A New Method of Perfusion Fixation for the Rabbit Femur

By

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Summary: We report a new method of perfusion fixation for the proximal one-third of the femur of the Japanese white rabbit. Fluids to flush the blood and fix the marrow were injected into the abdominal aorta and drained from the stump of the femur. The oozing of the fluids from the stumps guaranteed complete flushing and fixation. The new method facilitated fixation and decreased the volume of necessary fluids.

Scanning electron microscopy (SEM) images of bone marrow fixed using the new method and using the conventional method did not differ. Large fat globules were not observed in the SEM specimens produced using either the new or the conventional method.

The hip joint and proximal one-third of the femur are regions of special interest for orthopedic surgeons. Diseases arising from either congenital causes (Furguson, 1971) or fractures (Garden, 1974) and osteonecrosis as a result of either steroid administration (Pietrogrande and Mastromariano, 1957) or other causes (Jones, 1984; Welch and Charnley, 1970) frequently occur in these regions.

Recently, we began to study the effects of prednisolone upon blood vessels of the bone marrow of the rabbit femur using scanning electron microscopy (SEM). In the conventional method of perfusion fixation for the femoral bone, fluids to flush the blood and fix the bone marrow are injected into the abdominal aorta and drained from the inferior vena cava. However, this method has two difficulties. First, the complete fixation of the femoral bone marrow cannot be confirmed during the process of fixation. In the conventional method, the fixative fluid may only pass through routes with low-vascular resistance, taking the shortest path from the aorta to the inferior vena cava. Thus, tissues of importance may remain unfixed; this result only becomes apparent after fixation. Second, a large amount of a fixative is required using the conventional method. The conventional method fixes the pelvis and hind limbs equally, which weigh nearly 800 grams in total. This means that as much as 800 ml of fixative fluid is needed per rabbit. Selective perfusion of the tissues would likely decrease the amount of fixative required.

In the new method of rabbit femur perfusion presented here, we amputated the femur and used the femoral stump as the route for draining the perfusion fluid. The efficacy of the perfusion method was then examined by the injection of Japanese ink into the femoral bone and by observation of the bone marrow blood vessels using scanning electron microscopy.

Materials and Methods

Animals

Female Japanese white rabbits, weighing 3.0–3.5 Kg, were used for the experiments. The animals were purchased from Sankyo Lab Service (Sizuoka, Japan) and fed in a ward of the Animal Center, Kanazawa Medical University (KMU). The ward was air-conditioned and laboratory chow and water
were given ad libitum. The animals were treated according to the ethical regulations for the laboratory animals at KMU.

Methods of perfusion

The new method of perfusion fixation was used in five rabbits. Ten to fifteen milliliters of pentobarbital was administered, and the rabbits were anesthetized. The abdomen and thighs were cleaned with 70% ethanol and skinned. The thigh was tightly ligated with a thick cotton string at the distal two thirds of the femur and was amputated just distal to the ligation. The amputation was performed bilaterally. Then, the abdominal wall was opened, the small intestine was moved aside, the abdominal aorta was exposed, and a plastic catheter (Ga #21) was inserted into the abdominal aorta. The adipose tissue surrounding the aorta and inferior vena cava at the level of the catheter insertion was thoroughly removed and the areas were cleaned with 70% alcohol. The catheter was connected to an extension tube equipped with a three-way stopcock and a plastic bottle containing cold 0.9% saline solution and a bottle of 2% glutaraldehyde in phosphate buffer solution (PBS), pH 7.4. The point of insertion of the plastic catheter was located distal to the origin of the renal arteries, and care was paid lest the catheter tip should reach the bifurcation of the abdominal aorta into the common iliac artery. The plastic bottle was placed approximately 120 cm above the rabbit heart. The aorta and inferior vena cava were tightly ligated at the level of the catheter insertion. Additionally, the inferior mesenteric artery was also ligated near the origin to stop blood circulation to the large intestine. At first, 500 ml of 0.9% saline solution was perfused to flush the blood from the distal two thirds of the femur. When the blood exiting the stump of the femur became transparent, 200 ml of 2% glutaraldehyde was perfused to fix the femur.

In another five rabbits, the conventional method of perfusion fixation was performed to enable a comparison of SEM results. The rabbits were anesthetized, 800 ml of 0.9% saline solution was injected into the abdominal aorta to flush the blood, and the saline solution was drained from the inferior vena cava. About 800 ml of 2% glutaraldehyde was then perfused for fixation.

