A Technique for the Microscopic Study of Blood Vessels in Living Striated Muscle (Cremaster)

By Guido Majno, M.D., Virginia Gilmore, and Monika Leventhal

ABSTRACT

A technique is described whereby it is possible to study the vessels of a striated muscle (the rat cremaster) in vivo. In the anesthetized rat, a part of the scrotal skin is excised; the cremaster muscle is exposed and studied by epi-illumination. To improve the optical conditions, a white reflecting surface is brought behind the cremaster through the inguinal canal. Twitching of the muscle may be prevented by the use of a neuromuscular blocking agent. It is also possible to study the blood vessels of the fascia overlying the cremaster. The method may also be applied to the rabbit; it is recommended for short-term studies in which topographic requirements prevail. Its advantages and limitations are discussed.

ADDITIONAL KEY WORDS

inflammation Evans blue endothelium rat rabbit microcirculation permeability carbon black gallamine

The investigator who wishes to study the behavior of blood vessels in vivo has a choice of at least two dozen methods (1-7). It might appear superfluous to add another one to the list, were it not that the preparation here described—the cremaster of the rat and rabbit—represents a vascular bed which has been relatively less studied: that of striated muscle.

We were drawn to develop this technique by necessity, after having found that in two of the well-established models the blood vessels did not respond to histamine in the manner which is typical for mammalian skin and muscle. Earlier work (8) had shown that blood vessels induced to leak by histamine (or related compounds) can be readily identified at the level of light microscopy by the simple artifice of injecting carbon black intravenously. In these vessels, plasma escapes through gaps in the endothelium; the basement membrane remains intact and retains the carbon particles, which accumulate against it as on a filter, and thus cause the vessel to become "labeled" in black. Vascular labeling as thus defined is easily induced in rat skin and muscle. Hoping to study the dynamics of this phenomenon in vivo, we tested the bat wing and were unable to obtain any labeling at all (results to be published): thus this membrane, despite its cutaneous derivation, appears to behave in a manner quite unlike that of mammalian skin. In the hamster cheek pouch the labeling was very weak, possibly because the preparation, which is pinned out in drum-skin fashion, develops a tissue pressure high enough to reduce or prevent the escape of fluid from the blood vessels.

A method for the study of the cremaster in vivo has already been reported by Grant (3). The two approaches, which are substantially different, will be compared in the discussion.

Material and Methods

METHOD AS APPLIED TO THE RAT

In the rat the cremaster muscle is a laminar, bag-shaped extension of the internal oblique; the inguinal canal is patent and wide enough for the testis to slip in and out of the abdominal
FIGURE 1

Top: Rat table and accessories (see text). The wire ring W is simply taped onto the table. Bottom: A curarized rat under observation, with the spatula SP inserted through an abdominal opening. The plastic syringe C is attached to a cannula inserted into the jugular vein. The T-tube (T) is connected with a respirator (not shown).
cavity. The basic principle of our method is to cut a window in the skin of the scrotum, and to study the cremaster by a combination of epi- and transillumination. To hold the cremaster flat, and to improve the optical conditions, a light-reflecting surface is slipped behind the muscle: This is a white spatula, mounted on a holder and introduced into the scrotum through an opening in the abdominal wall. Since this spatula and its holder, fitted on a special table, are the keys to the method, they will be described first.

Table and Accessories (Fig. 1)

This table, which supports the rat or rabbit, can be of any shape as long as one end may be fitted under the objective of a microscope. We chose a Zeiss-Winkel stand stripped of stage and condenser, because its shape of a deep "C" allows ample freedom of movement to a table brought under the objective (Fig. 1, below). Main dimensions of the table are: length, 30 cm; width: front 30 cm, rear 15 cm; legs: front 11 cm, rear 8 cm (height can be increased by 5 cm by a telescoping mechanism and a screw-type foot). The front end (which fits under the microscope) is 3 cm higher because the spatula, coming from the abdomen into the scrotum, tends to point downward; and its legs are wider apart to accommodate the base of the microscope. On each side a vertical bar (V) is mounted. These two hold a third one horizontally (H); the latter can be raised or lowered by steel collars (with tightening screws, TS) fitted onto the vertical bars. Sliding along H is a metal clamp (CL): essentially a piece of brass through which two holes have been drilled, one to receive the horizontal bar (H), and another one beneath it, at right angles, to receive the handle of the spatula. Both holes are equipped with tightening screws.

