ROLE OF

THE NEUTROPHIL IN

POST-ISCHEMIC SKIN FLAPS

by

Chen Lee, M.D.

Department of Surgery

McGill University, Montreal

A thesis submitted

to the Faculty of Graduate Studies and Research,

in partial fulfilment of the requirements for

the degree of Master of Science.

NOV 1990

ABSTRACT

Near perfection of microsurgical techniques have permitted the routine clinical application of free flaps to reconstruct difficult wounds. Success of this versatile and powerful reconstructive tool is most often threatened by the consequences of vascular thrombosis and ischemia. Despite the need to revascularize an ischemic free flap, the post-ischemic injury has been reported to paradoxically escalate because of revascularization and reperfusion. Our limited understanding of the pathophysiologic events initiated by ischemia has thwarted therapeutic attempts at arresting this post-ischemic injury. In order to gain a better understanding of the post-ischemic injury, this thesis reviewed current theories. Special emphasis was placed on the concept of reperfusion injury. The existing literature has been largely supportive of a neutrophil mediated reperfusion injury in ischemic organs such as heart, skeletal muscle, and intestine. This provided the motivation to explore the pathophysiologic role of the neutrophil in post-ischemic skin flaps.

In a series of four experiments, this thesis evaluated neutrophil function, localization, depletion, and inhibition in ischemic cutaneous and myocutaneous flaps. Neutrophil function as measured by phorbol myristate acetate stimulated superoxide production was found to be enhanced on reperfusion of ischemic myocutaneous flaps. ^{111}Indium labelled neutrophils were found to localize in post ischemic cutaneous and myocutaneous flaps. Post-ischemic **cutaneous** flap survival was not improved by either cyclophosphamide induced neutrophil depletion or ibuprofen neutrophil inhibition. Post-ischemic **myocutaneous** flap survival was significantly improved by both cyclophosphamide induced neutrophil depletion and ibuprofen neutrophil inhibition. Thus, the cumulative evidence from these experiments yielded surprisingly different conclusions for cutaneous and myocutaneous flaps. The post-ischemic injury in cutaneous flaps was not mediated by the neutrophil. In contrast, the neutrophil did partially mediate the complex post-ischemic injury of myocutaneous flaps.

RESUMÉ

Les techniques microchirugicales sont maintenant si perfectionnées qu'elles ont permis l'utilisation courante de lambeaux libres pour soigner les plaies compliquées. Le succès de cette méthode si versatile et si puissante a été le plus souvent compromis par les complications thrombotiques et ischémiques. Malgré l'absolue nécessité de revasculariser un lambeau libre ischémique, certains auteurs ont suggéré que les lésions post-ischémiques étaient paradoxalement aggravées par la revascularisation et la reperfusion. Notre compréhension limitée des conséquences physiopathologiques de l'ischémie a contrarié les essais thérapeutiques destinés à empêcher le développement de lésions de revascularisation. Ces dernières, si l'on en croit la littérature, seraient induites par les polynucléaires neutrophiles lors d'ischémie cardiaque, musculaire ou intestinale. Ces données nous ont conduit à étudier le rôle physiopathologique des polynucléaires neutrophiles à la suite d'une ischémie au niveau de lambeaux libres.

Quatre protocoles expérimentaux dont les résultats font l'objet de cette thèse ont évalué la fonction et la localisation des polynucléaires neutrophiles ainsi que les conséquences de leur déplétion ou de leur inhibition au sein de lambeaux ischémiques cutanés et cutanéomusculaires. La fonction neutrophilique, mesurée par la production de superoxide en réponse à une stimulation par l'acétate de phorbol myristate, est augmentée lors de la reperfusion de lambeaux cutanéomusculaires ischémiques. Des polynucléaires marqués à l'Indium^{111} se concentrent dans les lambeaux cutanés et cutanéomusculaires à la suite d'une ischémie. La survie post-ischémique de lambeaux cutanés n'est améliorée ni par l'administration de cyclophosphamide (induisant une déplétion neutrophilique) ni par celle d'ibuprofène (inhibiteur fonctionnel des polynucléaires neutrophiles) tandis que chacun de ces traitements a un effet bénéfique sur la survie post-ischémique des lambeaux cutanéomusculaires. Les conclusions pouvant être tirées de ces

expériences sont donc trés différentes selon qu'il s'agit de lambeaux cutanés ou cutanéomusculaires. Dans le premier cas les neutrophiles ne semblent pas être impliqués. Dans le second ils sont partiellement responsables.

