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A TECHNICAL NOTE FOR THE SQUEEZED-FLOW PERFUSION METHOD: SENSITIVE MONITORING OF VASOMOTION OF MEDIUM-SIZED, ISOLATED BLOOD VESSELS

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We introduce an improved perfusion technique that allows sensitive detection of vasomotion as well as differential estimation of drug action upon luminal or abluminal application. The technique employs a short, isolated segment of medium-sized artery (internal diameter 0.7-4.0 mm) that would not generate sufficient resistance by conventional technique. By our method, the constant flow goes through a narrow gap between the intimal surface of the vessel and the outer wall of the blind-end tubing that occupies a large dead-space in the vascular lumen. Due to this narrow clearance, subtle vasomotion can be amplified onto the perfusion pressure, while not much flow-rate is necessary to maintain the baseline perfusion pressure. The method also allows application of drugs either from intimal or adventitial side. This technique should provide powerful means for physiological and pharmacological studies on blood vessels.

Key words: blood vessel, contraction, relaxation, perfusion pressure, Poiseuille's law, drug application

INTRODUCTION

Monitoring of contraction/relaxation responses of isolated blood vessels has been useful and essential not only for studying vascular physiology but also for studying vascular pharmacology. There have been scientific needs to determine the vasomotion quantitatively using isolated arteries of medium size (neither a conduit artery nor a resistance artery). To this end, helical-cut strips and circular-cut ring preparations are commonly used. However, these techniques have a definite limitation, i.e. they cannot distinguish the action of a drug on its luminal application from that on the abluminal application, because the drugs added in the bathing medium may get into the vascular tissue from both surfaces in these experimental techniques. In fact, it has been known that the sensitivity of vascular responses to some, if not all, of the vasoactive substances (e.g. noradrenaline) is higher when the agent is applied from intimal side than from adventitial side (1-5). To differentiate the responses to luminal and abluminal drug application, perfusion technique for an isolated vascular segment is effective and essential.

In conventional perfusion technique, the perfusion-pressure is monitored immediately upstream the vascular segment, or for more accuracy, the pressure-difference between inflow and outflow of the segment is measured. However, a segment of medium-sized vessels is too short and the lumen diameter is too large to generate sufficient resistance against the perfusate flow. To resolve this problem, in general, a back-pressure is applied, i.e. the outlet following the vascular segment is narrowed. This back-pressure increases the intra-segmental pressure and thereby wall tension, and thus amplifies the changes in vascular tension to permit sensitive monitoring. However, the back-pressure (usually 40-50 mmHg) makes the vascular tissue leaky and hence vessel tissues do not endure for a substantial period of experiment.

Instead of applying a passive tension (back-pressure) onto the vessel, it should be better for the segment to generate active tone, active resistance, and thereby active pressure. To this end, the

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effective cross-sectional area of the lumen should be narrowed by some space-occupying item within the lumen. We thought of inserting polyethylene tubing to spare most of the luminal space, and found that this was efficient, useful, and convenient. Because, in this new improved system, the perfusate flows through the narrow clearance, we refer to the technique as "the squeezed-flow perfusion technique."

MATERIALS AND TECHNIQUE

Overview of the system

Figure 1 depicts the overall system. Reservoir A contains the perfusate (physiological solution such as Ringer-Locke solution) which is thermo-regulated (37 ± 0.3 ℃) and aerated with carbogen gas (95% O_2 / 5% CO_2) to maintain the fluid's pH at 7.35-7.42. If necessary, perfusate can be replaced with the one containing certain drug of the known concentration. The switching of perfusate can be readily done by using a 3-way stopcock. The perfusate is driven by a peristaltic pump at a constant flow rate (e.g. 1.0 ml/min) through polyethylene tubing (PE-240, INTRAMEDIC™, Clay Adams, Parsippany, NJ, USA; see Table 1 for specifications) which forms most of the circuit except peristaltic tubing and the tubing for setting the vascular segment. A pulse damper is set just before the injection port and pressure transducer. The pulse damper consists of polyvinyl tubing (ca. 200 mm long) and a microfilter (filter pore size: 4.5 μm, Millex™, Millipore Co., Bedford, MA, USA). Air is slightly pressurized into the polyvinyl tubing by using a syringe through the microfilter. Because the air does not pass through the dry microfilter, the slightly pressurized and enclosed air functions as efficient pulse damper. Due to this efficient damper, pulsation is little enough to permit sensitive monitor-