Japanese ink injection experiment

In two rabbits, the blood was flushed as per the new perfusion fixation method and twenty milliliters of Japanese ink, diluted by an equal amount of 10% formalin, was injected after the amputation of the femur. The femurs were isolated and immersed in 10% formalin for two days. They were then rinsed in running tap water overnight and decalcified in Plank-Rychlo solution (7 g of AlCl₃, 8.5 ml of 37% HCl, 5.0 ml of formic acid, and dH₂O to make a 100 ml solution) for 24 hours; the femurs were then treated in 5% sodium sulfate solution for 12 hours and rinsed in dH₂O. The femur was serially cut into sections with a thickness of approximately 1 mm using a sharp, manually handled brain knife. The femoral slices were then dehydrated in a series of graded ethanol solutions, cleared in a solution consisting of a mixture of benzyl benzoate (315 ml) and methyl salicylate (380 ml), placed in a plastic medium (Enteran) and mounted under a glass coverslip.

Scanning electron microscopy (SEM)

The femurs obtained from ten rabbits, five fixed by the new perfusion method and another five fixed by the conventional perfusion method, were processed for SEM observation of the marrow blood vessels. The femurs were longitudinally cut along the long axis of the bone using a handy round saw from the level of the greater trochanter to the distal end of the third trochanter. The bone marrow was excised with a sharp knife blade and fixed in 2% glutaraldehyde in phosphate buffer solution (PBS), pH 7.4, for a few hours, rinsed in PBS, postfixed in 1% osmium tetroxide in PBS, and rinsed again in PBS. The marrow specimens were placed in 25% and 50% dimethyl sulfoxide (DMSO) in 0.1 M cacodylate buffer solution, pH 7.4, for an hour at the respective concentrations. Then, the bone marrow specimens were frozen in liquid nitrous oxide at −196°C and fractured by application of a razor blade. The bone marrow fragments were again osmified, rinsed, dehydrated in a graded series of ethanol, and infiltrated with isoamyl acetate. The
fragments were then critical-point dried, attached to aluminum stubs with the fractured surface upward, ion sputter-coated, and viewed under an electron microscope.

Results

Bone marrow blood vessels of the femur stained with Japanese ink

A blot of Japanese ink appeared on the cut ends of the femur about 90 seconds after the beginning of the ink perfusion. Almost the entire surface of the femoral cut ends was stained, more or less, within 10 minutes. Bone slices at 10, 17 and 30 mm from the highest point of the greater trochanter were all stained black, more or less (Fig. 1). The proximal region tended to be more densely stained than the distal femur near the femoral stump; however, ink particles were observed in all the sinusoidal areas, suggesting the even circulation of the fixative solution.

Bone marrow blood vessels viewed under scanning electron microscopy

Arteries

The outer diameter of the bone marrow arterioles was usually less than 200 μm (Fig. 2). The arterioles ran straight and branched into smaller vessels, the diameters of which were 10–20 μm. The arteriolar wall was formed of three layers: an inner mesothelial layer, a middle layer of compact tissue including muscle cells, and an outer adventitia layer of thick connective tissue. The luminal endothelial surface was continuously lined, and endothelial discontinuity was not observed. Numerous minute caveolae opened on the endothelial surface. The endothelium exhibited very short microvilli that delineated the borders of the adjacent endothelial cells. In spite of an extensive survey and comparison, no differences were found between the results obtained using the new perfusion method and those obtained using the conventional method.

Veins

The outer diameter of the primary veins (= sinusoids) was approximately 10 μm (Fig. 3). The veins usually joined together with an acute internal angle, forming V-shaped confluences, to form the secondary veins or venules, the size of which ranged from 30 to 200 μm in diameter. The secondary veins joined together and formed larger veins that exited the bone marrow along with the nutrition arteries. The primary and secondary veins did not differ remarkably in their vessel wall organization. The vessel walls consisted of a very thin layer of endothelial cells attached sporadically by preadipocytes and reticular cells. Rows of hemispherical fat cells were usually silhouetted on the vascular bed of the veins (Fig. 4). The venous endothelial cells showed a tendency to form round and oval pores varying in size from 10 to 50 μm in diameter. Fat cells resting underneath the endothelium were frequently visible through the endothelial pores. Erythrocytes in the venous lumen, often enveloped within cytoplasmic extensions of larger cells of unknown origin, were not uncommon.