PREFACE

This thesis represents original work generated by the author while he was training in General Surgery at McGill University, Montreal, Quebec, 1988-89. All experiments reported herein were conceived and executed under the supervision of Dr C.L. Kerrigan in the Microsurgical Research Laboratories at the Royal Victoria Hospital, Montreal. The superoxide assay crucial in experiment #1, was developed by Dr J.M. Tellado. The equipment necessary to perform this assay was generously supplied by Dr N.V. Christou. In experiment #2, neutrophil radiolabelling with ^{111}Indium was performed by Ms M.C. Blais of the Diagnostic Imaging Services, Mallinckrodt Canada Inc. Imaging of flap radioactivity was done by Ms L. Proulx and Dr R. Lisbona at the Department of Nuclear Medicine, Royal Victoria Hospital. In experiment #3, randomization and coding of drugs was accomplished with the assistance of Mr D. Bois of the Oncologic Pharmacy, Royal Victoria Hospital. The ibuprofen used in experiment #4, was kindly supplied by Mr R. Bourgault of the Upjohn Company. High performance liquid chromatographic determinations of swine serum ibuprofen levels were performed by Dr D. Freeman of the Clinical Pharmacology Resource Group, University of Western Ontario. Statistical analyses were performed on a desk top computer using software from SAS^{R} and MicroStat^{R}. This project was funded by a grant from the Medical Research Council of Canada (MA-7240).

ACKNOWLEDGEMENTS

Since commencing this project I have learned much about research, reconstructive surgery, computers, and farm animals. This is quite a feat for a computer illiterate person who did not know what a flap was, and had never touched a pig before. Only a special person such as Carolyn L. Kerrigan M.D. could teach the A B C's of computers, demonstrate how to handle a pig, and not sarcastically remark on my ignorance of flaps. Her unselfish generosity, warm friendship, and wise guidance were essential to the successful completion of this project. I now look to this elegant woman for continued friendship and guidance during my residency in Plastic Surgery.

I would like to also acknowledge the influence and contribution of the following individuals: José M. Tellado M.D., for the stimulating ideas and the technical assistance with the superoxide assay. Without his help the whole series of experiments might not have been possible; Philippe Poussier M.D., who has been like an older brother guiding a directionless sibling to the right path; Errol B. Marliss M.D., for initiating me to the research laboratory; Jonathan Meakins M.D. and John Marshall M.D., for the opportunity to participate as an elective student in surgical research; Juan Carlos Puyana M.D. and Luis A. Picard-Ami M.D., for their friendship and stimulating discussions from which ideas were conceived for this project; Ms. Genvieve Holding, whose good nature and warm words were always a cheerful welcome; and the people I cherish most, my Parents, who teach from another culture and generation that honour, respect, and hard work yields the satisfaction of life.

TABLE OF CONTENTS

OVERVIEW

Since Ambroise Paré (1517?-1590) first championed the arterial ligature to control hemorrhage, four centuries of advancements in vascular reconstruction now permit restoration of the anatomical continuity of severed vessels with diameters smaller than 1 mm. Success is routinely achieved in the replantation of amputated digits. In difficult wounds, the restoration of anatomical form and function is feasible by distant microvascular transfer of complex tissues (free flaps).

Ischemia is the most common threat that jeopardizes the success of a replant or free flap. Despite two decades since the first free flap, little progress has been made in the treatment of the 'critically' ischemic flap. However, a wealth of knowledge has been gained in the trenches of the research laboratory. Through experimental work a better understanding of the biology of cell injury has been achieved. These current concepts and theories on post-ischemic injury are described in the section 'Physiology of the Injury'. Special emphasis has been placed on oxygen free radicals and how they may pertain to biological systems. Mechanisms by which the neutrophil can give rise to tissue injury are also reviewed.

The essential focus of this thesis rests on the neutrophil as a potential etiologic force that drives the post-ischemic injury in free flaps. Four complimentary experiments were designed to answer the question, 'Does the neutrophil cause injury in the post-ischemic skin flap?'. The answer is not likely to effect immediate changes in clinical management. Nevertheless, such contributions represent the crucial initial steps in the evolution of sound scientific management.