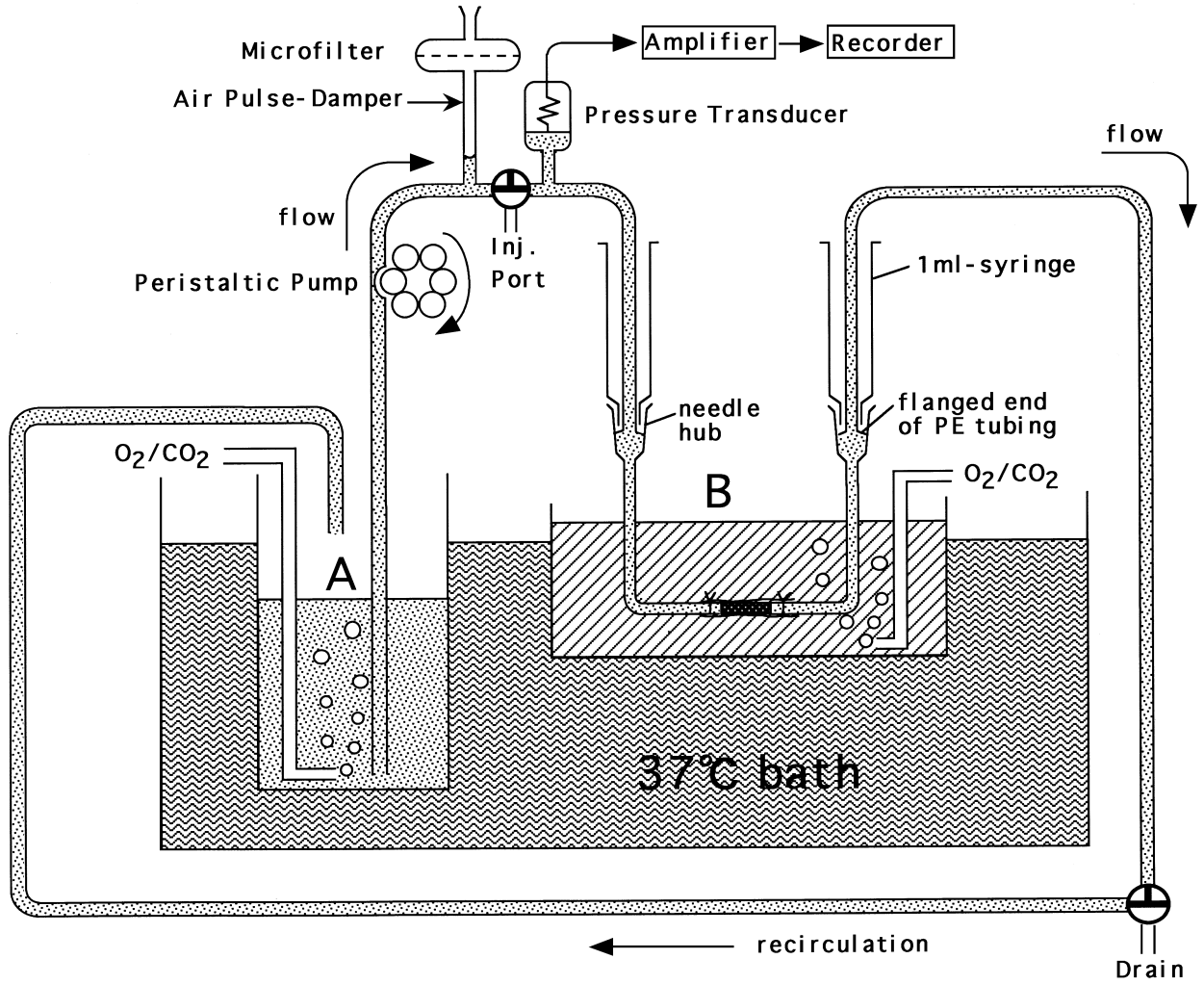

Fig. 1. Diagram of the overall system for squeezed-flow perfusion. Detail explanation is given in the text.

ing of the perfusion pressure.

The test drug solution which is prepared in a known concentration is injected through the injection port either by hand or by a micro-infusion pump (Harvard Apparatus, South Natik, MA, USA). Perfusion pressure is monitored with pressure transducer and polygraph system (Nihon Kohden, Tokyo).

