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Effect of Hemorrhage on Blood Flow to Marrow and Osseous Tissue in Conscious Rabbits

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EFFECT OF HEMORRHAGE ON BLOOD FLOW TO MARROW AND OSSEOUS TISSUE IN CONSCIOUS RABBITS

by

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ABSTRACT

The purpose of this experiment was to characterize cardiovascular responses in skeletal tissue 15 minutes and 16 hours following reversible hemorrhagic hypotension and to validate a quantitative method for measuring blood flow rates in marrow and bone.

Cardiac output and regional blood flow determinations were made using the radioactive microsphere technique. Microspheres (15 u dia) were injected into unanesthetized rabbits via a chronically implanted left atrial catheter. Blood flow and cardiac output measurements were made by injecting individual microsphere isotopes, each with a different label (^{85}Sr, ^{51}Cr or ^{141}Ce), at three specified time intervals: first, as a pretreatment control value; second, 15 minutes following a standardized non-fatal hemorrhage (20 ml blood/kg body wt); and third, 16 hours post-hemorrhage.

Results of four standard validations used to test reliability of the microsphere method as applied in this study showed close correlation between blood flow and microsphere distribution.

On the day of the experiment both central ear arteries were cannulated. One catheter was used for blood pressure and heart rate recordings. The other vessel provided arterial blood for pCO_2, pO_2, pH and hematocrit measurements and also served as the site for collection of free-flowing reference samples used to calculate cardiac output.

Each animal was sacrificed immediately following the third isotope injection and the heart, both kidneys, spleen and both femurs (marrow and bone separated) were removed, weighed fresh and ashed overnight at 550° C. Every tissue contained three isotopes, each representing a flow rate measurement during one of the time periods. Dissolved soft tissue samples and bone ash were counted and appropriate equations used to calculate percentage distribution of cardiac output, blood flow and tissue resistance for each time interval.

Fifteen minutes after hemorrhage there was a significant decrease in cardiac output, blood pressure, arterial pCO_2; while hematocrit and heart rate were significantly increased. These changes were accompanied by significantly reduced blood flows to the heart, kidney, spleen, whole bone, marrow and osseous tissue with corresponding resistance increases in all tissues except the heart. Spleen, whole bone, marrow and osseous tissue received a decreased percentage distribution of cardiac output while there was an increased percentage going to the heart. Renal flow fractions remained unchanged.

Sixteen hours following hemorrhage cardiac output, heart rate and arterial pCO_2 returned to normal while pO_2 increased and hematocrit decreased. Arterial pH was unchanged at both post-hemorrhage measurements. Heart and marrow blood flows were significantly increased and kidney flow rates continued to be lower than control values. Perfusion of whole bone, spleen and osseous tissue returned to pretreatment levels.

Tissue resistance decreased in the heart, spleen, whole bone and marrow but remained elevated in the renal vascular bed. Osseous tissue resistance decreased but not significantly. Percentage distribution of cardiac output increased in the heart, whole bone and marrow and decreased in the kidney while the spleen and osseous tissue values returned to normal.

Normal hemodynamic properties of bone showed parallel blood supplies to marrow and osseous tissue with higher vascular resistance in the latter. It was estimated that total skeletal tissue plus marrow received 16% of resting cardiac output.

The response 15 minutes post-hemorrhage demonstrated the characteristic decrease in regional blood perfusion with a relative preservation of flow to the heart. There was a uniform reduction of blood flow within the femur giving no evidence of preferential shunting away from osseous tissue in favor of marrow.

Sixteen hours after hemorrhage blood flow to the various soft tissues reflected continuation of a stress state where both vasodilatory metabolic and vasoconstrictive neurohumoral factors were still operative. A selective blood flow increase not seen in surrounding osseous tissue was observed in marrow. The response seen in marrow may be a preparatory mechanism for the increased metabolism associated with erythropoietic and reticuloendothelial activation and may represent a direct vasoproliferative effect of erythropoietin on marrow vasculature.

LITERATURE REVIEW

Introduction

Bone is a highly specialized form of connective tissue and it is the most complex of all body building materials. Its unique physical and chemical characteristics are reflected in the diversity of its functions. First, and probably the most obvious function of osseous tissue is in providing structural support and protection for bodily organs. The arrangement of collagen fibers and mineral crystals makes bone aptly suited to this function. Although three times lighter, bone has the tensile strength of cast iron with greater flexibility allowing for absorption of sudden impacts. For most humans, strength and unity of the skeletal system is maintained throughout life. Growth and remodeling occur in response to mechanical stress and are influenced by various hormones.

A second major function of the skeletal system is in mineral homeostasis. Bone is an important component for maintaining the magnesium, calcium, hydrogen ion, sodium and phosphate concentrations of blood within narrow limits. Here again, the structural features of bone, especially the relationship between collagen and mineral, are critical factors determining mineral availability. It has been estimated that the total surface area of the bone crystals in an averaged size human skeleton exceeds 100 acres.

Contained in the bone crystal are two storage pools of calcium intimately involved with mineral homeostasis. The first is called "exchangeable bone" and it is a readily available source of calcium which can rapidly respond to changes in plasma calcium equilibrium by simple membrane transfer of calcium. The second pool, which comprises the largest percentage, is called "non-exchangeable" or "deep bone" and it can be mobilized with time in response to hormonal or pathologic stimulation.

Both of the major physiological functions of bone are dependent upon an adequate blood supply and any alteration in blood flow can disrupt these normal processes. Knowledge concerning the adequacy of the vascular supply of bone and bone marrow under normal and pathological conditions is incomplete. The quantitative measure of bone blood flow is remarkably difficult because of the involved vascular pattern and the rigidity of the tissue. The design and application of methodologies dealing with this subject has provided a stimulating challenge for many students of bone physiology.

In discussions of bone circulation, the long bones of the extremities (i.e., femur and tibia) are the generally used model. These bones represent a basic component of the skeletal system and their size and accessibility make them more suitable for study.

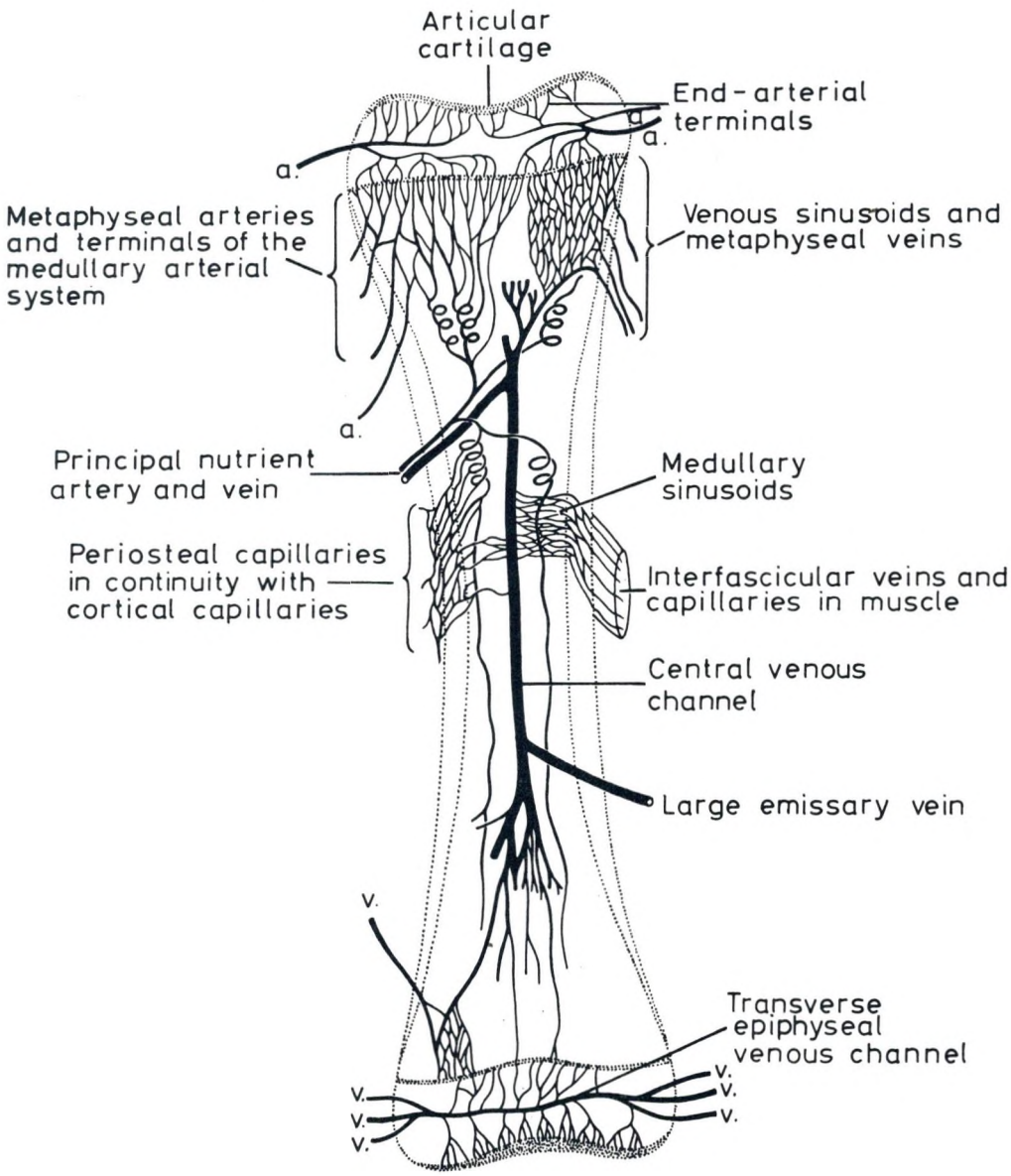
The present investigation involves the quantitative measurement of bone blood flow and its response to reversible hemorrhagic shock. The

following review will cover basic physiological and anatomical aspects of bone circulation including the different methodologies used for measuring bone blood flow. The circulatory dynamics of the growing skeleton represents an entire study in itself, therefore only the circulation of mature bone will be considered here. For detailed accounts of the developmental aspects of bone hemodynamics see Brookes (1) and Trueta (2). Included in this discussion will also be a short summary of the general response to reversible hemorrhagic shock.

Anatomy of Bone Blood Flow

A basic step in studying the physiology of blood flow is an anatomical description of the vascular distribution within an organ or tissue. The technique used in many organs, including skeletal tissue, involves the vascular infiltration of a contrast medium such as India ink or barium sulfate which can then be visualized within the arterial system. Based on such methods, the following description of bone blood flow can be made. A pictorial description is represented in Figure 1.

Regardless of species, long bones have three main sources of blood supply: 1) the nutrient vessels (usually one or two) which branch from the main limb artery and enter the diaphysis through a nutrient foramen, 2) the multiple metaphyseal arteries which supply proximal and distal ends, and 3) the periosteal vessels which run transversely to the long axis of bone (3). Arterial and venous distributions generally run together.


Articular cartilage
End-arterial terminals
a.
a.
a.
Metaphyseal arteries and terminals of the medullary arterial system
Venous sinusoids and metaphyseal veins
a.
Principal nutrient artery and vein
Medullary sinusoids
Periosteal capillaries in continuity with cortical capillaries
Interfascicular veins and capillaries in muscle
Central venous channel
Large emissary vein
v.
Transverse epiphyseal venous channel
v.
v.
v.
v.
v.
v.

A central vein drains nutrient arterial channels and parts of the diaphyseal cortex. In metaphyseal regions, veins normally accompany the local supplying artery.

Upon entering the diaphysis the nutrient artery immediately divides into ascending and descending limbs, each directed toward a corresponding metaphysis. Both divisions give off radially oriented branches which pierce the cortex. Terminal ends of ascending and descending branches help supply blood to the ends of the bones and anastomose freely with metaphyseal vessels (4, 5, 6). These anastomoses are important because they permit metaphyseal arteries to sustain inadequate medullary circulation when the nutrient artery has been ruptured by fracture or surgery (7, 8).

The role of periosteal and marrow vessels in supplying cortical bone is controversial. Most workers agree that cortex vasculature is joined to both periosteal and endosteal vessels, with more numerous and larger vessels coming from the latter (9). Branemark (10) showed that capillaries leaving marrow arterioles dip into Haversian canals and swing back into the marrow emptying into collecting sinusoids. Capillaries from marrow arterioles also drain directly into marrow sinusoids. This scheme provides separate flows to marrow and cortex from the nutrient artery. Recently De Bruyn et al. (11) observed few direct connections between marrow arterioles and sinusoids but centripetally oriented osteal vessels of marrow origin supplied the main drainage into the sinusoidal

network. Marrow was described as a portal system where sinus blood had already passed through diaphyseal cortex from both marrow and periosteal arterioles. Brookes et al. (12) argue that cortical blood does not come from the periosteum but strictly from marrow sources. Flow direction is entirely centrifugal, exiting through cortical capillaries. Trueta (2) supports the concept of a dual blood supply to bone cortex, each with a different function. The outflowing nutrient-marrow system would be responsible for erythropoiesis, while the inflowing periosteal-cortical circulation would be more closely tied with osteogenesis and mineral metabolism.