The spatula, made of a 5-mm brass rod, is visible in Figure 1 (above). Its flat end, obtained by filing two opposite sides of the rod, is 35 mm long, 5 mm wide, and 1 mm thick; its corners are rounded and both surfaces are covered with white glossy enamel.

Illumination

Two zirconium arc lamps (Fish-Schurman Corp.) are mounted on stands of adjustable height ("Lab-Jack," Cenco) at such an angle that their beams point downwards at approximately 45 degrees. These lamps produce an extremely bright focus with a relatively small amount of heat. By focusing the beams on a thermistor probe, it was found that heat production could be reduced to a negligible level by using water filters (in practice, flat flasks with a depth of about 20 mm). The addition of copper sulfate to the water was unnecessary.

Optical and Photographic Equipment

We used 8X and 6X oculars (Leitz or Zeiss), a Zeiss Planachromat 2.5 objective, and Leitz objectives designed for use in metallography (UM "5" and UM "10"). These objectives have a working distance of about 15 mm, and thus allow the beam of the lamps to fall on the preparation at an angle close to the vertical. The stated
enlargements are not accurate because they refer to the special conditions of metallographic work. We determined our final magnifications by photographing a millimeter scale; the range of enlargements in the illustrations is 29 to 158X.

For still photography we used a Leitz Micro-\textit{Ibso} attachment with a Leica back; for cinematography, a Bolex H 16 reflex camera run by an electrical motor which could be set for time-lapse as well as for speeds up to 14 frames/sec (Zieler Instrument Co.). The camera was mounted on the horizontal arm of a drill-press stand: The drill-press table, with its upright fitted with a collar of adjustable height, proved to be a very firm, steady, shock-proof photographic workbench. The films used were: for still photography, 35 mm, High Speed Ektachrome (Type B) and Kodak Plus-X Pan (shutter speeds 1/60"-1/125"); for cinematography, Ektachrome Commercial Type 7255.

Preparation of the Rat

\textbf{Anesthesia.} Sodium pentobarbital ("Diabutal," Diamond Lab.) 6 mg/100 g, was given intraperitoneally; this dose is sufficient to maintain surgical anesthesia for 45 to 60 min.

\textbf{Shaving.} The fur was clipped on the ventral side of the neck, over the abdomen, and over the left half of the scrotum and left groin (Fig. 2, left). The latter area was then moistened and carefully shaved with a hand razor; this prevents loose hair and scales from contaminating the microscopic field.

\textbf{Cannulation of the jugular vein (9) is required for the injection of carbon black. Heparin is not necessary.}

\textbf{Tracheostomy} is not absolutely necessary unless the animal must be maintained by artificial respiration. However, bronchial secretions tend to accumulate under prolonged anesthesia (especially in older rats) and these are easily removed if a tracheal cannula is in place.

\textbf{Exposure of the cremaster.} Using toothed forceps, the skin of the left groin in the shaved area is seized about 5 mm laterally to the penis. A skin fold is lifted, 8 to 10 mm high, and cut at the base with curved scissors (Fig. 2, left). The cut is continued, while the forceps maintains the traction, into the distal end of the scrotum. If the traction on the skin is sufficient one should be able to excise, together with the skin fold, most of the fascia covering the cremaster; the latter is then moistened with Krebs-Ringer-phosphate solution. Hemorrhage from the subcutaneous tissue is slight and stops promptly; none occurs from the cremaster, which has an independent vascular supply. Thereafter the edge of the skin, all around the cut, is raised with fine hooks so that it contains a pool of 0.3 to 0.5 ml of fluid over the exposed cremaster (Fig. 2, right). The hooks are fashioned from ordinary pins; these are attached to 15 cm lengths of thread bearing small lead (fishing) weights at the other end. The threads are passed over the ring of wire held horizontally over the cremaster (Fig. 1).