Reservoir B is the tissue bath containing the same physiological solution as that in the reservoir A and the medium is also thermo-regulated and aerated. We made the tissue bath by cutting a 20-ml disposable syringe to remove 1/3 of the syringe along its long axis. The blood vessel segment is set in the tissue bath by being supported with a pair of connection syringes (1 ml-disposable syringes). The syringes can be easily connected to needle hubs which are parts of squeezed-flow apparatus. The hole of the 1-ml syringe has been enlarged so that the circuit tubing (PE-240) can barely pass through the hole with their contact being water-tight. Then, the edge of the tubing is flanged by a flanging tool for liquid chromatography use (TOSOH, Tokyo). Thus, when the syringe is connected to the needle hub, the connection is water-tight. Reservoir B content can be replaced with drug solution, which is tested for its abluminal application. After passing through the vessel segment, the perfusate can be drained (single-passage flow system), or recirculated back to the reservoir A (recirculation system) depending on the purpose of respective studies.

Isolated vascular segment: the core part of the system

Figure 2 shows the core part of the system, "the squeezed-flow perfusion system." The upper panel shows a longitudinal-cut view along the flow direction. The lower panel is the oblique view of the same part as the upper panel. The perfusate comes from left-hand side through the polyethylene tubing. The flow then goes out of the proximal orifices (in-to-out orifices) because the tubing downstream here is obstructed with a clay-plugged needle. Therefore, the flow gets into the narrow clearance which is formed by the intimal surface of the vessel and the outer surface of the obstructed tubing. The perfusate then flows back into the outlet tubing through the distal orifices (out-to-in orifices). Because the flow squeezes down through a narrow clearance, the perfusion resistance is much greater than that created by the conventional perfusion technique. Due to this narrow clearance, subtle changes in vascular tone

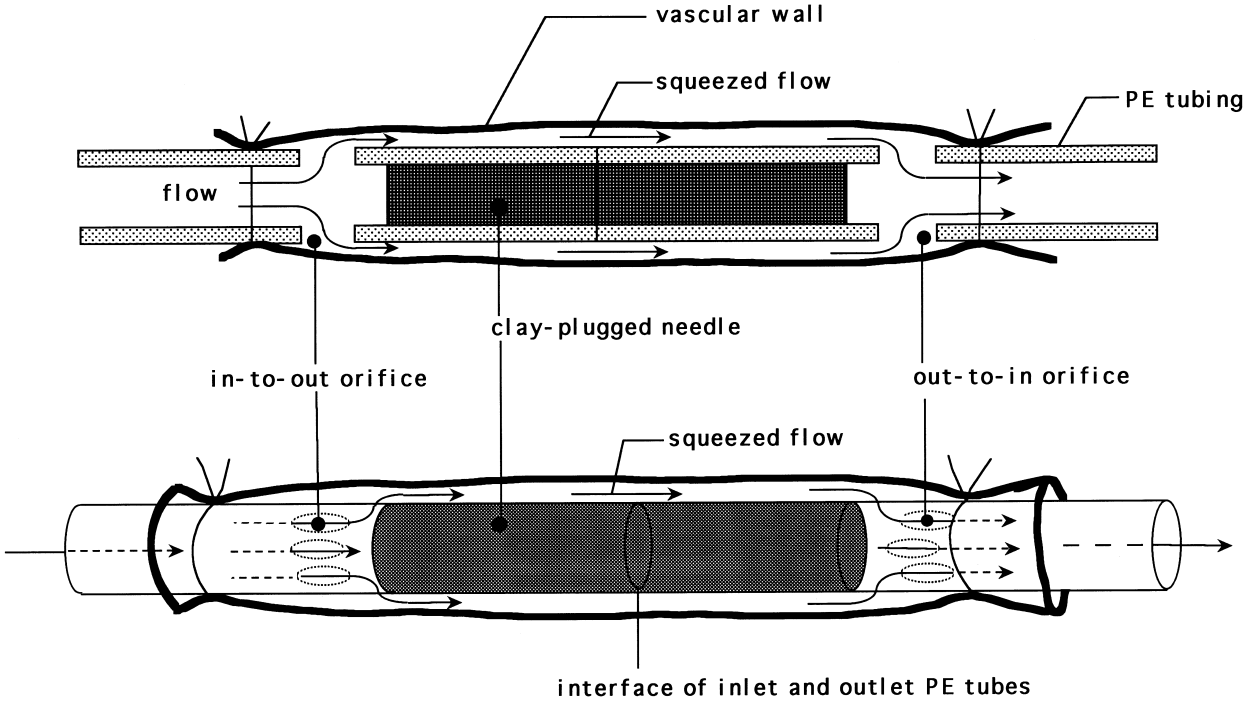

Fig. 2. The core part of the squeezed-flow perfusion system. The upper panel shows the cut view, and the lower panel illustrates the oblique view of the core part of the perfusion system. Detail explanation is given in the text.

should be amplified to considerable magnitude of changes in perfusion pressure, which reaches the reasonable ranges to be detected with sufficient accuracy.