The artery and its accompanying vein usually ran in parallel, and the artery was often surrounded in part by the vein (Fig. 2). The artery and accompanying large vein were close enough that the arterial adventitia was covered by venous endothelial cells (Fig. 4). In an extreme case, a thready arterial branch that traversed the lumen of the vein was completely covered with venous endothelial cells. These observations did not differ between the specimens produced using the new perfusion method and those produced using the conventional perfusion method.

Some investigators (Robb-Smith, 1941; Gurd, 1970) have suggested the possibility, in bone fractures, of the leakage of fat globules from ruptured fat cells in the bone marrow stroma into the venous system and the subsequent migration of the fat globules to the lung. Since femur amputation is an artificial fracture of the bone, we extensively surveyed the venous lumina of the specimens produced using the new perfusion method and compared the results with those of specimens produced using the conventional perfusion method. In the specimens obtained by both perfusion methods, small and smooth-surfaced globules, the diameters of which were less than 10 μm, were observed.

Fig. 3. The marrow sinusoids (S) were approximately 10 μm in diameter. The sinusoids joined together, forming a venule with a larger diameter. Fat cells and reticular cells were often seen attached to the basal surface of the venous endothelial cells.

Fig. 4. An arteriole (A) and its minute branches running along with an accompanying venule. The venule was fractured and its lumen was opened (VL). Many circular pores (P) were observed in the layer of the venous endothelium. Fat cells and other blood cells were visible through the pores (arrows). Note that the venous endothelial cells lined the adventitial surface of the arteriole and its branches (asterisks).
However, globules of a size large enough to occlude a lung capillary were not observed.

Discussion

We have described here a new method of perfusion fixation for the femoral bone marrow. In any kind of morphological studies that involve perfusion fixation, the investigators should pay attention to the pressure of perfusion. In our injection method, the perfusion pressure into the abdominal aorta was 120 cmH₂O, less than 10 mmHg. According to Tajima (1972), the mean blood pressures of Japanese white rabbits are 83 mmHg in the diastolic phase and 124 mmHg in the systolic phase. Our perfusion pressure was far less than the diastolic pressure; therefore, artifacts caused by a high injection pressure were unlikely. On the other hand, such a low pressure may not be sufficient to ensure the permeation of the injected fluid throughout the marrow sinusoids. However, the presence of Japanese ink particles confirmed that the fixative had thoroughly permeated the femur. The partial body weight between the levels of the first lumbar vertebra and the femoral amputation was estimated to be 400 to 500 gm. Since the weight of intravascular blood accounts for approximately 8% of the tissue weight in Japanese rabbits, the injection of 500 ml of 0.9% saline solution is equal to approximately 13 times the estimated total circulating blood volume. This volume is sufficient for a complete flushing of the intravascular blood. We used only 200 ml of 2% glutaraldehyde for fixation, but this volume was assumed to be more than ten times the volume necessary for the fixation because an injection of 20 ml of Japanese ink stained almost all areas of the tissue. Actually, the glutaraldehyde fixative oozed out from the stump of the femur when only 30 to 50 ml of the fixative was injected.

The SEM results did not differ for specimens produced using the new perfusion method and those produced using the conventional method. Judging from the time and volume of fluids necessary for perfusion fixation, the new method is superior to the conventional method. One issue of concern with the new perfusion method was the possibility of the artificial induction of fat embolism. Pulmonary embolisms are rare but serious complication of bone fractures (Robb-Smith, 1941). Many adipocytes in the marrow are believed to be injured in bone fractures, releasing triglycerides into the marrow venous blood. These fat globules can cause pulmonary fat embolism (Peltier and Lai, 1958; Gurd, 1970). If this is true, many fat globules would be present in the marrow sinusoids, since the amputation of the femur in our perfusion method would have the same effects as an artificial fracture of the femoral bone. However, in spite of an extensive survey of the venous lumina using SEM, we did not find any fat globules in the venous system of the bone marrow that were large enough to occlude the arterioles of the lung. While the possibility that mechanically released fat globules may lead to pulmonary embolism as a result of the bone amputation remains a concern, our new perfusion method appears to be advantageous in many ways.

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