BACKGROUND

VASCULAR RECONSTRUCTION

Organ failure resulting from vascular occlusion has proved to be catastrophic in all surgical disciplines. Until the 1800's, vascular reconstruction and restoration of flow had always been sacrificed for the sake of hemostasis. In 1899, the first end-to-end anastomosis of an artery in a human was performed.[59] Soon after, Carrel and Gutherie established the principles of modern vascular anastomosis by demonstrating experimental success with fresh and preserved homografts and heterografts for vascular replacement and bypass.[59] Despite sporadic clinical cases of vascular reconstruction, arterial ligation and amputation of gangrenous tissue remained the mainstay of surgery for critically ischemic organs until World War II. Development of new surgical techniques, better anaesthesia, and improved availability of blood opened doors to the era of **cardiovascular surgery**, where routine revascularization of a critically ischemic heart or limb became possible. When the principles of vascular reconstruction were applied to organ replacement, restitution of physiologic function by allograft was feasible in diseases of various etiologies. Combined with improved understanding of the immune system, successful **transplantation** of cadaveric 'spare parts' is now performed with increased frequency in end-stage diseases of the heart, lung, kidney, liver, and pancreas.

The birth of **microsurgery** took place in 1960, when vascular principles described by Carrel were employed under the operating microscope by Jacobson and Suarez.[57] With design of new microsurgical instruments and refinement of skills, patency in anastomoses of blood vessels with diameters less than 1 mm could be consistently achieved.[14] In 1965, Komatsu and Tamai[67] were the first to successfully **replant** a completely amputated thumb demonstrating the clinical utility of microvascular surgery. A renaissance in plastic and reconstructive surgery was heralded in 1972, by Daniel and Taylors'[27] report of the first successful distant transfer of a cutaneous **free flap** to cover a traumatic ablation injury to the posterior

tibial region of a lower extremity. Since then, there has been an exponential growth in the use of distant free tissue transfers to close a diversity of complex wounds ranging from extremity injuries, to radiation and decubitus ulcers.[118] Much of the disfigurement resulting from necessary en block resection of tumours in surgery of the head and neck, potentially can be ameliorated by improved design and increased use of complex composite flaps[21] as in facial bone reconstruction by employment of the osteocutaneous flap.[128] Functional salvage of a traumatized extremity may depend on distant transfer of well vascularized tissue to promote healing and eradicate infection.[17,84]

GRAFTS, FLAPS, and FREE FLAPS

Skin and bone **graft** survival depend on adequate plasmatic circulation of nutrients from the vascular bed in which it is placed. The presence of an undisturbed intrinsic vascular anatomy of a surgical flap imparts distinct advantages over grafts. First, the survival character of flaps far exceeds grafts in areas of acquired (radiation, atherosclerosis) or innate (bone, tendon) poor blood flow. Furthermore, bacterial clearance is enhanced in well vascularized flaps (eg. myocutaneous).[17] Finally, composite tissue flaps offer a variety of applications due to their desirable characteristics such as tissue thickness, composition, and complexity.[26] In contrast, skin or bone grafts are markedly restricted in complexity and application because of limited ability to draw sufficient nourishment by passive permeation from the recipient vascular bed.

All **flaps** can be classified based on method of movement, blood supply, and tissue composition.[26] Originally, skin flaps were classified based only on the method of movement of donor tissue to recipient site. Local movement could be achieved by advancement, rotation, or interpolation. Where local tissue was of insufficient

quantity or inappropriate tissue composition to reconstruct a defect, donor tissue from a distant site could be employed in the reconstruction. Distant movement was initially accomplished using ingeniously sequenced surgical procedures, where the skin flap of a mobile donor extremity was 'tubed' to preserve its blood supply, then approximated to cover a distant recipient bed; at a later procedure, distant transfer of the flap was completed by transection and revision of the tube base. Because safe distant movement demanded adequate neovascularization of donor flap to the recipient vascular bed, long lengths of time and multiple operations were obligatory.[124] With the introduction of microvascular surgery, distant movement by **free flap** was possible in a one stage procedure by vascular transection of the donor flap followed by microvascular anastomosis at a distant recipient site. Improved understanding of the anatomic distribution of vascular territories has permitted reconstruction of more complex wounds.[26] Procedures designed to achieve wound coverage by movement of a flap from one area to another can only be justified if loss of tissue and function at the donor site is inconsequential, relative to the therapeutic benefit achieved at the recipient bed. When this principle is optimized by proper preoperative planning and appropriate flap selection, essential coverage of vital structures in the recipient bed can be achieved with minimal tissue and negligible loss of function from the donor site.[118]