How to set a vessel segment to the squeezed-flow system

This section describes a practical guide for the new perfusion technique. Fig. 3 illustrates the procedure for setting up the segment.

Step 1) To prepare the isolated blood vessels (Fig. 3, 3rd row): Medium-sized artery (veins can also be studied by this method) is isolated from any organ or tissue of any animal species. All the branches are ligated with thin silk suture to protect from the perfusate leakage.

Step 2) To prepare the tubing which has inflow and outflow orifices, and a clay-plugged needle (Fig. 3, top row): A polyethylene tube with orifices and a stopper (clay-plugged) needle for the core part of the system are custom-made for optimum fitting to respective vessel preparations.

Step 2-1) To select the proper size of tubing (Table 1): At first, proper size of the tubing should be chosen. The optimum size of the tubing to be inserted into the isolated vessel segment is such that it snugly fits into the lumen of the vessel segment without involuntary injury onto the endothelium. You may try some of the ready-made PE tubing with a series of dimensions as shown in Table 1. To insert the tubing smoothly, it is better to blunt the razor-cut edge of the tubing in a small flame. If the tubing is too loose, then it would be difficult to obtain the sufficient resistance to the flow as well as the optimum base-line perfusion pressure. If too tight, then the endothelium may be scraped off.

Step 2-2) Determination of length of PE tube, length of stopper needle, and site to open orifices: After the proper size (outer diameter) is selected, the tubing is inserted through the vessel segment and then the length of the tubing is determined so that the tubing outlies approx. 15 mm on both sides of the vessel segment (as shown in the 4th row, Fig. 3). At this condition, the positions where orifices should be opened are determined, and the length of the stopper needle is also determined (as shown in the 2nd row, Fig. 3).

Step 2-3) To make orifices: For large size tubing (e.g. outer diameter >1.5 mm), orifices are opened with a sharp razor blade. For smaller tubing, orifices are made by high-speed drill of proper bore size which is attached to a hobby router. An important issue here is that the size of orifices should be adjusted so that the orifices per se do not generate any resistance to the flow. In other words, the size of orifices should be large enough so as to freely pass the perfusate, yet should not be too large to keep the physical strength and structure of the tubing. After the orifices are made, the tubing is cut into equal halves at the middle position of the inlet and outlet orifices (the position which is indicated as "interface" in Fig. 2; also see Fig. 3, the 2nd row).

Step 2-4) To make a stopper needle: To prepare a stopper needle, an injection needle which fits into the tubing (select the proper size according to the Table 1) is cut with proper length as determined as above. Both ends of the needle are blunted and the edges are rounded with a hobby router for easy insertion into the tubing. Then, both ends of the needle are tightly plugged with hematocrit sealant clay (Terumo, Tokyo). Onto this plugged needle, the equal halves of the tubing are inserted until their central edges are firmly attached together (Fig. 2, lower panel, the "interface" of the tubing).

Step 3) Insertion of the set tubing into vessel segment: A tip to do this step is that you should have a feeling to put the vessel segment onto the tubing rather than the feeling to insert the tubing into the vessel segment. The vessel segment is then tied up at both ends with silk suture (Fig. 3, the 4th row). Cautions should be adopted not to injure the endothelium, not to tie up the vessel over the orifices, and not to tie up too firmly in order to avoid crashing the lumen of the tubing.

Step 4) Mounting the core part to the holding syringes: Both ends of the above-set tubing are connected to blunt-end needles (Fig. 3, the bottom row) which have already been water-tightly connected by their hubs to 1 ml-syringes. The pair of syringes are then held with clamps so that the core perfusion segment is immersed in the bathing media in the organ bath (Fig. 1, reservoir B).