Medullary reaming and periosteal stripping have been used to destroy the corresponding blood supply to bone in order to assess its relative contribution. Using these methods Macnab (13) stated that cortical flow comes almost totally from branches of the nutrient artery. Rhinelander (14), Trueta (15), and others (16, 4) concluded that nutrient vessels supply one-half to two-thirds of the cortex, the remainder coming from periosteal sources.

Despite apparent contradictions regarding anatomic aspects of bone blood supply, one concept remains consistent throughout: bone is a highly vascular tissue with abundant anastomoses which provide a potential reserve supply in case of injury to one of the main afferent sources.

Methods for and Results of Bone Blood Flow Measurement

Numerous attempts have been made to assess the quantitative aspects of bone blood flow. The following section summarizes the methods and results from numerous investigations of bone blood flow during widely different physiologic and pathologic conditions.

It has often been stated that mature bone, because of its insignificant metabolic needs, requires a very small vascular supply. Early measurements of bone blood flow tended to support this concept. However, Rasmussen and Bordier (17) have described recent studies showing that mature bone is metabolically active with a blood flow rate much higher than previously assumed. Circulation through bone equals or exceeds that of resting skeletal muscle (18). This relatively high flow rate is thought to be related to bone's homeostatic function in mineral balance (19).

In ideal situations blood flow to a tissue can be measured directly by using flowmeters or collecting the venous effluent. Bone does not readily lend itself to this type of measurement because of the multiplicity of arterial and venous channels. Because of this, almost all attempts to measure bone blood flow have involved indirect methods; however, a few studies have utilized direct means. Drinker et al. (20) in 1922 devised a method of perfusion by cannulating nutrient vessels and collecting the venous outflow. Blood flow ranged from 2 to 60 ml/min under varying conditions. Cumming (21) measured marrow flow through

the rabbit femur by collecting blood from the nutrient vein and estimated a mean flow rate of 51 ml/min/100 g. He observed a wide variation which he thought due to age and marrow activity. Hypoxia and hypercapnia increased flow while epinephrine and norepinephrine decreased flow. Based on an estimated total body weight, marrow blood flow was calculated to be 7.6% of the resting cardiac output (22). Post and Shoemaker (23) studied relative changes in blood flow to the canine femur by cannulating both upper and lower venous efflux systems. They concluded that nutrient vessels were not as important as epiphyseal-metaphyseal vessels in draining blood from the femur. Shim and Patterson (24) used the direct method of venous cannulation to study control factors in bone blood flow. They reported a reactive hyperemia after femoral artery occlusion and concluded bone circulation was controlled by neural, hormonal and metabolic factors at both local and systemic levels.

Blood flow to bone can also be studied by methods which rely on certain blood flow dependent parameters such as temperature or intramedullary pressure. Larsen (25), while studying diaphyseal necrosis in 1938, was first to measure intramedullary pressure by inserting a water manometer into the femoral diaphysis of dogs. He reported normal values of 30 to 40 mm Hg. Stein et al. (26) observed rhythmical fluctuations in marrow pressure synchronous with respiratory phases. They also noted a significantly higher pressure within the diaphysis compared to the

epiphysis. Azuma (27) reported a similar pressure differential between epiphysis and diaphysis in mature dogs indicating a vascular barrier. However, Cuthbertson et al. (28) showed that when the nutrient artery was ligated, there was only a transient drop in diaphyseal pressure suggesting collateral circulation from metaphyseal vessels. This apparent discrepancy was further studied by Cuthbertson et al. (29) and they did not find a consistent difference between epiphyseal and diaphyseal pressures in adult dogs when measured over an extended period. It appears that the manner and degree of union of arterial fields on either side of the epiphyseal scar is variable but is sufficient to maintain marrow flow after nutrient artery ligation.

In all of the above studies it was assumed that marrow pressure was related to blood flow but there was some doubt as to the validity of this assumption (30). However, in a series of studies, Shaw (31, 32) showed good correlation between relative blood flow through marrow and marrow pressure. He used a heated thermocouple to measure blood flow while simultaneously recording intramedullary pressure. Injections of epinephrine, norepinephrine, acetylcholine, pituitrin and hexamethonium all produced a fall in pressure and blood flow, while histamine increased both. Valderrama and Trueta (33), using the same heated thermocouple technique, studied the relationship between muscle movement and bone circulation and stated that muscle action greatly influences direction and distribution of blood flow within bone. Thus muscle action during hind limb movement could alter flow and pressure within marrow. The heated

thermocouple method of measuring blood flow with the simultaneous recording of marrow pressure has recently been used to study the effects of compression and decompression on bone blood flow (34, 35). During compression marrow pressure and flow fell while the effects were reversed during decompression.

Michelsen (36, 37) has recently made a detailed study of bone marrow hemodynamics. Blood was infused into the nutrient artery at known rates and perfusion pressures while both intramedullary venous pressure and bone marrow pressure were recorded. At normal perfusion pressures flow ranged from 10 to 120 ml/min/100 g of wet marrow. His results showed that bone marrow has both an arterial and venous resistance and the relative contribution of these two systems to total outflow varies with perfusion pressure.

Within the last year a new method has been introduced to study bone marrow pressures utilizing a miniature implantable pressure transducer (38). Normal bone marrow pressure and medullary venous pressure were found to be nearly equal at about 17% of the systemic blood pressure. In this preparation, the primary determinant of marrow pressure was thought to be osseous venous resistance located at or near the points of exit from the marrow cavity. These studies show that the arterial vessels within marrow are responsive to vasoactive chemicals and can alter resistance to flow while the venous resistance vessels, which are also vasoactive, are located within bone. In conclusion, it can be stated

that the major concensus of the previous studies was that the circulatory dynamics of bone marrow are anatomically and functionally interconnected with the surrounding osseous tissue.

It should be emphasized that there are certain important limitations in the previously described studies. Although valuable for measurement of qualitative changes, little accurate quantitative data are obtainable with these techniques. Because of this it was deemed desirable to develop a method which could give reliable quantitative information on bone blood flow. Introduction of the isotope clearance technique utilizing the Fick principle (total exchange/arterial-venous difference) has produced extensive results from a number of investigations.

Frederickson et al. (39) measured blood flow to rat bone using a bone-seeking isotope (^{45}Ca). The authors reported values for rat femur ranging from 10 to 30 ml/min/100 g wet weight. The validity of the clearance method as an indication of osseous blood flow depends on the bone having consistent efficiency in taking up isotope from blood passing through it. Validation studies performed by various authors have shown incomplete extraction of bone-seeking isotopes. One group (40) found extraction ratios for ^{85}Sr and ^{45}Ca to be 43 and 55%, respectively. Tibial flows averaged 7.7 ml/min/100 g in young dogs and 5.6 ml/min/100 g in mature animals. Copp and Shim (41) found an extraction ratio of 76% for ^{85}Sr injected into the tibial artery of dogs and suggested that arteriovenous shunting might cause the lower extraction rate. Mean flow

rates were reported at 10 ml/min/100 g. Kane (18) feels a more likely explanation is that the kinetics of exchange of these isotopes between bone and blood is slow relative to the rate of their inflow. He suggests the calculated values from these studies could be corrected by dividing the isotope clearance by the extraction ratios giving flow rates close to 13 ml/min/100 g. Ray et al. (42) used ^{45}Ca and ^{85}Sr clearance rates corrected for incomplete extraction and found the mean flow to canine femur was 8.22 ± 0.46 ml/min/100 g.

Strontium-85 (43) and ^{86}Rb (44) clearance have been used to study the effects of denervation on bone blood flow. A 5 to 45% increase was seen on the treated side and was considered the result of decreased sympathetic tone in the hind limb. Semb (45) used the isotope clearance technique to show that blood flow to bone decreases initially following hind limb immobilization compared to the normal contralateral tibia (25 ml/min/100 g). Flow reached and exceeded normal after a week and was linked to an increased collateral return of blood from marrow and osseous vessels compensatory to the loss of the "muscle pump".

Shim et al. (46) using clearance techniques provided quantitative data on contributions of blood supplied by the different arterial systems of long bones. The nutrient artery contributed 46% of the normal supply of the entire rabbit femur (9.60 ± 0.47 ml/min/100 g) and at least 71% of the shaft including the marrow. Thirty-seven and 33% of the total inflow of upper and lower epiphysis respectively came from the nutrient artery.

This group also studied circulation through different regions in long bones (47). The average rates of blood flow of the femoral head, trochanter, diaphysis and condyle regions were 18, 10, 7 and 12 ml/min/100 g, respectively.

Sim and Kelly (48) demonstrated a close relationship between remodeling and osseous blood flow as indirectly determined by ^{85}Sr clearance. The results suggested a powerful metabolic factor regulating flow according to the tissues need for nutrients. Using the same techniques, Laurnen and Kelly (49) studied blood flow characteristics in healing fractures. They showed an increased circulation rate the first day following fracture which reached a maximum of 6 times normal at 2 weeks. Kane and Grim (50) measured hind limb blood flow simultaneously in dogs using ^{42}K and ^{86}Rb clearance and by venous outflow collection. The distribution of flow was also determined by a radioactive glass microsphere method. Potassium-42 clearance and the microsphere method agreed within 10% while the ^{86}Rb clearance was somewhat higher. Perfusion rates for canine femur were 12-18 ml/min/100 g.

Van Dyke et al. (51) estimated skeletal blood flow from clearance measurements of ^{18}F, assuming complete extraction by bone. They calculated total skeletal flow in rats to be 2.4 ml/min, which represents a minimum value because of the possibility of incomplete extraction. Recently Wootton (52) has compared this technique with the distribution

of ^{51}Cr-labeled microparticles (40-50 u dia) in the rabbit femur and found good comparisons between the two. The results showed that about 40% of the afferent blood flow to the rabbit femur flows through a capillary bed in the marrow before reaching bone mineral.

Arteriole blockade has been previously used by Brookes (53) to study cardiac output distribution in the rat. Ion-exchange resin particles labeled with ^{59}Fe (30-70 u dia) were used with flow rates to the total rat skeleton recorded at 27.55 ± 0.96% of the cardiac output.

Chromium-51-tagged red blood cells have also been used in quantitative bone blood flow studies (54). The method involves measurement of the amount of ^{51}Cr-labeled RBC's delivered to bone. Perfusion rate is determined by measuring the concentration of tagged cells in bone from the time they first flow into bone until equilibrium is reached. Using this method the rabbit tibia averaged 16 ml/min/100 g. Brookes (55) used this method to calculate flow to compact and cancellous bone and bone marrow. The results show that cancellous flow in the metaphysis had the highest flow rates followed closely by marrow then cortical bone. The differences in hemodynamics observed suggested differing local vascular factors present within the osseous circulation.

Tissue washout of radioactive iodoantipyrine has recently been used to study bone circulation (56-59). Blood flow to cortical bone in canine tibia was 0.76 ml/min/100 g with marrow flow being four times as great. About 70% of the total perfusion volume of the nutrient artery went to

diaphyseal cortex and varied directly with perfusion rate. It was also found that blood flow and mineral deposition in fractured bone are closely related and the main control mechanisms for mineral deposition under these conditions is blood flow and not a change in cortical bone capillary permeability.

The many problems and assumptions associated with use of the previous techniques led to the development of more reliable methods. The relatively new method for measuring regional blood flow using radioactively labeled microspheres has provided a mechanism for studying flow to small or unaccessible regions. The size, composition and specific gravity of the new carbonized microspheres are more uniform than the labeled microparticles mentioned previously. Within the circulation the spheres behave similar to red blood cells and are trapped by capillary beds in proportion to blood flow (60-66). This technique provides an excellent means for studying bone blood flow since it is independent of the metabolic activity of bone.

Boelkins et al. (67) utilized the microsphere technique and reported values for the tibia and femur blood flow in egg-laying chickens. The relatively high flow rates in this species was thought to reflect the importance of the skeleton as a source of eggshell calcium. In another recent study, Boelkins et al. (68) showed rapid blood flow changes in the avian skeleton associated with plasma calcium alterations after injection of parathyroid hormone. A decreased flow was associated with the hypocalcemic phase, while the flows increased during the hypercal-

cemic response to parathyroid hormone. These results emphasize the importance of blood flow in mobilizing mineral stores from bone. Lunde and Michelsen (69) used 15 u diameter microspheres labeled with ^{85}Sr to measure flow rates in the rabbit femur. Cortical diaphyseal bone averaged 1.0 ± 0.7 ml/min/100 g while mean marrow flow was 25.0 ± 14.6 ml/min/100 g. Maloney et al. (70) utilized microspheres to study the relative changes in bone marrow perfusion after nutrient artery ligation and marrow regeneration. Their findings confirmed the presence of a highly effective collateral circulation to the medullary cavity and that marrow regeneration is associated with an increased blood flow.