Flap Complications

Flaps or portions of flaps that fail, thus compromising the essential coverage of the recipient bed, have been shown to have disastrous consequences. A failed flap will often require multiple secondary procedures, increased duration of hospitalization, and prolonged convalescence. In addition, the loss of soft tissue coverage at the recipient bed may expose vital vascular structures, predispose to sepsis, and can culminate in either extremity amputation or even loss of life. Although failure can be precipitated by infection or wound dehiscence, the most common cause is **ischemic** necrosis.[40]

The estimated incidence of ischemic necrosis in local flaps causing major complications (defined by a flap that failed to accomplish the purpose for which it was designed) ranges from 6 to 10% and 7 to 24% for minor complications (defined by a flap in which perfect primary healing had not occurred, but the purpose was achieved).[28,69] **Local flaps** primarily suffer partial ischemic complications at that portion most distal from the blood supply, corresponding to where flap dimensions may have been created too large relative to the vascular territory by which it is nourished. Kerrigan[60] showed that **partial ischemia** resulted simply because of inadequate distal delivery of arterial blood within the flap's **intrinsic** microvascular bed. As no surgical method of revascularization exists to correct partial ischemia of a failing flap and since pharmacologic attempts to increase arterial blood flow have been largely unsuccessful in improving survival, prevention has been the prime method of minimizing the incidence of partial ischemia.[26,62,94]

Prevention by meticulous preoperative design to ensure that flap dimensions are not 'too large' or utilization of the **delay procedure** are the only effective means of minimizing the risk of partial ischemia.[26] The delay procedure was devised to improve skin flap survival by incising two longitudinal sides of a potential flap followed by undermining of the intervening skin thus creating a bipedicle flap. Three weeks after the flap had been sutured back in situ, it could then safely be converted to a single pedicle flap with improved survival of its anatomic length. The mechanism, has been in part attributed to a redistribution of nutrient blood flow within the intrinsic vascular network of the flap as a response to the hypoxic stress of the delay procedure.[58,95,125]

Since the inception of clinical microvascular surgery, few reports have dealt with the incidence of ischemic necrosis in **free flaps** alone. In a small series of free groin flaps, Serafin[116] noted that thrombotic occlusion of the microvascular anastomoses was responsible for a 22% and 14% incidence of major necrosis and

minor necrosis, respectively. Furthermore, the incidence of thrombotic complications in small vessels approached 10 to 15% in other clinical series of free tissue transfers[73,118] and replantations.[127] Focal thrombosis of the microanastomoses to a flap with resultant arrest in nutrient blood flow has proved to be the most common complication causing free flap ischemia and failure. Contrary to partial ischemia, **global ischemia** brought on by vascular complications **extrinsic** to a flap, as in the case of anastomotic thromboses, remain amenable to revascularization by microvascular reconstruction. However, a clear distinction must be made from partial ischemia where the prime problem lies in insufficient distal delivery of blood in the intrinsic microvasculature. The later is not amenable to surgical revascularization. It is the purpose of this thesis to improve our understanding of global ischemic injury as sustained by free flaps.

PHYSIOLOGY of the INJURY

By historical perspective, failure to re-establish vascular perfusion in ischemic organs had always culminated in tissue necrosis and organ failure. Even with the technology to revascularize virtually any globally ischemic organ, tissue necrosis has continued to be a serious problem in the post-ischemic period. Until recently, this necrosis had been ascribed solely to the insult sustained during the period of **ischemia.** The possibility that organ failure occurred as a direct result of **reperfusion** (defined as restoration of nutrient blood flow) was and is still largely ignored because this is at paradox with the absolute necessity of reperfusion in the ultimate survival of ischemic tissue.[13] Numerous investigators have since demonstrated the undeniable existence of a paradoxical second injury that occurs early during the period of reperfusion in many organ systems.[42,44,49,53,75,120,142,143] In animal experimentation, reperfusion injury has been shown to be superimposed and additive to the injury sustained during the ischemia itself.[53] Furthermore, reperfusion injury has been

demonstrated to be alterable by pharmacologic modalities and thus has served as one focus of attention in studies attempting to minimize the post-ischemic organ injury.[2,8,15,25,42,43,49,89,109]

Therapeutic interventions to improve post-ischemic flap survival can potentially be achieved at two levels.