\textbf{Introduction of the spatula.} An abdominal incision 1 cm long is made along the midline using a thermocautery (if a knife is used instead, blood will smear the spatula and disturb the visual field). The spatula, mounted on its clamp, will then be directed towards this opening and slid downwards into the scrotum. To avoid snaring the epididymal fat body or other structures, the upper surface of the spatula should be gently pressed against the ventrolateral aspect of the abdominal wall, and pushed downwards in this position until its tip appears in the cremasteric sac. The handle of the spatula is then lowered and adjusted so that the exposed part of the cremaster is horizontal. The preparation is now ready for direct study, unless the following step is also required.

\textbf{Neuromuscular block and artificial respiration.} To obtain a perfectly still field, devoid of twitching, we injected 10 mg/100 g of gallamine triethiodide (Flaxedil) into the peritoneum; respiration ceased in 2 to 3 min, and was then maintained with a piston-type respirator set at 70 cycle/min (Harvard Apparatus Rodent Respirator Model 680).

\textbf{Technical hints.} The critical step of the entire procedure is the excision of the skin over the cremaster. An easy mistake is to exert too little traction on the skin fold, with the result that the cut is not deep enough to remove most of the fascia; hence the visibility of the vessels in the cremaster is impaired. Once the first cut is made it is very difficult to excise more fascia without injuring the cremaster (an alternate solution is to use these "missed" preparations for studying the vessels of the fascia, rather than those of the cremaster). Another possible mistake is to force the spatula downwards when it has hooked some abdominal structure: The immediate result is a disturbance in the circulation of the cremaster; the spatula should slide downwards with little or no resistance. It should not exceed, in width, about \% the width of the testis. Wider spatulas can be used; they stretch the muscle and give better visibility, but a carbon injection will show considerable vascular labeling along the edges. Since the width of the photographic field at the lowest power is 2.1 mm, there is little point in using spatulas wider than 4 to 5 mm.

These preparatory maneuvers can be accomplished, with practice, in 15 to 20 min.

The final aspect of the rat on its table under
the microscope is shown in Figure 1. The rat lies on a paper towel (which will absorb any fluid spilled from the pool) and it is kept warm by a 60-watt lamp. The pool of Krebs-Ringer phosphate solution is constantly checked, and occasionally changed, with a glass pipette; a supply of Krebs-Ringer phosphate solution at 35°C is kept at hand. The microscopic field is selected by shifting the rat table.

Shellac-free "biological ink" prepared by Pelikan Werke (sold by J. Henschel and Co., Inc.), 0.1 ml/100 g, is used for injections into the jugular vein.

Observations are carried out while the preparation is under a thin layer of fluid (1 mm is sufficient, more will decrease resolution) which has the function of abolishing highlights as well as of keeping the tissue moist. Optically the fluid causes an apparent raising of the object. If desired for photographic purposes, the fluid can be removed without danger for a few seconds only. To test the effect of a drug on the preparation the pool of Ringer's is replaced with a solution of the drug in the same medium. (For long-term experiments Grant's constant irrigation device (3) may be practical; we did not find it necessary.)

