74, 75].

2.5.3 Arterial Blood Gases

Differences in arterial oxygen and carbon dioxide tension are other factors that could contribute to the biological variation. In one of the first microsphere studies on ocular blood flow, it was shown

that hypercapnia increases retinal as well as uveal blood flows in cats [4]. By the use of the microsphere technique, choroidal vasodilatation during hypercapnia has also been observed in the rabbit [14]. A recent study in the rat, however, indicates that the response is different in this species, as hypercapnia had no effect on choroidal blood flow, but hypocapnia decreased blood flow [91]. Retinal blood flow was decreased by hypocapnia and increased by hypercapnia in the rat [91]. It is well known that the arterial oxygen tension significantly affects retinal vascular tone; hypoxia causing vasodilatation and hyperoxia, vasoconstriction. The effect on uveal vessels is less or absent, however [26]. The number of studies done with microspheres is few, but it has been shown that retinal blood flow is increased by arterial hypoxia in the cat [2] and that the effects of hypoxia on uveal blood flows are minor in the rabbit [48].

It may be worth noting that different anesthesia may affect acid–base balance differently. In our experiments done under urethane anesthesia in rabbits, base excess (BE) was mostly negative, indicating metabolic acidosis. A sodium bicarbonate solution was routinely given to adjust BE and pH to normal values. In the experiments with inhalation anesthetics, BE was mostly positive, indicating metabolic alkalosis. Hence, the arterial PCO_2 was allowed to be slightly higher than normal (Table 2.5), to keep arterial pH closer to normal.

2.5.4 Other Possible Causes for Biological Variability

Age, gender, seasonal and hormonal variations, and diseases are other factors that could contribute to biological variability, but the influence of these factors on the measurements of ocular blood flows are largely unknown. In most of the studies from the Department of Physiology, Uppsala University (Table 2.2), both sexes of animals were used, which could have contributed to the variability. As regards age, the rabbits were mostly about the same age, as they were ordered to be a certain weight/age from special breeders. For cats and monkeys, there could be a much larger variation in age within and between different studies. At the time of the earlier studies, there was no law on approved breeders of laboratory animals in Sweden. As cats therefore could be obtained from several different breeders and even private persons, their age was largely unknown. Similarly, the monkeys were wild-caught animals of unknown age. There is one study for which we know the exact age of every animal though. In this study, ocular blood flows determined in Abyssinian cats with hereditary retinal degeneration were compared with blood flows in normal cats. As the disease progresses slowly, we tried to find age-matched controls. The results showed that uveal blood flows were negatively correlated with age in the Abyssinian cats, most likely as a result of the progression of the disease, but not in the controls. However, the

age range was narrower (0.5–4.8 years compared to 0.9–8.1 years) for the controls and not as evenly spread [60]. Thus, a decrease with age in uveal blood flow in normal cats cannot be completely excluded. A decrease in pulsatile ocular blood flow by age has been observed in humans [72], and choroidal blood flow, measured by Laser Doppler flowmetry, is decreased with age in pigeons [29].

Diseases that had not yet given any clinical signs of illness in the animal could also contribute to the biological variation. Over the years, it happened on two or three occasions that we had to stop doing experiments in rabbit, due to respiratory diseases in the animals. The rabbits seemed healthy, when sitting in their cages, but during the experiments, it was impossible to adjust their acid–base balance to normal values. Sampling of blood from an ear artery, in the conscious animals, revealed respiratory insufficiency.

2.6 Summary for the Clinician

The microsphere method is a reliable method for determination of ocular blood flows in experimental animals. The advantages with the method are: (1) it measures blood flow directly, (2) it is suitable for measuring blood flow in small pieces of tissue and in inaccessible tissues, and (3) it does not disturb the normal circulation, if the experiments are properly designed. Radioactive microspheres have been the most commonly used type of microsphere for ocular blood flow determinations as well as for regional blood flow determinations in general. Due to the health and environmental issues connected with the use of radioactive material, the use of radioactive microspheres has declined during the last decade. Colored and fluorescent microspheres have to some extent replaced the radioactive ones, but these are more labor intensive. A promising new development is the use of neutron-activated microspheres, which have the same advantages as radioactive microspheres but lack the disadvantages.

For reliable measurements with the microsphere method, the size and number of microspheres should be optimized for the tissue under investigation. In larger species (e.g., rabbit, cat, monkey), the most commonly used size and injection number are 15 μm and $1\text{--}2 \times 10^6$, which is a good compromise if one wants to measure uveal as well as retinal blood flows and cannot use more than one size in an experiment. If retinal blood flow and/or blood flow in the optic nerve head are the primary interest, smaller spheres (10 μm) and a larger number of spheres are preferred. In smaller species (e.g., rat), spheres with a diameter of 10 μm are recommended for measurement of choroidal blood flow and 8- μm spheres for retina and optic nerve. The "400 spheres/piece of tissue rule" is difficult to achieve for the retina and anterior uvea, but doing more experiments can compensate for the paucity of microspheres in the tissues. Furthermore, biological variation contributes more to the error in the measurements than the paucity of microspheres. Differences in arterial blood pressure, anesthesia, and arterial blood gases contribute to the variation of reported values on ocular blood flows. Other factors such as diseases, age, gender, and hormonal and seasonal variations could contribute to the variation as well.

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