Physiological Characteristics of Bone Blood Flow

The accumulated data indicate that bone blood flow is controlled by neural, hormonal and metabolic factors. Sympathetic nerve stimulation decreases bone perfusion by local arterial vasoconstriction. This response is also seen following injection of endogenous vasopressor hormones. Both parasympathetic nerve stimulation and injected acetylcholine lower bone blood flow largely by decreasing systemic blood pressure.

Various metabolic factors including acid metabolites, pH, pCO_2 and pO_2 influence blood flow to bone. It must be remembered that the long bone is an organ system composed of cortical bone, cancellous bone and marrow. Each component has its own metabolic requirement which is reflected in the differing flow rates. The close adjustment of blood flow

to the various energy-dependent activities within bone emphasize the importance of a metabolic factor coupling flow to tissue needs.

It appears blood flow to bone is quite rapid for a tissue composed of relatively few cells. This puzzling fact may be explained when one considers the possibly important relationship between blood flow and bone mineral homeostasis. Parathyroid hormone, which is known to stimulate mineral mobilization from bone, also may influence bone blood flow (68). Future research may reveal that the many factors controlling blood mineral levels also affect bone blood flow.

Hemorrhagic Shock

Circulatory shock means a decreased cardiac output to a point where tissues do not receive an adequate blood supply. Anything which reduces venous return, such as diminished blood volume, decreased vasomotor tone or increased systemic resistance, can result in shock. Hemorrhage is probably the most common cause of hypovolemic shock. If shock is severe enough to cause deterioration of the circulatory system then a state of progressive or uncompensated shock begins and death ensues. There are, however, several basic mechanisms which attempt to return cardiac output and arterial pressure to normal levels. These include (1) the baroreceptor reflex which stimulates the sympathetic nervous system; (2) a central nervous system ischemic response which causes an even more powerful sympathetic stimulation; (3) reverse stress relaxation property of vascular smooth muscle which allows vessels to contract

resulting in better filling of the circulation; and (4) various compensatory mechanisms that return blood volume back toward normal including increased fluid retention by the kidney, increasing absorption of fluid by the gastrointestinal tract, absorption of fluid from interstitial spaces of the body, and increased thirst and salt craving.

There has been a volume of literature published which describes regional blood flow changes during the above described hemorrhagic conditions. Much of the data variability on responses in various circulatory beds arises from differences in experimental design, species, and depth and type of anesthesia (71). Perhaps the most reproducible results are an increased fraction of cardiac output to heart, brain and liver during hypotension, demonstrating selective preservation of flow to vital organs (72-74). The initial response to hemorrhage in other tissues is thought to be related to the degree of alpha-adrenergic innervation. Greater numbers of alpha-type receptors means greater vasoconstriction and hence flow reduction. However, this model does not always hold, for superimposed upon these neuronal effects are hormonal and local metabolic factors which demonstrate variable potencies within different regions of the vascular tree.

STATEMENT OF THE PROBLEM

Estimates of total skeletal blood flow have been as high as 25 percent of resting cardiac output with approximately one-third of this supplying the bone marrow. Relatively little is known of the circulatory dynamics of this highly vascular organ system in response to hemorrhagic hypotension. Blood flow to marrow becomes increasingly important in periods of anemia or blood loss because of its hematopoietic function. Furthermore, it is not known if the high bone-blood perfusion rate associated with active hematopoiesis is specific for marrow tissue or involves an increased flow through the surrounding bone. Separating marrow and osseous flow rates has been difficult due to lack of an appropriate method.

The purpose of this investigation was two-fold. First, to establish a valid, quantitative method for separating marrow and bone blood flow. Second, to utilize this method to compare hemodynamic changes in bone versus marrow following mild hemorrhagic shock in unanesthetized rabbits.

MATERIALS AND METHODS

Animals

White New Zealand rabbits (Oryctolagus cuniculus) weighing 2.9 - 3.7 kg were used in all experiments. The animals were raised in a germ-free isolated environment and purchased from Broken Pine Rabbitry (Chelsea, Alabama). Each animal was housed in an individual cage with access to food and water ad libitum.

Experimental Protocol - Overview

The purpose of this study was to characterize cardiovascular changes in bone and bone marrow following reversible hemorrhagic hypotension using the radioactive microsphere technique of regional blood flow measurement. Microspheres (15 u dia) were injected into unanesthetized rabbits via a chronically implanted left atrial catheter. Individual microsphere isotopes, each with a different label, were injected at three time intervals. First, as a control value; second, 15 minutes following a standardized, non-fatal hemorrhage; and third, 16 hours post-hemorrhage. Cardiac output, blood pressure, heart rate, blood gas, blood pH and hematocrit values were all measured at the time of each microsphere injection. Each animal was sacrificed immediately following the third isotope injection and the heart, both kidneys, spleen and both femurs (marrow and bone separated) were removed and prepared for counting.

Blood flow, percentage distribution of cardiac output and tissue resistance were calculated for each time period. Because all three determinations were made in the same rabbit, additional groups of animals corresponding to the second and third measurements were eliminated.

Surgical Preparations

A previously reported technique of chronic left atrial catheterization for injection of microspheres was used (75). Briefly, animals were weighed and anesthetized with sodium pentobarbital (30 mg/kg) via a marginal ear vein. The trachea was intubated orally and respiration was maintained mechanically (Harvard Apparatus Respiratory Model 661) at 30 strokes/min and 65 cc/stroke. The left thorax was shaved, washed and draped for sterile surgery. A 3-4 cm vertical incision was made between the third and fourth ribs directly over the heart. Thoracic and intercostal muscles were carefully separated and a self-retaining retractor was placed between the ribs to expose the heart. The pericardial sac was cut and the atria gently grasped with non-crushing forceps while a small incision was made in the appendage tip. A 25-30 cm long polyvinyl catheter (0.03 cm ID) fitted with a small terminal cuff was inserted through the atrial opening and secured to the atrial muscle with a stay suture (4-0 Ethiflex). The forceps were released and the catheter tie was checked for oozing of blood. After removing the retractor, the ribs were brought together using 2-0 chromic suture and the muscle layers were closed with 3-0 silk. A dorsal neck incision was then made and a

12-gauge needle inserted to guide the catheter subcutaneously from the chest exit to the neck for exteriorization. Final closing of chest and neck (3-0 silk) incisions was followed by removal of the endotracheal tube and an intramuscular injection of procaine penicillin (150,000 units). The exteriorized catheter was flushed with heparinized saline (500 units/ml) and a small plugged needle was inserted to prevent back-flow. The animals were allowed to recover for 4-12 days.

On the day of the experiment the animal was placed in a moderately restrictive plexiglass restrainer and both ear arteries were dissected out under local anesthesia (Lidocaine). A 15-20 cm long polyethylene catheter (PE-50) was inserted and secured into each artery.

Blood Pressure and Heart Rate Measurement

Blood pressure and heart rate were monitored via the right ear artery catheter which was connected to a P23A Statham pressure transducer. All tracings were recorded on a Grass Model 7C Polygraph. Mean blood pressure was determined directly by jacking the blood pressure pre-amp into a second channel calibrated for mean pressure display.

Blood Gas, pH and Hematocrit Measurement

Arterial blood was sampled from the left ear artery catheter using a 1.0 cc syringe and immediately analyzed (IL pH/Blood Gas Analyzer, Model 313) for pH, pCO_2, and pO_2. A portion of the arterial sample was

placed in heparinized capillary tubes, spun down (Clay-Adams Micro Hematocrit Centrifuge) and hematocrit values read on a micro-hemato reader (Clay-Adams).

Hemorrhage

The shock model used in this study was adapted from previously reported designs (76, 77) based on withdrawal of a constant fraction the total blood volume. Homeostatic mechanisms controlling flow re justments which occur during reversible hemorrhagic shock were all to operate without outside interference (i.e., maintenance of consta hypotension or reinfusion of shed blood).

Blood from an ear artery was continuously withdrawn at the rate 1.91 ml/min (Harvard Apparatus Pump, Model 901) until 20 ml/kg body weight was collected. Bleeding time ranged between 30 and 45 minutes depending upon animal weight. This amount of blood loss roughly corresponds to 38% of total blood volume in rabbits (78, 79).

Radioactive Microspheres

Isotopes

Radioactive microspheres, 15 ± 5 u in diameter were purchased from the Nuclear Products Division of 3M Company, St. Paul, Minnesota. Microspheres were suspended in 20 ml of sterile 20% dextran. Isotopes used and their respective specific activities were cerium-141 (^{141}Ce),

10 mCi/g; chromium-51 (^{51}Cr), 30 mCi/g; and strontium-85 (^{85}Sr), 10 mCi/g.

To reduce random errors in distribution to an individual tissue at least 400 microspheres should be present within that tissue (64). Estimation of the number of spheres can be made based on tissue activity if the counts per sphere are known.

Estimation of counts/sphere

A small aliquot (0.05 uCi) of each microsphere isotope was diluted in 20 ml of 10% dextran containing 0.5% Tween-80. After vigorous shaking on a vortex mixer, 0.05 ml of this solution was drawn into a 1.0 cc plastic syringe and spotted on the margin of millimeter graph paper. Using a glass slide for streaking, the microspheres were spread across a small segment of the graph paper. In this way, between 200 and 400 microspheres could be deposited on one square centimeter (cm^2) and easily visualized under a dissecting microscope. For each isotope the number of microspheres present on three - one cm^2 areas was counted in triplicate and averaged. The radioactivity of these paper squares was then counted and the counts per minute (CPM) divided by the number of spheres giving CPM/sphere as follows: ^{85}Sr, 33 CPM/sphere; ^{51}Cr, 12 CPM/sphere; and ^{141}Ce, 30 CPM/sphere. A half-life plot was made for each isotope so that the CPM/sphere could be determined at any time.

Injection procedure

The stock injection vial was vigorously shaken on a vortex mixer for 30 seconds. An aliquot of microspheres was drawn into a

1.0 cc plastic syringe and further agitated by moving the spheres back and forth through two 1.0 cc syringes connected via a 3-way stopcock. The microspheres were then transferred to a small volume (2 cc) glass injection vial filled nearly to the top with saline; a small air bubble was retained to facilitate mixing.

The injection vial had 22- and 18-gauge stainless steel barrels epoxyed into the top. The left atrial catheter was connected to the 22-gauge hub via a polyvinyl catheter (0.03 cm ID) with two 3-way stopcocks in between. The 18-gauge hub was connected via a polyvinyl catheter (0.04 cm ID) to a 10 cc syringe filled with 0.9% saline at 37° C.

Just prior to injection the vial was counted for total radioactivity. At the time of injection the vial was agitated on a vortex mixer for 30 seconds, inverted and constantly shaken while approximately 8 ml of the warm saline was injected through the vial flushing the microspheres into the heart. The injection lasted about 60 seconds and was followed by a 1 ml 37° C saline flush from the 3-way in the left atrial catheter. Blood pressure and heart rate were monitored continuously during the isotope injection. The injection vial was then counted again to determine residual microsphere activity which was subtracted from pre-injection activity to give total injected counts. This value was used in the estimation of cardiac output.

Cardiac output and blood flow determinations

Cardiac output and regional blood flow were determined using the microsphere reference sample technique (66, 80, 81). The method is

based upon the fact that cardiac output (CO) equals a reference sample flow rate (F) multiplied by the ratio of total injected counts (D)/reference sample counts (d): CO = F (ml/min) x D/d. Fractional distribution of cardiac output to an organ equals d'/D where d' represents total tissue counts. Blood flow (BF) to individual tissues can then be calculated as follows: BF = CO x d'/D.

In the present experiments reference samples were obtained by collecting blood from the free-flowing left ear artery. Average reference sample collection time was 1.5 minutes and commenced approximately 5 seconds before and finished approximately 30 seconds after injection of microspheres. Blood was collected and weighed (Mettler Model PN1210 Balance) in three tared plastic vials. Reference flow rates (ml/min) were calculated by dividing collection time (min) into total blood weight (corrected for specific gravity). To permit settling of microspheres into a point source for counting, blood samples were hemolyzed by adding 1 cc deionized water and centrifuged for 10 minutes at 2000 RPM. Cardiac output was then calculated as given above and, following tissue counting, percentage distribution of cardiac output and tissue blood flows were then calculated.