1) Early detection and prompt surgical revascularization to minimize the duration of the ischemic insult.[26,48,130]

2) Attempts to increase tissue tolerance to ischemia and reperfusion injury.

Short of early detection and revascularization, are there other methods which can improve outcome after an ischemic insult? Post-ischemic organ salvage potentially can be achieved by increasing tissue tolerance to either the ischemic or reperfusion insult. Clearly, if adjunctive nonsurgical therapy in improving the injury was possible, benefit would span a diverse set of clinical problems encountered in microvascular, cardiovascular, and transplantation surgery. A comprehensive understanding of the basic physiology of both the ischemic and reperfusion insults will lead to improved methods of increasing tissue tolerance to injury.

Ischemic Injury

Ischemia simply defined, is inadequate blood flow to meet the needs of the affected organ or tissue. The exact mechanisms responsible for ischemic organ failure have not been fully clarified. The functional essence of organs depend on adequacy of both,

1) the metabolic activity of parenchymal cells

and

2) a patent microvascular bed to deliver nutrients.

Dysfunction at either the cellular or vascular level must be considered in evaluating the mechanism of ischemic organ failure.

Cellular response

The most obvious disturbance observed in an ischemic insult has been the rapid exhaustion of molecular oxygen with resultant cellular hypoxia. In mammalian cells, aerobic glycolysis by tetravalent reduction of molecular oxygen has been the prime mode by which an adequate cellular store of high energy adenine triphosphate (ATP) is generated. With inadequate blood flow, this essential store of ATP is depleted at a rate proportional to the metabolic activity of the hypoxic cell. Thus, different degrees of ischemic tolerance exhibited from various tissues can in part be explained by the metabolic activity characteristic of each cell type; for instance, cellular **hypoxic tolerance** is different amongst neurons (most sensitive), myocytes (intermediate), and keratinocytes (least sensitive).[105]

Attrition of the cellular ATP stores can be offset to some degree by anaerobic glycolysis. However, this compensatory mechanism is limited as anaerobic glycolysis has been shown to be far less efficient at ATP regeneration; furthermore, formation of acidic 'waste' products such as lactic acid, inorganic phosphates, and purine nucleosides may add greater stress to the already fragile homeostasis of hypoxic cells. The depletion of ATP and accumulation of 'waste' products may mediate loss of the selective permeability necessary in the 'raison d'être' of biologic membranes, with resultant mitochondrial dysfunction, cell swelling, and death if uncorrected.[9,22]

Vascular response

In addition, the accumulation of anoxic cellular by-products may mediate changes within the microvasculature of tissues that are subjected to global ischemia. Endothelial cell swelling, perivascular swelling, and loss of vascular tone with leakage from the intravascular to the interstitial space have been documented to increase the vascular resistance of tissues subjected to a global ischemic insult.[105] Although the primary vascular occlusion may have been extrinsic and focal, prolonged insult will result in the accumulation of by-products thus initiating diffuse alterations within the

intrinsic vasculature of the tissue resulting in functional closure of the microvascular bed, hence precluding any chance of parenchymal cell reoxygenation. Under such circumstances, a pure ischemic insult ensues at the level of the microvascular bed and hypoxic parenchymal cell death proceeds regardless of whether the primary event causing the initial global ischemic insult can be reversed. This altered microvascular response may indeed explain the **'no reflow'** phenomenon previously described in free flaps.[34,83]

Ischemic preservation

When a critically injured global ischemic organ is destined to fail regardless of whether vascular perfusion is restored, it can be deemed to have exceeded its **ischemic threshold.**[53,61,150] Despite what has been described herein, the basic mechanism and elements of the injury are largely undetermined. We know only that ultimate survival of ischemic organs demands at the very least a patent intrinsic microvasculature to deliver sufficient oxygen and that the hypoxic tolerance of its parenchymal cells has not been exceeded. Methods to effectively prolong the ischemic threshold can serve to temporize injury until such time that reperfusion and reoxygenation can be accomplished. To this goal, methods of preservation during global ischemia have been directed at reducing cellular metabolic rate and maintaining intrinsic microvascular patency.