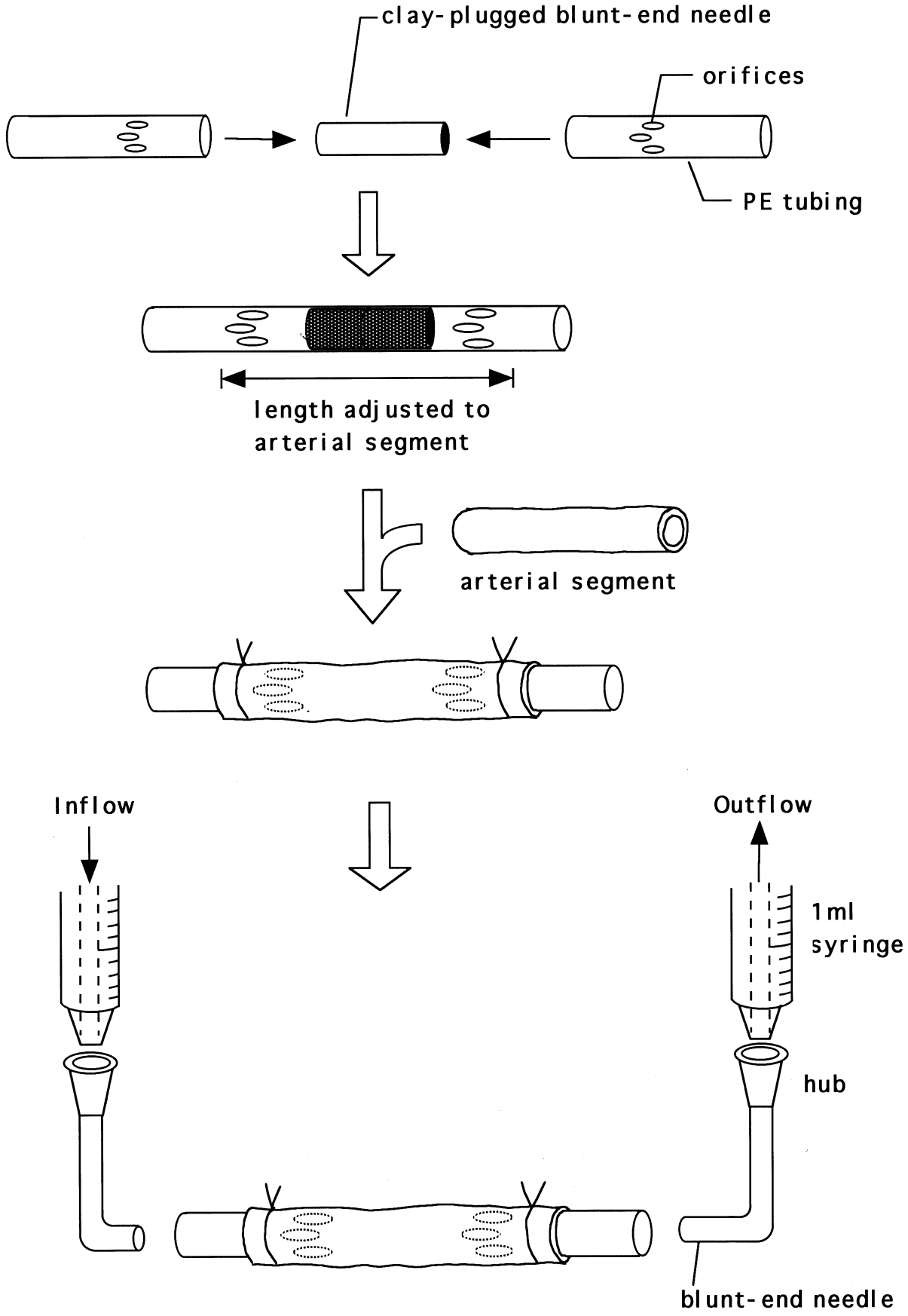

Fig. 3. Step-by-step procedures for setting up the vascular segment. Detail is explained in the text. See the section "How to set a vessel segment to the squeezed-flow system."

Table 1. Dimension of PE tubing with various sizes and respective size-fitting needles

PE#		10	20	50	60	90	100	160	190	200	205	240	260	280	320
diameters (mm)	inside	0.28	0.38	0.58	0.76	0.86	0.86	1.14	1.19	1.40	1.57	1.67	1.77	2.15	2.69
	outside	0.61	1.09	0.965	1.22	1.27	1.52	1.57	1.70	1.90	2.08	2.42	2.80	3.25	3.50
gauge of needle which fits into tubing		30	27 26	23	21	20	20	18	18	17	16	15	15	14	12

Adapted from the data sheet of Intramedic polyethylene tubing (Clay Adams, Parsippany, NJ, USA)

DISCUSSION

Advantages of the new method

We explain how efficiently this improved system may work. The advantage of the squeezed-flow perfusion system is twofold: firstly the considerable amplification of changes in perfusion pressure, and secondly the capability for differential application of drug solution either from luminal or abluminal side.