Sample preparation

Following the 16 hour post-hemorrhage blood flow and cardiac output measurements, the animal was sacrificed with an overdose of pentobarbital. After checking the atrial catheter for correct placement,

the heart, spleen, both kidneys and both hind limbs were immediately removed. Both femurs were cleaned of surrounding soft tissue and cut longitudinally using a small rotary saw (Dremel Model 380). The marrow was then manually scraped from the diaphysis. All tissues were weighed fresh (Mettler H64 Analytical Balance) in tared crucibles and ashed overnight at 550° C in a muffle furnace (Blue M Co.). The residual ash was weighed and dissolved in 4-7 ml of 2.0 N HCl. This made it possible to transfer the largest organ into one or two small counting vials, even when the ash residue was not completely dissolved. The counting vials consisted of disposable plastic tubes (12 x 75 mm, Falcon #2063) which were placed in plastic scintillation vials fitted with an adapter cap (Packard Instrument Co. #6000152).

Ashed bone was ground to a fine powder and placed directly into multiple counting vials. Each vial was loaded with exactly 0.5 g of ash to maintain consistent counting geometry.

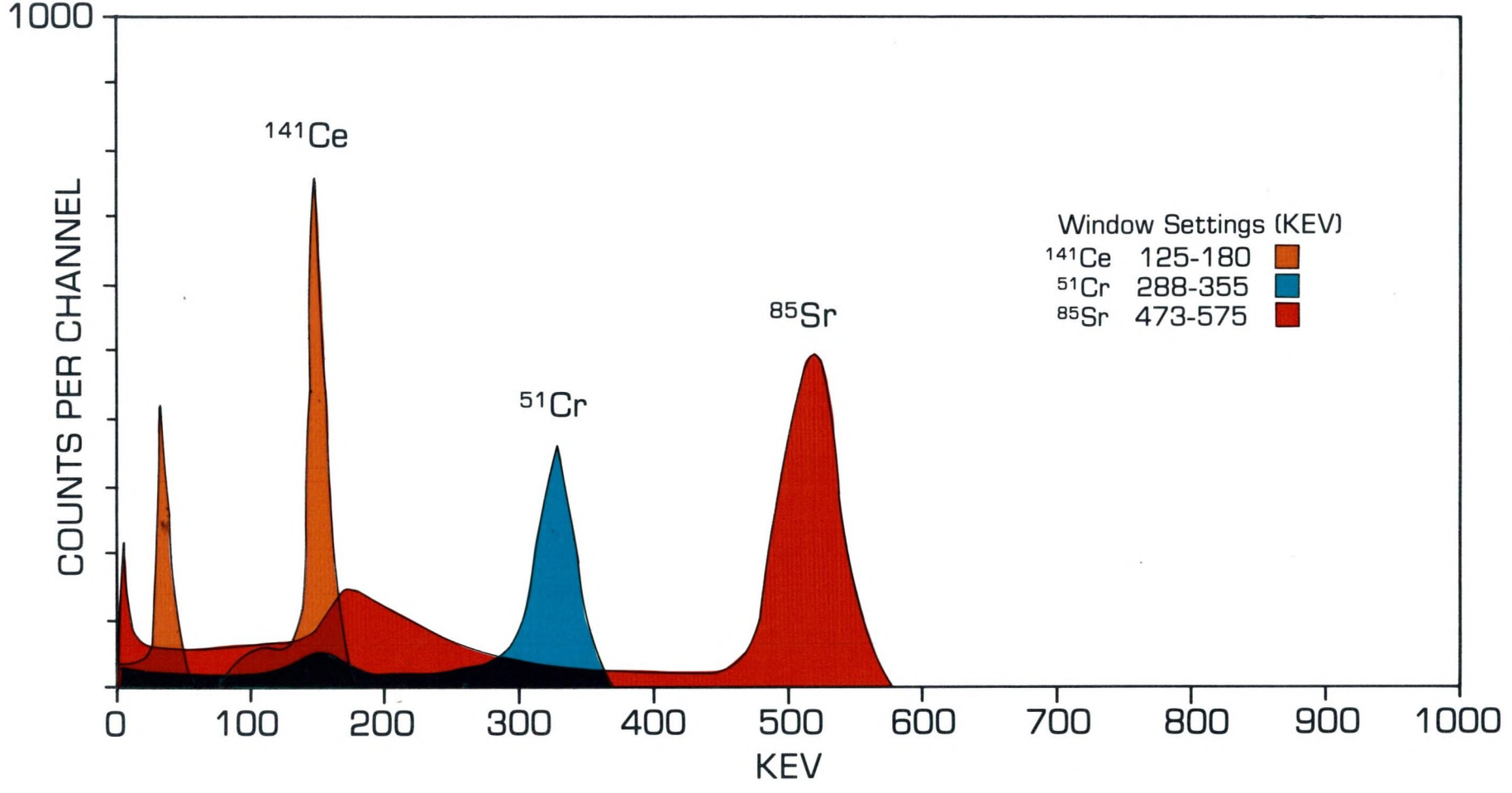
Counting procedures

All radioactivity was counted in a Packard Model 5986 Auto-Gamma Scintillation Spectrometer. This instrument is equipped with a 1024-channel pulse height analyzer permitting isolation of an energy range within a given spectra. The entire energy spectra of an isotope and any areas of interest can also be visualized directly from a TV monitor which allows easy adjustment of proper window settings for isotope separation. The counter was connected to a tape cassette/printer (Texas Instruments, Silent 700) for hard copy data printout.

Total injection vial, residual injection vial and blood sample activities were counted with an open window setting of 1-1000 kev. Counting times were normally 5, 20 and 100 seconds, respectively. All tissue samples were counted for 500 seconds.

Each tissue sample contained three isotopes representing measurements made at the three specified time intervals. It was necessary therefore to quantify the amount of each isotope present for calculation of blood flow and percentage distribution of cardiac output. To do this, the method of measuring and separating nuclides, previously published by Rudolph and Heymann (60), was used. Because none of the three isotopes used in this study had any appreciable activity above its major peak (Figure 2), calculation of relative amounts of each nuclide in mixtures was greatly simplified.

A pure sample of each isotope was initially counted with an open window of 1-1000 kev. Next, the pure samples were recounted with the appropriate sample kev energy windows set as follows: ^{85}Sr, 473-575 kev; ^{51}Cr, 288-355 kev; and ^{141}Ce, 125-180 kev. Knowing the total activity of each isotope, sample peak-to-total window ratios could then be calculated for all three window settings. Using this information the activity of each isotope could be determined. In the example given (Table 1), 45.41% of total ^{85}Sr activity falls within its peak energy channels with no contamination from other isotopes. Therefore, when counting mixed isotopes, the activity in channels 473-575 is multiplied


1000
COUNTS PER CHANNEL
141Ce
51Cr
85Sr
Window Settings (KEV)
141Ce 125-180
51Cr 288-355
85Sr 473-575
0
100
200
300
400
500
600
700
800
900
1000
KEV

by the corresponding peak-to-total ratio (1/.4541) giving total ^{85}Sr counts. Activity for ^{51}Cr was derived similarly with subtraction of ^{85}Sr counts appearing in the ^{51}Cr window. This calculation was again used for ^{141}Ce, the only difference being that two subtractions were necessary for ^{85}Sr and ^{51}Cr spillover into ^{141}Ce peak channels. The counting equations are also summarized in Table 1.

In using the microsphere method it is necessary to make corrections for different sample heights and geometries to minimize errors in counting (82). In the present study, three different counting geometries were used as explained below.

Blood samples represented point sources where all activity (microspheres) was located on the bottom of the vial. Problems associated with nuclide separation were not encountered because each blood sample usually contained only one isotope and was counted with an open window (1-1000 kev).

Ashing at 550° C destroys microspheres resulting in a homogeneous distribution of isotope throughout the sample. Because counting geometries of the liquid soft tissue samples and solid bone ash samples were different, it was necessary to formulate different sets of counting equations for each.

Soft tissue standards were prepared by grinding a representative organ (i.e., kidney) into a semi-liquid with a mortar and pestle and dividing it into three samples. Microspheres were added and mixed so

TABLE 1

CALCULATION OF COUNTING EQUATIONS

Isotope	Window Settings (key) 1-1000	473-575 A	288-355 B	125-180 C
^{85}Sr	84369[a]	38312 45.41%	4157 4.93%	8127 9.63%
^{51}Cr	7837	---[b]	4908 62.63%	873 11.14%
^{141}Ce	43863	---[b]	---[b]	29980 68.35%

[a] All values represent counts/min
[b] No counts above background

Total ^{85}Sr activity = 2.202A

Total ^{51}Cr activity = $1.597B - \frac{2.202A(0.049)}{0.626}$

$= 1.597B - 0.173A$

Total ^{141}Ce activity = $1.463C - \frac{(1.597B - 0.173A)0.111 - 2.202A(0.093)}{0.684}$

$= 1.463C - 0.282A - 0.260B$

that each sample contained a different isotope. Standards were ashed, prepared as described for soft tissues and counted. The counting equations were then calculated as just described. To establish equations for bone ash, aliquots of each microsphere isotope were added to samples of previously ashed bone and thoroughly mixed. Each sample was then counted as previously described and the equations calculated.

The position of the sample in relation to the counting crystal was also a critical feature. Sample heights for the two tissue geometries (dissolved samples and ash) were initially set using standards and remained constant throughout the experiment. Optimal counting rates were determined by adjusting the sample level knob on the instrument and observing the count rate. The established heights were 0.5 cm for dissolved samples and 1.5 cm for ash samples.

Validation Studies

Two different approaches were used to validate the assumption that microspheres were evenly mixed with blood and distributed to tissues in direct proportion to flow following left atrial injection.

In two anesthetized rabbits with chronically implanted left atrial catheters each central ear artery and both tibial arteries were cannulated (PE-50) for reference sample collections. Multiple cardiac output determinations were made utilizing free-flowing ear and tibial vessels. Estimates were made with each microsphere isotope under high and low cardiac output situations. Comparison of cardiac output values based on

different reference sites was used as evidence for adequate mixing and uniform distribution. Reference samples were collected and cardiac output calculated as previously described.

In the second validation experiment all three microsphere isotopes (^{85}Sr, ^{51}Cr, ^{141}Ce) were combined in a single injection vial and injected simultaneously into the left atria of a conscious rabbit. The animal was sacrificed, multiple tissues removed and counted, and flow rates calculated for each isotope. Theoretically, flow rates should be identical and the results demonstrated excellent agreement within the different organs.

Any errors in the counting equations were randomized by rotating the injection sequence of the three isotopes in each experiment.

Statistics

The experimental design of this study allowed paired-sample testing to be used for data analysis. Changes in cardiac output, blood pressure, blood gases and pH, heart rate and hematocrit were measured with the paired-sample t-test. Percentage of cardiac output, distribution, blood flow and tissue resistance were considered non-parametric data (skewed distributions and unequal variances); therefore, changes were assessed using the Wilcoxon paired-sample test (83). All sample statistics were done on a Wang 700 Series Advanced Programming Calculator.

RESULTS

Validation of Microsphere Technique

Basic to the microsphere technique is the assumption that complete mixing of spheres occurs in the left ventricle and distribution is proportional to blood flow. The four verification methods used here showed this assumption was valid.

The first technique used to demonstrate adequate mixing was to measure cardiac output based on the reference samples obtained from sampling points anterior and posterior to the heart. Estimates of cardiac output (Table 2) based on simultaneously collected reference samples containing microspheres from free-flowing ear and tibial arteries were not significantly different. The second validation technique involved comparison of blood flow to paired organs. Analyses of blood flow between right and left kidney and right and left femur gave correlation coefficients of 0.921 ($P < 0.001$) and 0.918 ($P < 0.001$), respectively (Figures 3 and 4). The third technique involved comparing a measured flow rate with one calculated on the basis of a second reference sample. A highly significant correlation was found ($r = 0.942$, $P < 0.001$) when measured tibial artery blood flow rates were compared with the corresponding blood flow values calculated on the basis of microspheres sampled from a free-flowing ear artery (Figure 5). The fourth technique

involved correlating blood flow rates estimated by simultaneous injection of all three microsphere isotopes. Comparison of blood flow rate estimates from different tissues yielded the following correlation coefficients: ^{85}Sr vs ^{51}Cr, 0.997; ^{85}Sr vs ^{141}Ce, 0.999; and ^{51}Cr vs ^{141}Ce, 0.996. All values were significant at the 0.001 level. These data indicate adequate mixing of microspheres with a strong relationship between distribution and blood flow.