METHOD AS APPLIED TO THE RABBIT

The basic anatomical relationships of the cremaster are the same in the rabbit as in the rat; thus we found it possible to expose the cremaster, introduce the spatula and visualize the vessels essentially as described above. The rabbit, however, offers several drawbacks. Its scrotum is relatively smaller than that of the rat; in our 2-kg rabbits the testes corresponded in size to those of 250-g rats. In these larger rabbits the muscle is covered by a thick fascia that cannot be removed with the skin as can be done in the rat; visibility is therefore impaired, making it necessary to work with very young animals. The largest vessels are always on the dorsal aspect, and thus out of sight. Finally, the carbon did not appear to mix with the blood stream as well as in the rat; myriads of microscopic emboli became visible immediately after the injection, most of them, under normal conditions, having no circulation. The largest vessels are not always accessible, since they frequently run along the lateral aspect of the testis; when seen they appear slightly deeper than the superficial capillaries, since they lie between two layers of muscle fibers (3). The circulation should be lively; if it appears sluggish, this is usually the effect of a technical error.

Results and Discussion

With a 6x ocular and a 5x UM objective, in a typical preparation, the field is very bright. The muscle fibers, rather surprisingly, are too transparent to be seen: The field seems to be occupied by nothing but blood vessels against a bright yellowish background. Many capillaries are clearly visible, but some of them, under normal conditions, have no circulation. The largest vessels are not always accessible, since they frequently run along the lateral aspect of the testis; when seen they appear slightly deeper than the superficial capillaries, since they lie between the two layers of muscle fibers (3). The circulation should be lively; if it appears sluggish, this is usually the effect of a technical error.

Evaluation of operative trauma. The extent of damage, if any, produced by the preparative manipulations and subsequent exposure is easily gauged. (1) Direct damage to a small vessel will usually cause stasis with permanent stoppage of flow. This can be seen in fascial vessels at the periphery of the surgical field; it is easily recognized and usually irrelevant. (2) Vessels are more often induced to leak indirectly, i.e., by mechanical trauma to mast cells with subsequent liberation of chemical mediators affecting the venules (8, 11, 12). A minimal amount of stretching is enough to bring about this type of trauma. It can be detected by injecting intravenously a small amount of the carbon suspension (0.05 ml/100 g); leaking venules are almost instantly labeled in black (8). This carbon is cleared from the circulation so fast that within a few minutes its concentration will drop below the level required for vascular labeling, even though the skin of the rat still appears grey; a second and larger dose of carbon can then be given, as required by the experimental protocol.

The extent of surgical trauma thus revealed varies somewhat from animal to animal; it is usually so small that it cannot be recognized at low powers, and wholly negligible if compared with the effect of a local application of histamine (see illustrations in the following paper) (10). It should not exceed a few grains of carbon at one or two spots in two or three venules; within these limits one need not discard the animal but merely choose a clear field. Another alternative is to wait 30
to 60 min for this minimal amount of vascular leakage to wear off (this is discussed later). Appropriate controls showed that the major factor in producing operative trauma is not the cut in the skin, nor the exposure, but the introduction of the spatula.

Effect of the carbon injection. When carbon black is injected intravenously for the purpose of vascular labeling nothing unusual is noticed if it is infused slowly, except that the blood becomes somewhat duskier. Striking effects are observed if the injection is sudden: Within 4 to 5 sec all the arteries, and the arteries alone, appear black (Fig. 3-1). A photograph at this instant represents the equivalent of a purely arterial injection. One to three seconds later the larger veins appear longitudinally striated in red and black, each streak representing the input of a single venule (Figs. 3-2, 4-1; see also ref. 10). This occurs, of course, because each tributary venule carries a mixture in varying proportions of blood and carbon; laminar flow keeps the single streams separated, and since the entire system of vessels lies essentially in one plane, each stream is seen on edge. Within a few seconds this striation disappears; capillary flow, in the meantime, sometimes becomes sluggish and remains so for 10 to 15 sec, suggesting that the rapid injection of carbon black may cause a transient disturbance of the microcirculation. On two occasions a photograph showed the advancing edge of the carbon, in a vein, to be shaped as a long wedge (Fig. 4-1) reminiscent of the parabolic shape described in arteries (13). Transient "rolling" of leukocytes along the venular walls was sometimes apparent (10); we felt that this, too, bore some relation to the rapid injection of carbon. In other respects our observations on the normal circulation were identical with those of Grant (3).