Amplification of pressure change by the new system

Firstly, we explain how much the pressure changes obtained by conventional perfusion technique may be amplified if the new technique is used. Here Poiseuille's law is applicable (6). It gives the hydraulic resistance equation

$$R = (Pi - Po)/Q = 8\eta l/\pi r^4$$

where R is the resistance, Pi is the pressure at the inflow, Po is the pressure at the outflow, Q is the flow, η is the viscosity of the fluid, l is the length of the vascular segment, and r is the radius of the vessel segment. Thus, the resistance, R, is inversely proportional to the fourth power of the radius, r.

Now, let the inside diameter of an isolated artery be 2.5 mm under a constant flow and at non-stimulated condition (Fig. 4, panel A), and let the vessel constrict in response to a drug so that its diameter becomes 2.4 mm (Fig. 4, panel B). According to the Poiseuille's law, the resistance before vasoconstriction (R_1) is $a/1.25^4 = a/2.44$, while the resistance after constriction (R_2) is $a/1.2^4 = a/2.074$, where the factor "a" is a constant because it is assumed that the length of the vascular segment (l), the viscosity of the perfusate fluid (η), and the flow rate (Q) are practically not changed throughout the experiment. Therefore, the ratio of resistance after/before constriction (R_2/R_1) is 1.17, meaning that the resistance increases 1.17 times and the net increase is only 17%. In the Fig. 4, S_L stands for the cross-sectional area of the effective (the live, not the dead) lumen space through which the perfusate fluid flows. When the vessel at the state (A) constricts to become the state (B), S_L decreases to 92.2% that of the control state. Such a little decrease in the lumen area should be hardly reflected on the increase in resistance.

Next, consider the same magnitude of constriction (inner diameter changes from 2.5 mm to 2.4 mm) in the squeezed-flow perfusion system. Because the resistance to flow is already high due to the narrow clearance even at the basal condition (under the basal tone of the segment), the new perfusion system requires much less flow rate (Q) to generate a basal perfusion pressure that is sufficiently high for sensitive monitoring. Thus, in general, the constant "b" is much less than the constant "a" as in a conventional system. Let the inner diameter of the vessel decrease from 2.5 mm (panel C) to 2.4 mm (panel D). The hatched area within the vessel lumen is the dead-space occupied by the obstructed tubing (e.g., it is PE-205 tubing in the panels C and D of Fig. 4). Therefore, the effective lumen area, S_L, is 1.51 mm^2 (100% control state) before constriction and 1.126 mm^2 (74.6% of the control) after constriction response. Thus, the decrease in S_L is exaggerated in the squeezed-flow system than in the conventional perfusion system when the vessel segment exerts the same magnitude of decrease in the diameter. This change should result in a 79% increase in the resistance ($R'_2/R'_1 = 1.79$). Thus, a subtle increase in resistance in the conventional system (17% increase) can be amplified into as high as ca. 80% increase (4.6-fold amplification) if the new technique is applied: this means a 4.6-fold

amplification of the pressure-rise in the new system compared with the pressure-rise in the conventional system.

There might be a critique that a 4.6-fold amplification is not sufficiently high as has been expected. However, careful consideration is needed for the fact that the basal flow for the conventional perfusion system (Q_1) is much higher than that for the squeezed-flow perfusion system (Q_2). Hence, if the flow rate Q_2 equals Q_1 (i.e., if the constant "a" equals the constant "b" in Fig. 4), then the resistance and thereby pressure-rise can be extremely exaggerated. In Fig. 4, the panel E shows a vessel with its lumen's cross-sectional area being equivalent to the effective (the live, not the dead) lumen area of the vessel shown in panel C. The radius of the vessel in panel E is 0.347 mm, and that of the panel C is 1.25 mm. Similarly, the radius in panel F is 0.300 mm, and that in panel D is 1.2 mm. If Poiseuille's law applies, and if the same constant flow is given to both large and small vessels, then the resistance generated by the vessel C and E is 168 times that generated by the vessel A ($R_1^4/R'_1{}^4 = 1.25^4/0.347^4$). Similarly, the resistance generated by the vessel D and F is 256 times that generated by the vessel B ($R_2^4/R'_2{}^4 = 1.20^4/0.30^4$).

It might also be criticized that Poiseuille's law is not applicable to the squeezed-flow system because shape of the stream is not cylindrical but hollow-fiber-like. However, for simplicity, we believe it may be allowed to apply the law for the following reasons. 1) The flow is virtually steady-flow because the pulse-damper used here is so effective that the pulsation due to peristaltic pumping is offset almost completely (this can be confirmed with some examples of real tracing which are reproduced in Ref. 5). 2) Although it flows through a narrow clearance, it is a laminar flow which goes along smooth surfaces of vascular endothelium and polyethylene tubing. 3) Both viscosity of the perfusate and flow speed was sufficiently low to permit the approximation to the law. By the same reasons, we regarded the flow in panel (C), Fig. 4 as equivalent to that in panel (E), also regarded the flow in panel (D) as equivalent to that in panel (F), although shear stress is different due to the difference in flow shape. Notwithstanding our rough approximation, the advantage of the squeezed-flow method to amplify vascular resistance for sensitive monitoring of vasomotion should deserve practice.