Number of Microspheres in Samples

To reduce random distribution error it is imperative to inject adequate numbers of spheres so that a minimum of 400 microspheres lodge in each tissue (64). The mean and standard deviation of total injected microspheres for each measurement was 403,449 ± 151,706. The tissue containing the lowest number of spheres was always marrow. Estimates (mean ± SD) for total number of microspheres present in marrow during the control, 15 min post-hemorrhage, and 16 hrs post-hemorrhage periods were as follows: 621 ± 195, 571 ± 330 and 1032 ± 425, respectively.

Cardiovascular and Blood Gas Parameters

Cardiovascular and blood gas parameters are summarized in Table 3. Fifteen minutes after hemorrhage cardiac output, mean blood pressure, pCO_2, and hematocrit all decreased significantly ($P < 0.01$). Heart rate was the only parameter that increased significantly ($P < 0.05$). Blood pO_2 and pH did not change.

Sixteen hours post-hemorrhage mean blood pressure ($P < 0.05$) and hematocrit ($P < 0.01$) remained significantly decreased. Arterial pO_2 showed a slight but significant ($P < 0.05$) increase over control levels. Cardiac output and heart rate returned to control levels and blood CO_2 and pH were unchanged.

Percentage Distribution of Cardiac Output

Table 4 gives the percentage cardiac output distribution data which is graphically represented in Figure 6. Distribution of cardiac output to whole bone ($P < 0.01$), osseous tissue ($P < 0.01$), marrow ($P < 0.01$) and spleen ($P < 0.05$) significantly decreased 15 minutes after bleeding. There was a slight but significant ($P < 0.05$) increase to the heart. Distribution to the kidney decreased slightly but not significantly.

The 16 hour post-hemorrhage measurement revealed significant increase in cardiac output distribution to whole bone ($P < 0.05$), marrow ($P < 0.01$) and heart ($P < 0.05$). The kidney showed an opposite reaction with a significant drop from control levels ($P < 0.01$). Spleen and osseous tissues both returned to control values.

Tissue Blood Flow

Tissue blood flows (ml/min/100 g) are summarized in Table 5 and graphically represented in Figure 7. All tissues had significantly decreased flow rates 15 minutes following hemorrhage (P values between 0.05 and 0.01). After 16 hours there was a significant increase in blood

flow to the heart ($P < 0.05$) and marrow ($P < 0.01$) while blood flow to kidney remained significantly reduced ($P < 0.01$). Blood flow to spleen, whole bone and osseous tissue returned to pre-hemorrhage values.

The percentage of whole bone blood flow going to marrow and osseous tissue is described in Figure 8. Sixteen hours after hemorrhage there was a significant increase ($P < 0.01$) in the percentage of whole bone blood flow going to marrow.

Tissue Resistance

Local tissue resistance data is presented in Table 6 and Figure 9. Initial responses to hemorrhage in kidney ($P < 0.01$), spleen ($P < 0.05$), whole bone ($P < 0.01$), marrow ($P < 0.05$) and osseous tissue ($P < 0.01$) were significant increases in resistance to flow. Only the heart showed no change in resistance. This reaction reversed itself 16 hrs post-hemorrhage when resistances in the heart ($P < 0.05$), spleen ($P < 0.05$), whole bone ($P < 0.05$) and marrow ($P < 0.01$) decreased significantly. Renal resistance, however, remained significantly elevated ($P < 0.05$).

Control Studies

Table 7 shows relative blood flow changes in non-hemorrhaged animals injected with microspheres at identical time intervals used in the experimental protocol. These data agree with previous reports on physiological reactions of multiple microsphere injections (61, 84). Although the values showed considerable variability with time, no con-

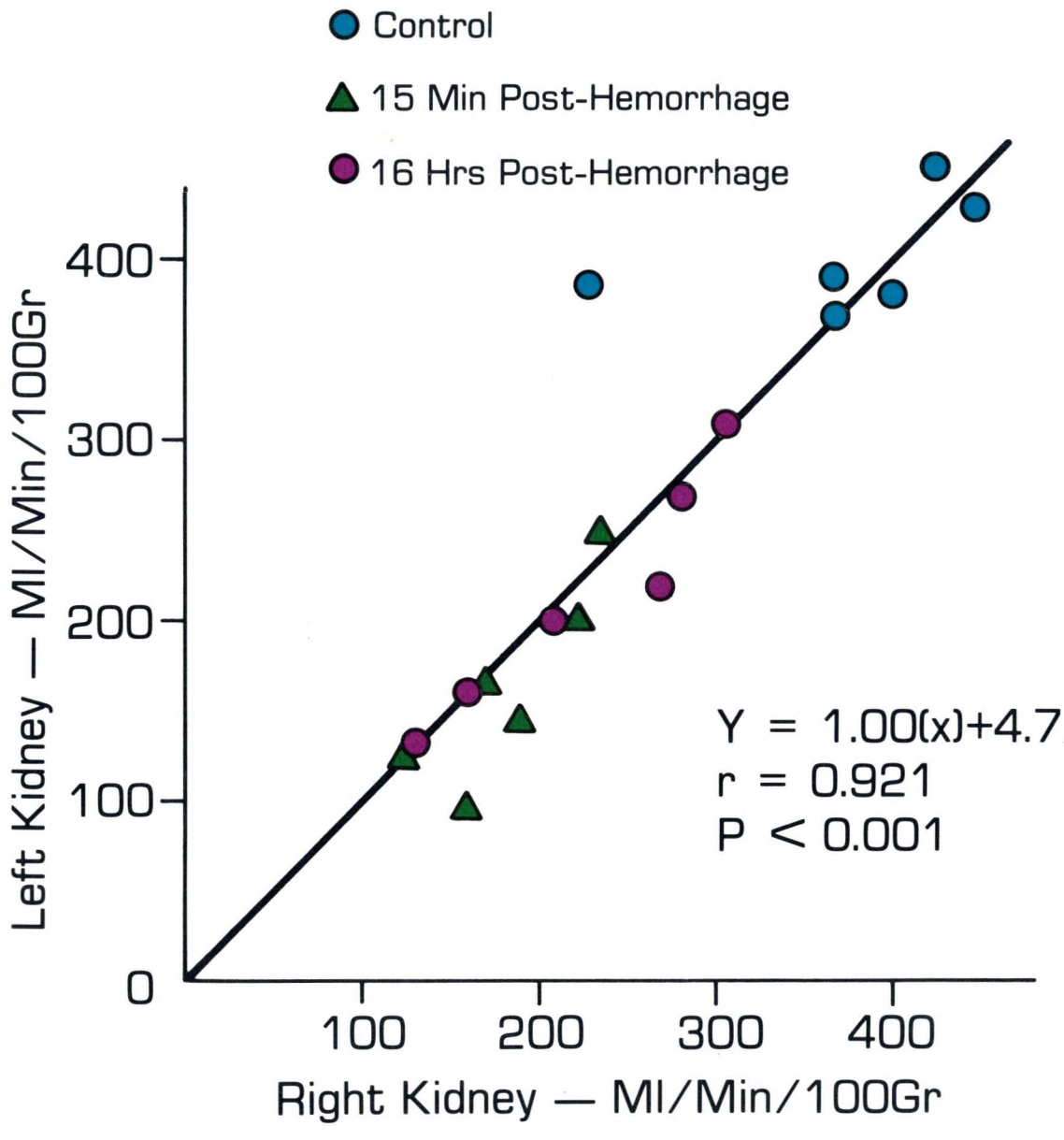
sistent changes, which might indicate a microsphere effect, were observed. Also, the changes were not consistent with those observed after hemorrhage. Since each animal served as its own control in the hemorrhage studies, more control animals were not run.

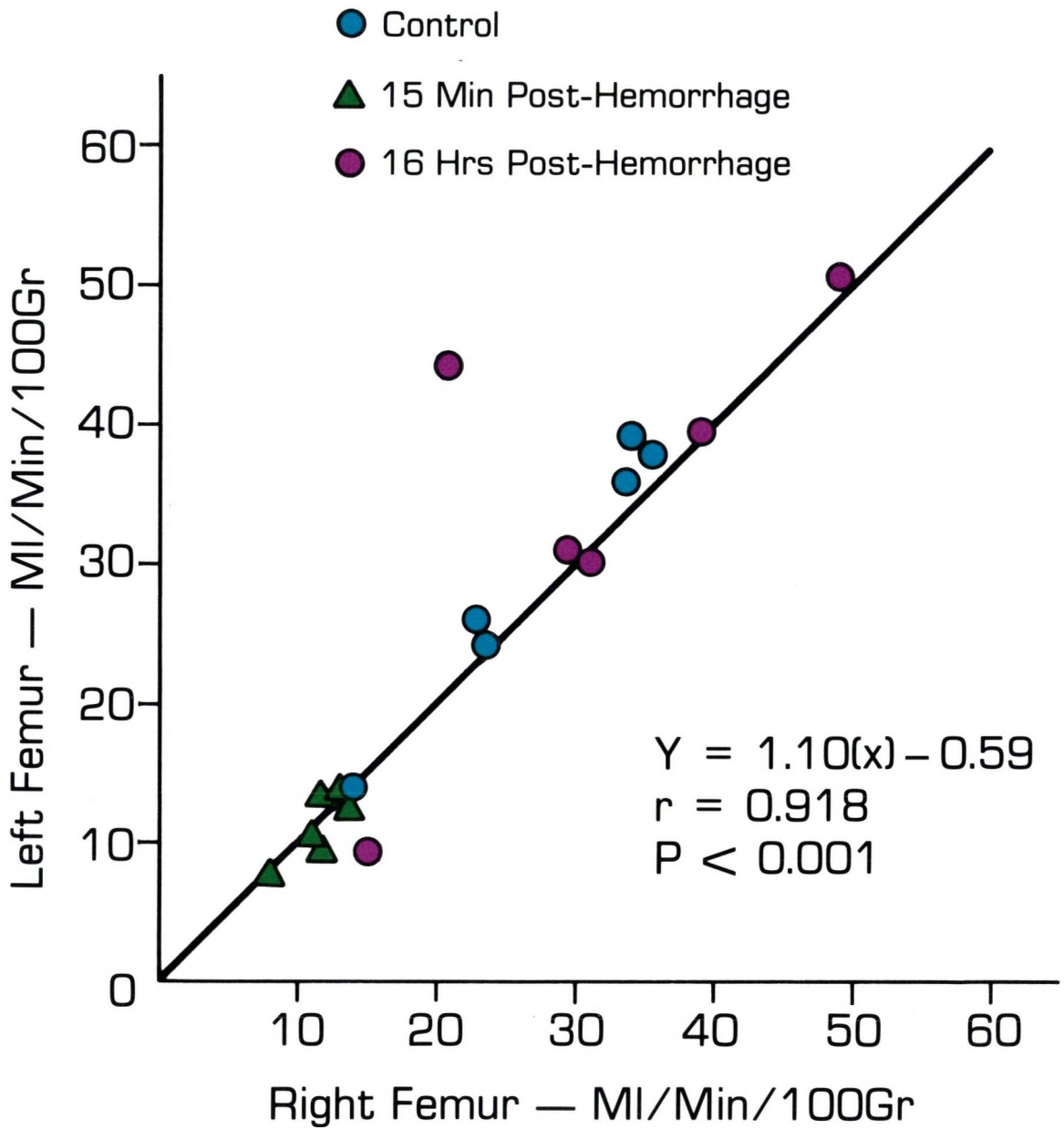
TABLE 2

CARDIAC OUTPUT BASED ON MULTIPLE REFERENCE SAMPLES

Isotope	Reference Sample Site[a]			
	Right Ear Artery	Left Ear Artery	Right Tibial Artery	Left Tibial Artery
^{85}Sr	311[b]	322	349	318
^{141}Ce	308	---[c]	318	304
^{85}Sr	250	238	266	243
^{141}Ce	231	203	222	212
Mean ± S.D.	266 ± 47		279 ± 50	

[a] All samples collected from free-flowing catheters
[b] Cardiac output in ml/min
[c] Sample lost due to inadequate blood flow


Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage
Left Kidney — Ml/Min/100Gr
0
100
200
300
400
100
200
300
400
Right Kidney — Ml/Min/100Gr
Y = 1.00(x)+4.7
r = 0.921
P < 0.001


Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage
Left Femur — Ml/Min/100Gr
Right Femur — Ml/Min/100Gr
0
10
20
30
40
50
60
Y = 1.10(x) – 0.59
r = 0.918
P < 0.001