Interventions designed to reduce either the basal or functional metabolic activity have demonstrated improved cellular tolerance to a hypoxic stress. Hypothermic induced reduction of **basal** metabolic activity of cells has been the most reliable clinical intervention by which ischemic organs are preserved.[10] Under clinical scrutiny, hypothermic ischemic organ preservation has proved to be effective in both replant[50] and transplantation surgery.[10,148] This principle has been most exemplified in congenital neonatal cardiac surgery where complete circulatory arrest under profound hypothermic conditions has been employed successfully for up to 60

minutes.[19,64] Modalities of decreasing **functional** metabolic cellular activity have demonstrated success in cardiac bypass surgery; where potassium mediated cardioplegic arrest of the beating heart is routinely used to minimize ischemic injury.[146] In transplantation, development of complex perfusates such as University of Wisconsin[10,144] and Collin's[145,146] solution have further augmented the hypothermic preservation of ischemic organs. Inhibition of anoxic cellular metabolism by perfusate stabilization of biological membranes and paralysis of ATP catabolism are possible mechanisms to explain the success of **artificial perfusion** in ischemic organ preservation.[126]

Attempts to improve intrinsic patency of the microvasculature under conditions of global ischemia have had limited success. Since the vascular response to ischemia has been partly attributed to the accumulation of cellular 'waste' products, its clearance by simple mechanical **washout** using various perfusates has been proposed as a potential method to preserve patency of the intrinsic vasculature in ischemic organs.[10,105,145] However, Harashina and Buncke[47] have tested various washout solutions for microvascular replantations. They obtained better results when a washout solution was not used compared to washout of the vascular bed with heparinized saline or Collin's solution. Artificial tissue perfusion and washout have not enjoyed widespread use in clinical free tissue transfers.

Reperfusion Injury

No method exists to completely arrest the ischemic injury and indefinitely prolong organ preservation. Thus, ultimate survival is dependant on revascularization and reperfusion before the ischemic threshold of the organ has been exceeded. Although revascularization and reperfusion are necessary to terminate the ischemic injury, it by no means ensures survival as the post-ischemic organ must then endure a second insult initiated paradoxically by the reperfusion itself. Alterations in calcium metabolism, activation of autolytic processes, and production of reactive

oxygen free radicals have been proposed as various potential mechanisms giving rise to injury in the early reperfusion period.

Calcium metabolism

Cheung has proposed that reperfusion injury after an ischemic insult is mediated by cytosolic **calcium overload** with unrestrained activation of autolytic cellular processes.[18,51] The hypoxic insult of ischemia and resultant depletion of ATP stores can impair the functional integrity of biologic membranes facilitating extracellular free calcium to leak into the cell. However, calcium mediated injury may not manifest during an ischemic insult because the limited free calcium load in the stagnant local extracellular microenvironment may be insufficient to trigger autolytic processes within the ischemic cell. When reperfusion occurs, the new influx of free calcium may overwhelm the already impaired ability of the post-ischemic cell to regulate calcium movement across its plasma membrane.[9,18] The resultant massive cytosolic calcium influx and calcium overload will then activate a wide range of calcium mediated autolytic processes (proteases, nucleases, adenyl cyclase, sodium-potassium-ATPase, glycogen phosphorylase, phospholipase). Further depletion of valuable energy supplies can be precipitated by calcium mediated uncoupling of oxidative phosphorylation in mitochondria.[9,18,22]

Drug studies with slow calcium-channel blockers such as nifedipine and verapamil, have yielded encouraging results in post-ischemic myocardial salvage.[15,90,107] However, these studies have failed to delineate the exact mechanism of salvage. An accelerated calcium mediated cell death during reperfusion is a plausible and attractive theory, but the basic premise that cytosolic calcium overload mediates reperfusion injury of ischemic organs remains largely unsubstantiated and requires further elucidation.[18]

OXYGEN FREE RADICALS

As the specific goal of this thesis was to examine free radical injury in the post-ischemic flap a detailed review is warranted. Free radical fuelled redox reactions have been postulated to play a fundamental role in a wide range of biologic disorders. Tissue injury in disease processes such as **ischemia/reperfusion,** circulatory shock,[52] radiation vasculitis, pulmonary oxygen toxicity,[13,122] thermal burns,[38,131] and even normal processes of aging have been postulated to be mediated by the destructive energy of free radicals.[22,97,129,140]