Use of curarizing agents. The cremaster is a striated muscle, and as such it tends to twitch. This is an unpredictable event; it does not impede observation, but makes it more difficult. Often it does not occur at all, or subsides after 10 to 20 min of observation; but it does usually occur whenever the muscle is submitted to some experimental procedure, the effect of which is to be recorded. Even if it does subside, the first few minutes may be
lost for photography. The obvious remedy is to use a neuromuscular blocking agent. Grant (3) recommends the local application of succinyl choline chloride (100 mg/ml). Unfortunately the drug is very rapidly destroyed in situ and if higher amounts are applied locally, enough may be absorbed to cause death (3); we prefer to curarize the animal as a whole. The result is a perfectly motionless field. Possible side effects should also be considered. Some curarizing agents are histamine liberators (14, 15); this could be critical, especially if the purpose of the study is to investigate the effects of histamine. We used gallamine, which is said not to liberate histamine (14), and we observed no sign of histamine liberation when gallamine was given systemically. However, in rats prepared with intravenous Evans blue, subcutaneous doses of 0.1 ml of a 20 mg/ml solution caused intense blueing, and some blueing was also produced by a tenfold dilution. Thus gallamine does cause some vascular leakage when applied topically in the rat, and one should be careful to avoid spilling the drug into the scrotal cavity. The same caution should be exerted when pentobarbital is injected ip, because we found that it, too, would cause blueing if injected into the skin. We sometimes had the impression that the circulation was livelier in the anesthetized animals which were also under the influence of the neuromuscular blocking agent. This may be another side-effect of gallamine, which is said to cause occasional tachycardia and mild hypertension through parasympathetic inhibition (12). With regard to vascular reactivity, we concur with the thorough study of Grant (3), who found no difference between curarized and noncurarized cremasters.

Study of the fascial vessels. As mentioned earlier, the vessels of the fascia can also be studied. This is, anatomically, a very special structure; the term "fascia" is not strictly accurate. It consists of flattened villi, 10 to 15 mm long and 1 to 2 mm wide, applied to the external surface of the cremaster. Each villus is supplied by a main artery emerging from the cremaster, and accompanied by several interconnected veins. Arteriovenous anastomoses are numerous (see ref. 10); the overall arrangement suggests that these structures may be involved in thermoregulation. Since most of their vessels lie in one plane, interspersed with a single layer of fat cells, they lend themselves well to study in vivo. However, they are also more easily injured by exposure than those of the cremaster, presumably because they lie closer to the connective tissue which is being cut; thus, after having chosen a given group of vessels, it is essential to test their permeability by injecting carbon black intravenously.

Survival of the preparation. Since our contemplated studies (10) required acute experiments lasting 5 to 10 min, we tested the preparation for possible signs of deterioration within the first hour after exposure. In 4 animals, carbon black was injected 5 to 7 min after exposure, and the cremaster was studied continuously for the next 22 to 26 min. In one cremaster, not a speck of carbon black was deposited (Fig. 4, ref. 10); in the others, there were a few sparse grains in rare venules. At that time, carbon was again injected; 2 min later there was no sign of increased injury. Then histamine or bradykinin was applied, to make sure that the vessels were still reactive; intense labeling developed. This sequence is illustrated in Fig. 2 (10). This indicates that the preparation remains in excellent condition for about half an hour.