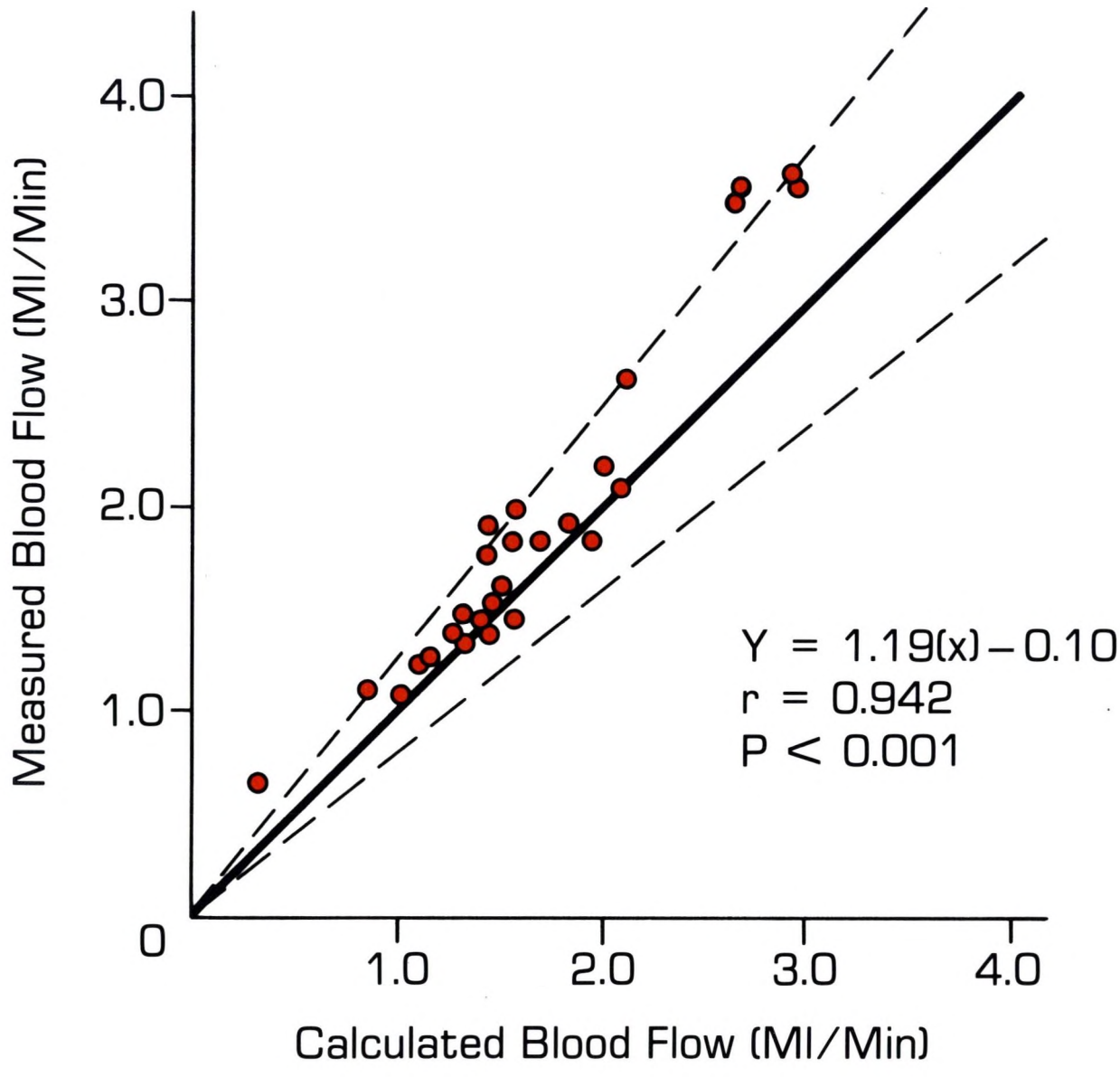

Measured Blood Flow (Ml/Min)
4.0
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
Calculated Blood Flow (Ml/Min)
Y = 1.19(x) – 0.10
r = 0.942
P < 0.001

TABLE 3

CARDIOVASCULAR AND BLOOD GAS PARAMETERS BEFORE AND AFTER HEMORRHAGE

	Control	15 Min Post-Hem	16 Hrs Post-Hem
Cardiac Output (ml/min/kg)	165 ± 24[a]	93 ± 10**	158 ± 36
Blood Pressure (mm Hg)	91 ± 12[b]	58 ± 10**	69 ± 9*
Heart Rate (beats/min)	281 ± 19	316 ± 30*	290 ± 17
Blood pO_2	87.2 ± 8.1	93.9 ± 6.9	92.8 ± 7*
Blood pCO_2	28.6 ± 3.4	21.4 ± 5.5**	28.4 ± 4.3
Blood pH	7.57 ± 0.02	7.55 ± 0.03	7.58 ± 0.03
Hematocrit	35 ± 4	27 ± 2**	21 ± 1**

[a] Mean ± S.D.
[b] Each value represents six determinations

* $P < 0.05$ based on comparisons with control values using paired t-test
** $P < 0.01$

TABLE 4

PERCENTAGE DISTRIBUTION OF CARDIAC OUTPUT BEFORE AND AFTER HEMORRHAGE[a]

	N		Control	15 Min Post-Hem	16 Hrs Post-Hem
Heart	6	Mean	3.2	4.3*	5.2*
		Range	2.5 - 4.2	2.8 - 6.5	3.6 - 6.7
Kidney	12	Mean	8.8	6.9	5.3**
		Range	4.7 - 13.0	4.5 - 11.2	3.4 - 6.4
Spleen	6	Mean	2.7	0.7*	3.2
		Range	1.3 - 3.8	0.3 - 1.9	2.2 - 4.8
Whole Bone	12	Mean	0.52	0.37**	0.63*
		Range	0.34 - 0.66	0.26 - 0.48	0.33 - 0.86
Marrow	12	Mean	0.14	0.11*	0.24**
		Range	0.01 - 0.19	0.06 - 0.17	0.09 - 0.33
Osseous Tissue	12	Mean	0.38	0.26**	0.39
		Range	0.21 - 0.56	0.16 - 0.44	0.30 - 0.53

[a] Equals fraction of total injected counts found in each organ

* $P < 0.05$ based on comparisons with control values using the Wilcoxon paired-sample test
** $P < 0.01$

TABLE 5

TISSUE BLOOD FLOW (ML/MIN/100 G) BEFORE AND AFTER HEMORRHAGE[a]

	N		Control	15 Min Post-Hem	16 Hrs Post-Hem
Heart	6	Mean	194	153*	285*
		Range	148 - 260	92 - 255	189 - 325
Kidney	12	Mean	387	173**	220**
		Range	233 - 451	95 - 253	132 - 312
Spleen	5	Mean	774	94*	810
		Range	352 - 1181	62 - 225	625 - 966
Whole Bone	12	Mean	28.5	11.4*	32.6
		Range	13.8 - 39.7	7.8 - 13.6	9.6 - 51.2
Marrow	12	Mean	29.7	13.7**	48.2**
		Range	22.3 - 36.7	8.0 - 20.3	18.0 - 77.3
Osseous Tissue	12	Mean	21.6	8.4**	20.6
		Range	8.6 - 30.6	4.7 - 12.3	9.6 - 30.0

[a] Blood Flow = $\frac{\%\ \text{Distribution CO} \times \text{CO}}{\text{Organ Weight}}$

* $P < 0.05$ based on comparisons with control values using the Wilcoxon paired-sample test
** $P < 0.01$

TABLE 6

TISSUE RESISTANCE/100 G TISSUE BEFORE AND AFTER HEMORRHAGE[a]

	N		Control	15 Min Post-Hem	16 Hrs Post-Hem
Heart	6	Mean	0.47	0.49	0.24*
		Range	0.38 - 0.63	0.25 - 0.93	0.18 - 0.34
Kidney	12	Mean	0.23	0.40**	0.32*
		Range	0.19 - 0.32	0.19 - 0.69	0.19 - 0.49
Spleen	5	Mean	0.14	0.96*	0.08*
		Range	0.06 - 0.28	0.20 - 1.18	0.06 - 0.12
Whole Bone	12	Mean	3.72	5.25**	2.61*
		Range	1.89 - 6.94	3.37 - 7.00	1.46 - 6.78
Marrow	12	Mean	3.16	4.99*	1.72**
		Range	2.04 - 4.56	2.84 - 9.23	0.71 - 3.60
Osseous Tissue	12	Mean	5.34	8.10**	3.59
		Range	2.68 - 11.75	4.68 - 11.63	1.68 - 6.78

[a] Resistance = $\frac{\text{Mean Blood Pressure}}{\text{Blood Flow/Min/100 G}}$

* $P < 0.05$ based on comparisons with control values using the Wilcoxon paired-sample test