Direction of drug application

There has been reported a kind of squeezed-flow perfusion method which was called "the stainless cannula inserting method" (7, 8). The method employed an injection needle which had several side holes and a plugged end (Fig. 5, left). The perfusion system was efficient to sensitively detect the vasomotion on a constant flow. In fact, it was that method to which we added our original modification for improvement. What we have modified is to correct the drawback of their system that the perfusate goes out of the vascular wall after passing through the narrow clearance and thereby the perfusate is mixed up with the outer bathing medium (Fig. 5, left). Due to this mixing, it is impossible to apply a certain drug exclusively from the inside of the vessel. In contrast, our system can do that (Fig. 5, right). Using our new system, it is possible to differentiate the pharmacological effect on vasomotion of a certain drug which is applied intra-luminally from that applied extra-luminally (5).

In addition, our system provides the convenient way to simultaneous monitoring of vasomotion (pharmacodynamic study) and the fate of substances such as uptake, processing, and degradation within or on the surface of the vessel wall (pharmacokinetic study) (9). Moreover, our system can apply a field stimulation by placing a pair of platinum plate electrodes along the vessel segment. Using such system, we can stimulate nerves innervating the adventitia and the media of the vessel wall while applying acetylcholine from intimal side or adventitial side. A typical application of such squeezed-flow perfusion system combined with field stimulation is reported previously (5). Taking advantage of the new perfusion system in combination with field stimulation, the paper demonstrated the asymmetrical effects of intra- and extra-luminally applied acetylcholine on the sympathetic liberation of noradrenaline.