Four other rats were prepared, their cremasters were exposed, and left untouched for 1 hr. Then carbon black was injected; to our surprise, carbon labeling was practically nil; two cremasters were flawless, and one or two grains of carbon were found in the others. The preparation, therefore, rather than deteriorating during the hour of exposure, had apparently "healed" any leaks that may have been caused by surgical trauma. This is in line with the known facts. Surgical trauma, in our case, signifies mainly stretching of the tissue with injury to mast cells and "histamine-type" vascular leakage; the latter lasts, in rats, no longer than 15 to 30 min (12). Testing with bradykinin after 1 hr pro-
FIGURE 4

Effect of intravenous carbon black (rat cremaster). Top, 6 sec after the injection: the arteries A', A are completely black; some carbon is beginning to arrive from the venule X (note laminar flow) into the larger vein V. In the latter the flow is two-directional, as indicated by the curved arrows. At the "watershed" area there is some turbulence (T), but laminar flow resumes.

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duced excellent vascular labeling (10), which indicates that vascular reactivity was normal. The only difference in these animals was a certain pallor of the cremaster, corresponding to a considerable narrowing of the arteries and a lesser perfusion of capillaries, whereas the diameter of the venous vessels was little altered (3). Grant suggests that this more vasoconstricted state represents the normal resting state of the muscle (3); we are more inclined to believe that it depends on the general anesthesia, because the carbon black took longer to appear in the arteries (6 to 7 min instead of the usual 4 to 5 min). Rats were not tested at periods longer than 1 hr, but Grant (3) found that his cremaster preparation could be followed "unchanged, or almost so, for several hours": Carbon labeling, however, was not used to test the permeability of the vessels. We cannot over-stress the importance of this preliminary test, in assaying the state and reliability of any preparation of living, exposed blood vessels—not only the cremaster.

Comparison with the method of Grant. The cremaster preparation of Grant (3) is basically similar to ours, except that no spatula is used; the vessels of the cremaster are visualized against the whitish background of the testicular albuginea. His method is simpler and easier. It has allowed a large number of pharmacological observations, which have proven that the blood vessels of the cremaster respond to drugs in the same way as those of other striated muscles. This has cleared the path for further use of the cremaster in studies of vascular pathology. We are also indebted to Grant for a detailed anatomical description of the cremaster, including muscular layers, vascular supply and innervation (16).

On the other hand, in the absence of the spatula, the field is curved, thus smaller; it oscillates with the respiratory movements; and the reflecting surface of the albuginea, with its large, tortuous veins, does not afford the luminosity and clarity which can be obtained with a lacquered metal surface. Thus our method is preferable if photographic records are required; it is just as safe if the utmost care is exerted in introducing the spatula.

Like most other preparations of live blood vessels, our own can scarcely be called ideal. It offers, however, the following advantages. Striated muscle is very rich in blood vessels; thus it is possible to have, within a relatively high-power microscopic field, vessels of a wide range of calibers and very nearly within the same range of focus. This is especially important when the effect of a drug on different types of vessels must be recorded (this was our primary requirement in the following article). The field of observation can be relatively wide (as much as 5 to 6 by 15 mm in a large rat) and includes many capillary fields, each with its relatively distinct arterial supply and venous drainage; thus it is particularly suited for studies in which topographic considerations are important, rather than studies on flow within single vessels. The method can also be used for studying the vessels of adipose and fibrous tissue; despite the detailed description, it is relatively simple; and after the study in vivo, it is easy to excise the muscle and to preserve it as a permanent mount for study by transillumination (8).

Although we do not recommend that a live muscle be exposed for hours, it is possible to use the method in long-range studies by producing a given lesion in the cremaster (in the intact animal) and then to expose it for microscopic study when required, even more than once if necessary. This we have done successfully in a study of oxygen gradients in healing wounds (17). The effect of histamine, bradykinin and serotonin on the vessels of the cremaster will be reported in the following paper (10).

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Within a short distance, as shown by the typical spur-shaped leading edge of the carbon, above (S), and by the confluence with a venule, below (small arrow). The artery A showed some spasm (narrowed segments), a rare event. Bottom, 2 min later the carbon is evenly distributed in the bloodstream; no venular labeling has developed, indicating that no damage has been produced by the operative procedures.
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References


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