** $P < 0.01$

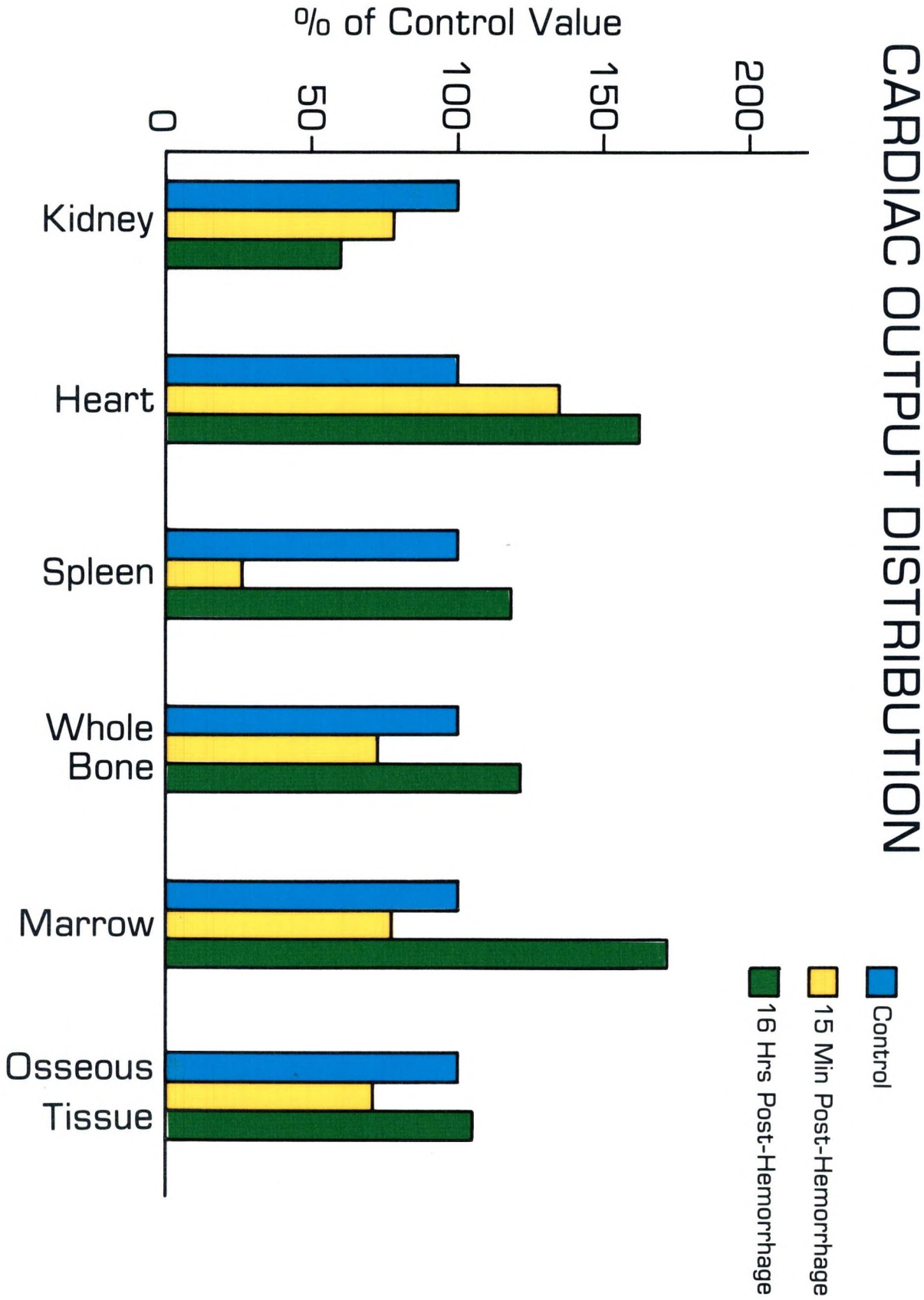
TABLE 7

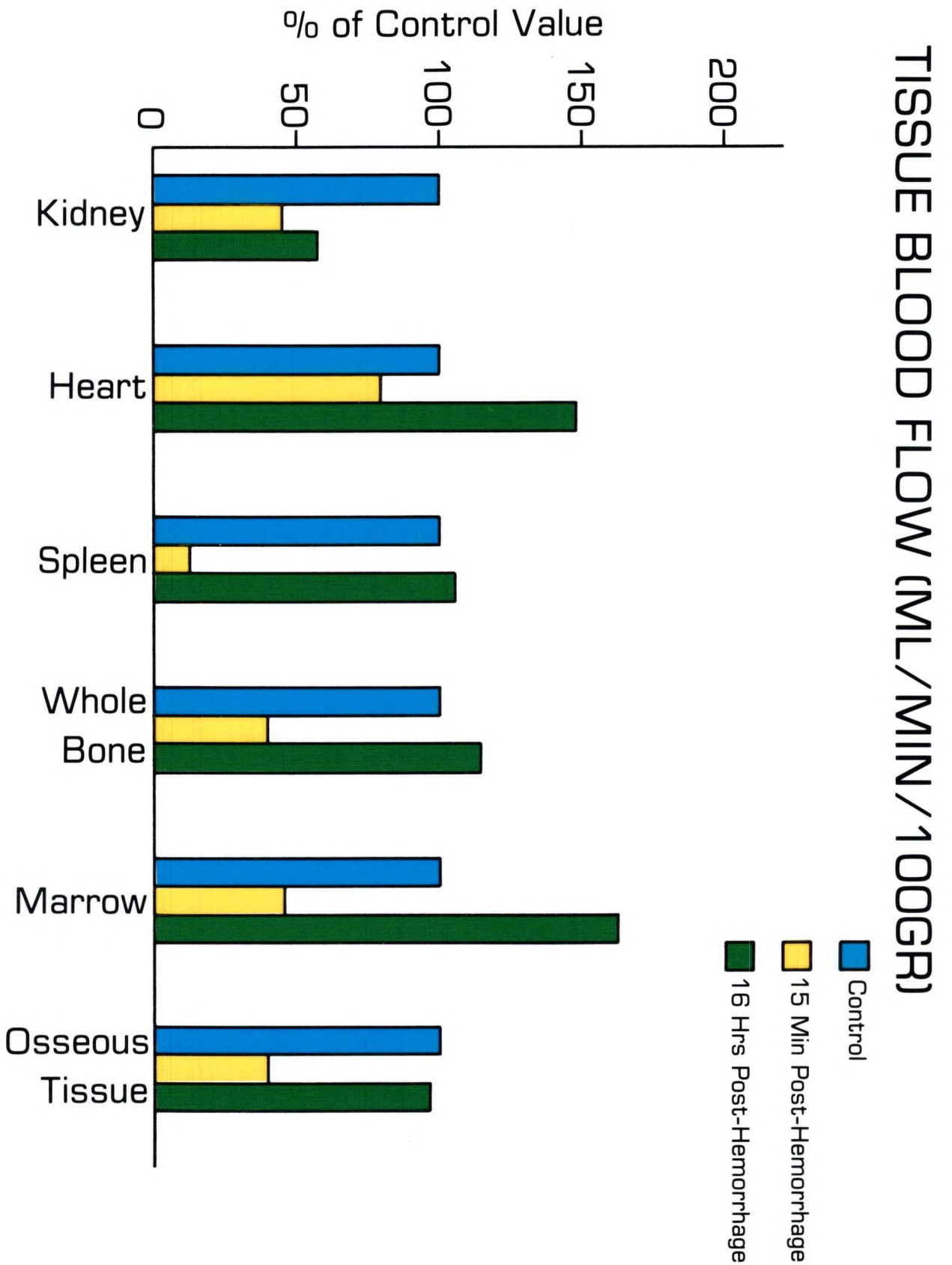
TISSUE BLOOD FLOW IN CONTROL ANIMALS

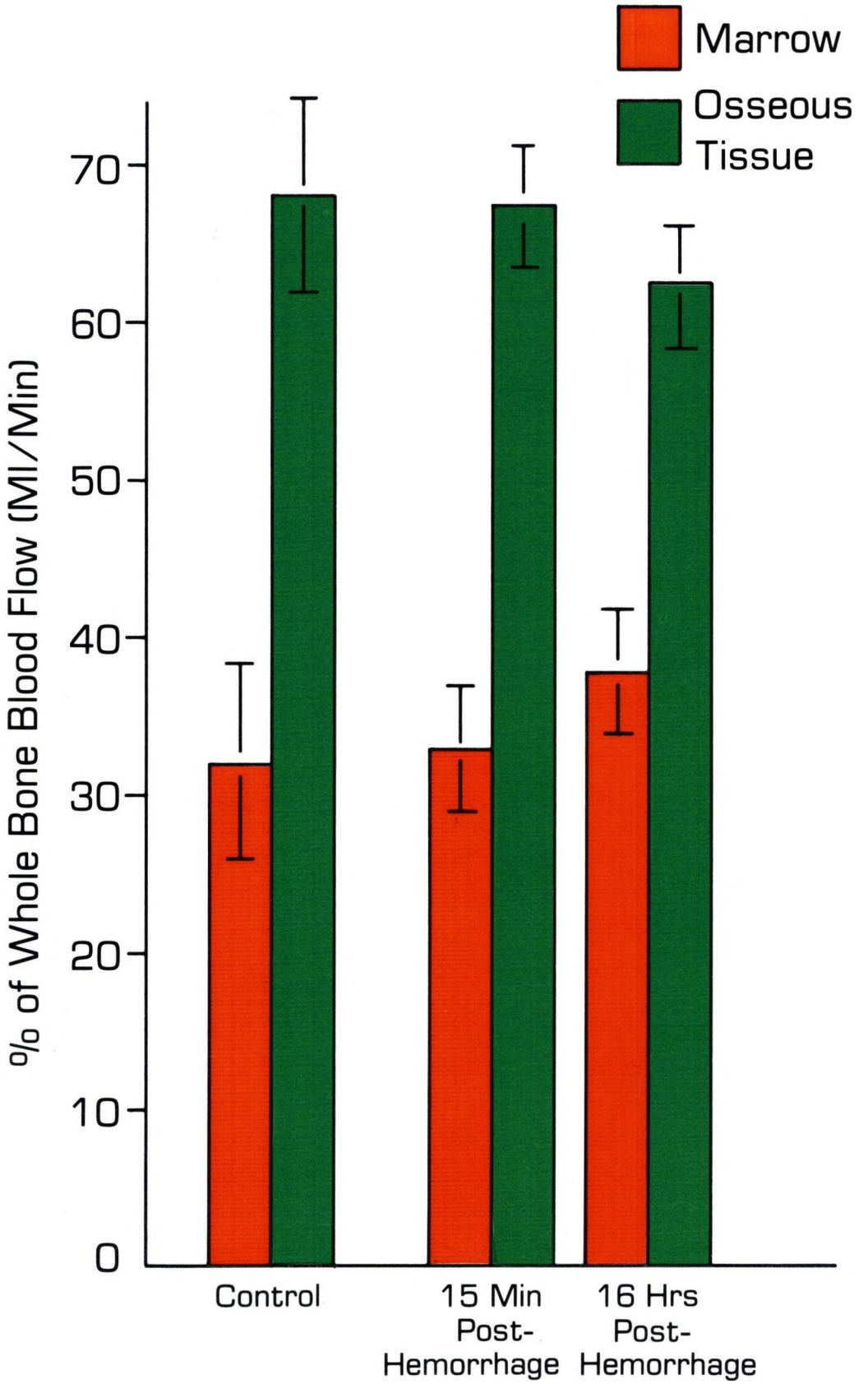
Tissue	First Estimate[a]	Second Estimate[b]	Third Estimate[c]
Heart	100	97	80
Kidney	100	147	90
Spleen	100	111	61
Whole Bone	100	102	84
Osseous Tissue	100	98	73
Marrow	100	118	129
Total Output	100	82	81

For comparison, changes are expressed as a percentage

[a] Control value
[b] Corresponds to 15 minute post-hemorrhage value
[c] Corresponds to 16 hour post-hemorrhage value


CARDIAC OUTPUT DISTRIBUTION
% of Control Value
0
50
100
150
200
Kidney
Heart
Spleen
Whole Bone
Marrow
Osseous Tissue
Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage


TISSUE BLOOD FLOW (ML/MIN/100GR)
% of Control Value
0
50
100
150
200
Kidney
Heart
Spleen
Whole Bone
Marrow
Osseous Tissue
Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage


Marrow
Osseous Tissue
% of Whole Bone Blood Flow (Ml/Min)
0
10
20
30
40
50
60
70
Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage


TISSUE RESISTANCE
% of Control Value
0
50
100
150
650
Kidney
Heart
Spleen
Whole Bone
Marrow
Osseous Tissue
Control
15 Min Post-Hemorrhage
16 Hrs Post-Hemorrhage

DISCUSSION

The purpose of this investigation was to apply the microsphere technique to measurement of bone and marrow blood flow before and after hemorrhagic hypotension. In the following discussion five major topics will be covered: first, validation of the microsphere technique and its application to bone studies; second, hemodynamics of the normal bone; third, the general cardiovascular response to hemorrhage; fourth, the specific effects of hemorrhage on bone and marrow blood flow; and fifth, possible mechanisms to explain the response. Since previous studies have dealt in detail with the general response to hemorrhage in the rabbit, the major emphasis will be on changes in bone and marrow.

Validation of the Microsphere Technique and Application to Bone

The skeleton is a large tissue mass weighing some 10 to 12 kg in the average sized human. The metabolic activity and vascular supply of this organ are greater than usually assumed (85). Enveloped within the skeleton is bone marrow which functions as both a reticuloendothelial and hematopoietic organ (86) and occupies a total tissue volume equal to the liver (87). Thus, skeletal blood flow supplies the nutritional needs of the active cells in both osseous tissue and marrow while also serving as a transport vehicle for bone mineral turnover. The major difficulties have been separating and quantitating bone and marrow blood flow due to the

lack of an appropriate method. The radioactive microsphere technique seems ideally suited for studying relatively inaccessible areas such as bone and marrow.

There are several advantages to the microsphere method as applied in the present study for measurement of bone blood flow. The technique is easily used in conscious animals and multiple blood flow measurements are possible without apparent effects on cardiovascular function. Theoretical assumptions regarding unstable blood-tissue interchange of isotopes or diffusible indicators used in many bone blood flow studies are not necessary. The microsphere method also allows separate flow measurements to bone and marrow which is not possible with other techniques.

One potential problem could be the presence of large numbers of operating arterio-venous shunts. These shunts permit microspheres to bypass areas where they normally would trap resulting in underestimated flow rates. It has been reported that a significant number of 15 u diameter microspheres are shunted through the rabbit hind limb, but this has been largely attributed to arterio-venous anastomoses in skeletal muscle (88). Based on anatomical observations of marrow microvasculature (10, 89) and previous studies using microspheres in bone (50, 69), it was concluded that nearly all of the blood in bone and marrow flowed through vessels of capillary dimensions and that extraction of microspheres was essentially complete. Thus, it appears that microspheres are suitable for studying bone blood flow.

The microsphere technique has undergone repeated validation procedures (88, 90-92) and has been used extensively in the rabbit (61, 72, 88, 93, 94). However, it is standard procedure to determine whether adequate mixing and uniform distribution occur under the conditions of a particular experiment. From this study there were several pieces of data which reflect uniform mixing and distribution of microspheres. There was agreement of cardiac output values estimated from multiple reference samples taken at distant sites (Table 2). Also, calculated versus actual estimates of flow showed excellent agreement (Figure 5). Simultaneous injection of all three microsphere isotopes provided highly comparable flow rate estimates to multiple tissues. Finally, flow rates to paired organs, the kidneys and femurs, were not significantly different (Figures 3 and 4).

In dealing with a tissue such as bone, which has relatively low flow rates, it is imperative that adequate numbers of microspheres become trapped in the tissue (64). Thus, estimates of counts/sphere were made and total numbers of microspheres in each tissue were calculated and total injected microspheres adjusted appropriately. In this way, there were always a minimum of 400 microspheres in bone and marrow samples. The above precautions increased the precision and reliability of the results presented in this study.

Hemodynamics in Normal Bone

Results from this study provide data on normal circulatory hemodynamics of bone in unanesthetized rabbits. On a weight basis marrow perfusion is greater than bone, but actual flow rates to marrow (0.71 ml/min) were about 37% of flow to the surrounding osseous tissue (1.94 ml/min). Vascular resistance in cortical and cancellous bone was greater than in marrow (see Table 8), possibly due to the unusually long length of cortical capillaries (1). The resistance data also show that the vascular beds of marrow and surrounding bone are in parallel. This assumption is based on applying the resistance data to standard formulas which differentiate series versus parallel flow patterns (95). This information supports previous anatomical (10) and physiological (58) evidence for separate blood supplies to marrow and bone. Assuming that femoral bone represents 1/25 of the entire skeletal mass (42), it was calculated that approximately 12.5% of cardiac output is distributed to the osseous portion of the total skeleton. Based on similar calculations, assuming marrow equals roughly 1.7% of the body weight in rabbits (96), the fractional flow distribution to total marrow tissue is about 3.6% of cardiac output. Thus blood flow to the skeletal system including marrow represents roughly 16% of cardiac output. This is comparable to flow fractions going to both kidneys.