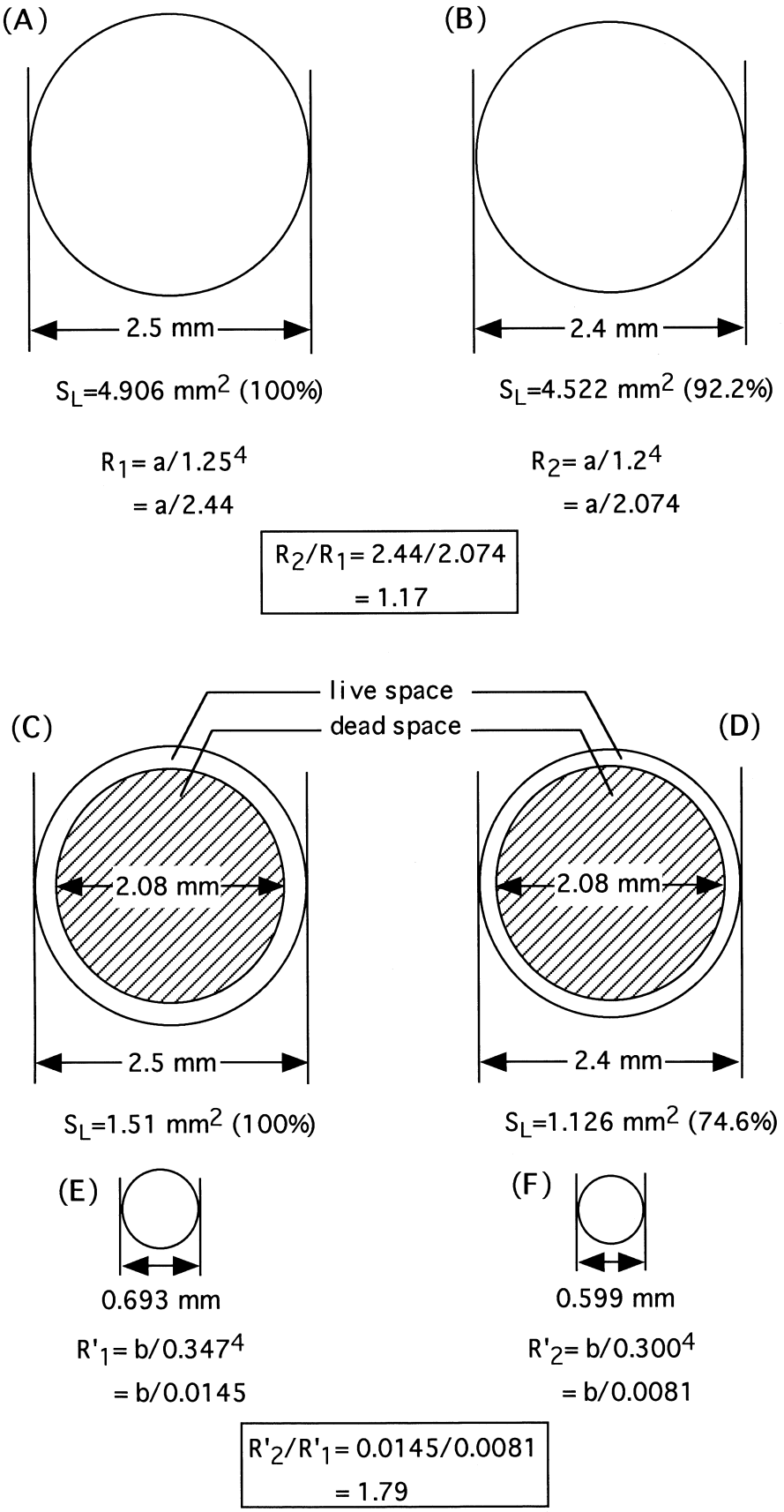

Fig. 4. Examples of calculation in application of the Poiseuille's law. See the text for detail explanation. SL: cross-sectional area of the live (effective) vascular lumen space through which the perfusate flows. R_1, R'_1, R_2, and R'_2: resistance against the constant flow. a and b: constants derived from Poiseuille's equation.

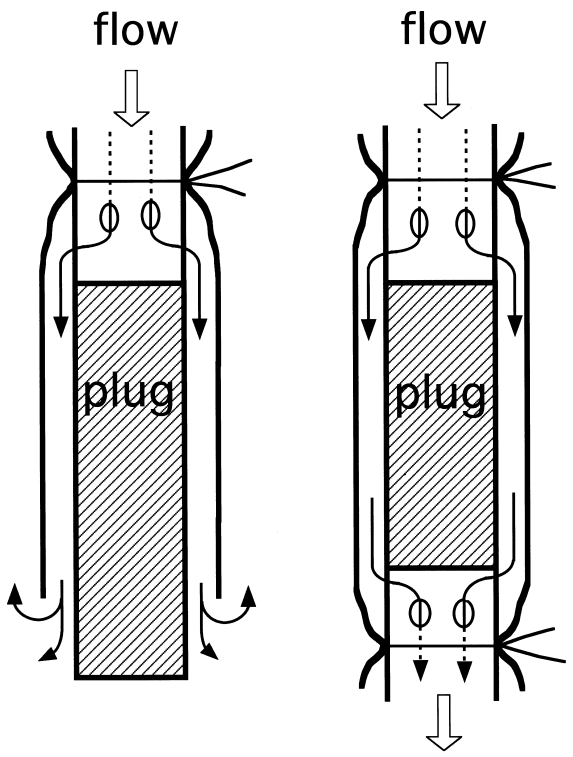

Fig. 5. Comparison of the stainless steel cannula inserting method (left) and the squeezed-flow perfusion method (right). The left panel illustrates the method described in Refs. 7 and 8. The major drawback of their method is that the outflow of the vascular lumen is mixed up with the outer bathing medium, and thus their method is unable to apply a drug exclusively from the intimal side.

Other features of the new method

It is not expensive at all to prepare the squeezed-flow perfusion apparatus. The standard polyethylene tubing is commercially available, and its specifications are precisely documented as shown in Table 1. One can choose the optimum-sized tubing by trying to insert the tubing into the vessel lumen. The hand-crafting of the cut-edge of the polyethylene tubing (smoothing, rounding, flanging, etc.) is easy. It is also easy to select the fitting size of the injection needles which are generally available for clinical use at a reasonable cost. Furthermore, it is not difficult to cut an injection needle to make blunt ends and to round the sharp edges by a hobby router equipped with cutting and grinding attachments.

After preparing the parts as above, it is easy to assemble the core part of the system. This helps avoid any involuntary injury onto the vessel segment especially onto the endothelium.

Concluding Remarks

Finally, in these days of molecular biology, those studies using the technique as above may appear classical or even outdated. However, the molecular biological studies should be powered and integrated if combined with the pathophysiological studies of the organ- and whole body-levels. In view of many advantages of the new technique, we recommend the new method as the very routine of cardiovascular physiology and pharmacology.

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