Comparison of blood flow data reported here with that in the literature can be done, however, variation should be expected. Values from

previous bone blood flow studies show marked variability due to different techniques, type and plane of anesthesia, and the extent of surgical intervention. Also, some studies do not separate marrow flow from osseous flow. Flow rates reported here are based on conscious animals with limited surgical intervention. Table 9 summarizes previous reports of bone and marrow blood flow. Marrow flow rates of 29 ml/min/100 g obtained here fall well within the reported range for this tissue. Estimates of bone blood flow appear to be generally higher than previous reports. This may reflect a greater sensitivity of osseous vessels to the effects of anesthesia. It seems likely that the blood flow values for marrow and bone reported here are accurate and reasonable for the unanesthetized rabbit.

In addition, cardiac output and regional flows to other tissues were in general agreement with previously reported values (61, 80, 88, 94). This gives further support to the validity of bone and marrow estimates.

Cardiovascular Response to Hemorrhage

Hemorrhage hypotension sets into motion a train of compensatory adjustments within the cardiovascular system designed to maintain tissue perfusion rates. Initial circulatory changes involve stimulation of neuronal and humoral induced vasoconstriction which does not develop uniformly in all tissues but acts to shunt blood toward certain vital organs. The results of the present study vividly demonstrate this immediate response pattern to acute blood loss.

TABLE 8

PREVIOUSLY PUBLISHED BLOOD FLOW RATES TO MARROW AND OSSEOUS TISSUE (ML/MIN/100 G)

Marrow	Osseous Tissue	Whole Bone	Investigator	Species	Method & Conditions
25 ± 14.6 femur	1.0 ± 0.7 cortical diaphysis		Lunde & Michelsen 1970 (69)	rabbit	15 u microspheres-anesthesia and surgery
10 - 120			Michelsen 1968 (37)	rabbit	perfusion technique-anesthesia and surgery
41 ± 18			Cumming & Nutt 1962 (22)	rabbit	venous effluent collection anesthesia and surgery
3.1 tibia	0.76 cortical diaphysis		Kelly 1973 (58)	dog	I-labeled 4-iodoantipyrine washout anesthesia and surgery
29 femur	17 cortical diaphsis		Brookes 1967 (55)	rat	^{51}Cr-tagged RBC's-anesthesia
		12-18 femur	Kane & Grim 1969 (50)	dog	^{42}K and ^{86}Rb clearance unanesthetized and no surgery
	8.22 ± 0.46 femur		Ray, Kawabata & Galante 1967 (42)	dog	^{85}Cr and ^{45}Ca clearance anesthesia and surgery
29.7 ± 5.7	21.6 ± 8.4 cortical and cancellous	28.5±9.0	Syftestad 1976	rabbit	15 u microspheres-unanesthetized and no surgery

The initial (15 min post-hemorrhage) observed fall in cardiac output was greater than the reduction in blood pressure indicating that sympathetic reflexes are geared more at maintaining blood pressure than cardiac output (95). A corresponding drop in hematocrit is consistent with the rapid hemodilution reported in rabbits (79). The elevated pO_2 (not statistically significant) seen 15 minutes post-hemorrhage most likely resulted from a stimulated respiratory rate as evidenced by the significant fall in blood pCO_2. These data are consistent with a respiratory compensation of mild metabolic acidosis known to occur during hemorrhage (97).

The observed tachycardia was possibly due to a massive reflex sympathetic discharge occurring immediately following hemorrhage. These reflexes cause dilation of the coronary vasculature which is also known to have powerful autoregulatory potential during hypoxia (98). Although actual perfusion rates in the heart decreased 15 minutes post-hemorrhage, the increased fraction of cardiac output indicated an attempt to preserve myocardial blood flow. The above two mechanisms undoubtedly contributed to this relative preservation of coronary blood flow following the acute hypotensive crisis in the present study.

The striking vasoconstriction and reduced flow to the spleen are predictable, based on the abundant supply of alpha-type adrenergic receptors within this tissue. Changes in renal flows and resistance agree well with a previous report where a similar amount of blood was

removed (99). The reduced flow was attributed to a synergism of sympathetic nerve activity and humoral effects including those of the adrenal cortex.

Changes in the status of the circulatory system 16 hours following bleeding reflect continuing compensatory reactions initiated in the early post-hemorrhagic period. Spontaneous restoration of blood volume is indicated by a return of cardiac output to near normal levels with the observed peripheral vasodilation tending to delay the rise in arterial blood pressure (100). An increase in plasma volume compensated for the depleted red cell volume as evidenced by the further reduction in hematocrit (101). The decrease in renal blood flow may have contributed to this blood volume recovery by reducing urine formation, thus conserving body fluids (99). The reason for an increase in arterial pO_2 is not known.

Isovolemic anemia can influence cardiac output and tissue resistance (102), but the mechanisms responsible for regional blood flow changes seen in this study seem unlikely to be due to a single physical determinant such as a fall in blood viscosity (103). Blood flow in the spleen returned to normal but cardiac perfusion increased to 147% of the control value 16 hours post-hemorrhage. This pattern is consistent with the concept that increasing metabolic and functional demands lead to varying degrees of active dilation from accumulated metabolites and may be of particular importance in the heart during recovery from hypotension. Additional details on mechanisms involved in the redistribution of cardiac

output in the various soft tissues following hemorrhagic shock are extensively covered in recent reviews by Zweifach (104) and Thal et al. (105).

Effect of Hemorrhage on Bone and Marrow Blood Flow

Details concerning circulatory changes occurring in bone and marrow following hemorrhage have been largely overlooked. Several studies have shown decreases in relative flow and increases in resistance in whole bone or marrow following blood loss (106-108). These experiments provided no quantitative data and no distinction was made between marrow and bone blood flows. They suggested that circulation in marrow undergoes active vasoconstriction during hemorrhagic shock. Results presented here support this view for both marrow and bone. Fifteen minutes post-hemorrhage the marrow flow dropped to 46% of control values while flow to osseous tissue decreased to 39% of the pre-hemorrhage level.

Bosch (109) has made a detailed study of calcium-45 plasma clearance in canine tibia and postulated that cortical capillaries are solely in series with marrow resistance vessels. Any increase in resistance shunts more blood through marrow with a smaller flow into cortical nutrient capillaries. Therefore, during vasoconstriction, as occurs following hemorrhage, more of the total femur flow should be directed away from bone into marrow. Data presented here do not support this hypothesis. In the present study marrow received 32% of total femur blood flow under normal conditions. Immediately after hemorrhage, this

percentage did not change indicating that reduction in flow to osseous tissue and marrow were proportionally the same. It appears that the complex anatomical and functional arrangement of resistance vessels respond to hemorrhage in such a way as to preserve the normal flow distribution within bone and marrow immediately following vasoconstriction. This situation may change several hours post-hemorrhage.

There is an established relationship between increased bone blood flow and active hematopoiesis in marrow. Michelsen (110) found a significant increase in marrow flow a week after phenylhydrazine induced anemia. Several related studies (111-113) have shown a striking similarity between blood flow and distribution of red marrow in the skeleton but no distinction could be made between flows in marrow and osseous tissue. The rate of blood flow to bone was thought to be important in proliferation of hemoglobin synthesizing marrow. It was further implied that hematopoietic stimulating factors produced in bone may be carried directly to marrow via the bone-bone marrow portal vessels. Of fundamental importance to these considerations is whether the high bone blood perfusion rate associated with active marrow is simply a reflection of active blood flow in the marrow cavity or involves increased flow rates to osseous tissue. In the present study the marked increase in marrow flow 16 hours after hemorrhage was not seen in the surrounding bone (Table 5). Consistent with this was a significant increase in the percentage of total femur blood flow going to marrow (Figure 8) indicating a

selective vascular effect in this tissue. This gives evidence that in certain situations marrow and osseous tissue may have separate control mechanisms. The importance of the marrow response is further emphasized by comparing the magnitude of the blood flow change to that seen in the heart (Figure 7). There is actually a greater percentage increase in marrow flow 16 hours post-hemorrhage than in myocardium.

In looking at whole bone data (bone + marrow) the importance of separating the two components becomes apparent (Table 5). When marrow flow was separated from whole bone the significant increase in blood flow 16 hours post-hemorrhage was obvious. However, the effect was masked in the whole bone data by the large flow to osseous tissue which did not change. Thus, it is critical to separate these two components to accurately assess sites and mechanisms by which factors can influence total bone blood flow.

Prior to describing a possible mechanism for the observed response, the control experiments should be mentioned. The control data (Table 7) indicate that neither restraint, the microspheres, nor the particular isotope used account for the observed changes in blood flow. Although there were changes in some tissues with time, they were not consistent with the changes following hemorrhage. Changes in cardiac output due to restraint are consistent with a previous report (61).

A possible explanation for the changes caused by hemorrhage may involve the glycoprotein hormone erythropoietin produced by the kidney

which can directly stimulate red cell production in bone (114, 115). The magnitude of erythropoietin release is dependent upon the severity of renal hypoxia (116-118). A 50% reduction in kidney blood flow following hemorrhage as seen in this study is known to result in peak erythropoietin titres 12 to 24 hours later (119).

Although erythropoietin was not measured directly, the observed increase in marrow perfusion rates 16 hours post-hemorrhage may have been related to the reported vasoproliferative effects of increased circulating erythropoietin. Greater numbers of arterioles and dilation of sinusoids in bone marrow and spleen following bleeding or direct erythropoietin injection have been documented (120, 121).

Hypoxia alone can cause dilation of vessels resulting in increased blood flow; however, it has been shown that bone marrow oxygen levels do not change significantly following hemorrhage (122, 123). The increased vascularization has been observed even in the absence of active erythrocyte production, suggesting the increased blood flow was possibly a preliminary step to erythropoiesis (124). Associated with increased erythropoiesis is a parallel reticuloendothelial activation in marrow which may be important in defense against circulating endotoxin following acute blood loss (125, 126).

Thus, the increased perfusion of marrow 16 hours after hemorrhage may have been stimulated by a direct vascular effect of erythropoietin to help meet the metabolic demands of erythropoiesis and reticuloendothelial activation. This reaction provides optimal environments for red

blood cell proliferation and host defense stimulation which are both necessary homeostatic mechanisms in recovery from hemorrhagic hypotension.

SUMMARY AND CONCLUSIONS

1. Cardiovascular responses in skeletal tissue following reversible hemorrhagic hypotension were studied using the radioactive microsphere technique of regional blood flow measurement.
2. Individual microsphere isotopes, each with a different label, were injected at three time intervals: first, as a control value; second, 15 minutes following a standardized, non-fatal hemorrhage; and third, 16 hours post-hemorrhage.
3. Values for blood pressure, heart rate, blood gas and pH, hematocrit, cardiac output, blood flow, percentage distribution of cardiac output and tissue resistance were calculated for each time period.
4. Results of four standard validations used to test reliability of the microsphere method as applied in this study showed close correlation between blood flow and microsphere distribution.
5. Fifteen minutes after hemorrhage cardiac output, blood pressure, arterial pCO_2 and hematocrit decreased and heart rate increased. Blood flows to heart, kidney, spleen, whole bone, marrow and osseous tissue decreased with corresponding resistance increases in all tissues except the heart. Spleen, whole bone, marrow and osseous tissue received a decreased percentage distribution of cardiac output while the percentage going to the heart increased.

6. Sixteen hours following hemorrhage pO_2 increased and hematocrit decreased. Heart and marrow blood flows increased and kidney flow rates remained low. Tissue resistance decreased in the heart, spleen, whole bone and marrow but remained elevated in the kidney. Percentage distribution of cardiac output increased in the heart, whole bone and marrow and decreased in the kidney.
7. Normal hemodynamic properties of bone showed parallel blood supplies to marrow and osseous tissue with higher vascular resistance in the latter. It was estimated that total skeletal tissue plus marrow received 16% of resting cardiac output.
8. The response 15 minutes post-hemorrhage demonstrated characteristic decreases in regional blood perfusion with relative preservation of myocardium. There was a uniform reduction of blood flow within the femur giving no evidence of preferential shunting away from osseous tissue in favor of marrow.
9. Sixteen hours after hemorrhage there was an increase in blood flow to marrow but not surrounding osseous tissue. Blood flow to the various soft tissues reflected continuation of a stress state where both vasodilatory metabolic and vasoconstrictor neurohumoral factors were still operative.
10. The vascular response in marrow may represent a direct vasoproliferative effect of erythropoietin in preparation for hematopoietic and reticuloendothelial activation.

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