Basic Chemistry

A **free radical** is chemically defined as any molecule or atom in which there is an unpaired electron in its outer shell. Such an electron configuration is unstable and characteristically is highly energetic, facilitating free participation in redox reactions, where reduction or oxidization can stabilize its electron configuration. The participation of thermodynamically reactive molecular oxygen in redox reactions has special significance in aerobic biologic processes. Its reduction can be used beneficially to liberate cellular energy (mitochondria) or to generate toxic free radicals important for host defense (microbial killing).[13,121] By accepting four electrons, oxygen is detoxified and ultimately reduced to water; however, partial reduction can occur with the formation of highly reactive toxic oxygen free radical intermediates (table 1).

Table 1.	**Oxygen Derived Reactive Intermediates**
O_2^-	Superoxide radical
H_2O_2	Hydrogen peroxide
$HO^\bullet$	Hydroxyl radical

The primary product generated from univalent reduction of molecular oxygen is the **superoxide** radical. Uncontrolled biological production of the superoxide radical has been implicated as the initiating event precipitating cell injury in a diversity of pathologic processes.[13,22,30,52,87] Its derivative compounds can freely diffuse across membranes (hydrogen peroxide) and are capable of damaging vital cellular membranes by directly oxidizing organic molecules (hydroxyl radical).[112,115] Although hydrogen peroxide is not a free radical, it is fundamentally integrated into the chemical reactions of biologic systems which generate or propagate other free radicals.[87] The hydroxyl radical, one of the most toxic oxygen derived metabolites, is generated in biologic systems by an iron catalyst (table 2).[121] Iron chelation by deferoxamine has been a proposed method of inhibiting hydroxyl radical production in biologic systems.[1,2,149]

Table 2 **Formation of the Hydroxyl Radical by an Iron Catalyst**

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^\cdot$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + HO^\cdot$$

Much of the microbial killing activity of leukocytes is dependent on the production of a chlorinated oxidant, hypochlorous acid.[18,31,65,66] This potent bactericidal agent is generated by an enzyme (myeloperoxidase) catalyzed reaction of hydrogen peroxide with chloride. It is interesting to note that under pH controlled laboratory conditions, hypochlorous acid (Dakin's solution) will solubilize whole tissues; yet under clinical use in open infected wounds, tissue injury has been found to be minimal without the loss of the beneficial bactericidal effect.[18] In a physiologic environment, this has been explained by the tendency of hypochlorous acid to react with endogenous molecules that contain free amino groups to generate the long-

acting reactive metabolites monochloramine and dichloramine (table 3). These metabolites have less host toxicity, however will maintain their bactericidal effect by oxidizing sulfhydryl residues to disrupt proteins.

Table 3. **Myeloperoxidase (MPO) Catalyzed Formation of Chlorinated Oxidants**

$H_2O_2 + Cl^- \xrightarrow{MPO} HO^- + HOCl$	Hypochlorous acid
$HOCl + RNH_2 \longrightarrow H_2O + RNHCl$	Monochloramine
$HOCl + RNHCl \longrightarrow H_2O + RNCl_2$	Dichloramine

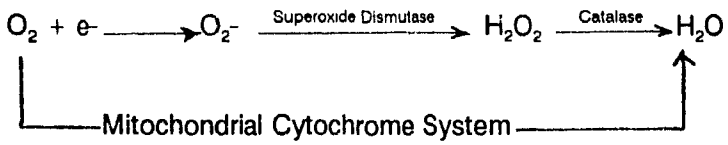
Evolution of Cellular Defences: Scavengers and Antioxidants

How is it that such a potentially toxic molecule as oxygen, became so firmly entrenched in biochemical functions vital to the essence of aerobic cellular life? In an atmosphere initially void of molecular oxygen, it is thought that the first life form was similar to today's blue-green algae.[13,66] Glucose was photosynthesized and energy was metabolized anaerobically by fermentation. Molecular oxygen produced by photosynthesis was excreted and accumulated as a waste product in the surrounding environment. As oxygen accumulated in the atmosphere and threatened primitive life, selective pressure on living organisms to evolve and thrive demanded these primitive cells to adapt by the acquisition of mechanisms to detoxify oxygen.[13]

Perhaps the most primitive mechanism to detoxify the reactive intermediates of oxygen has been antioxidants which are consumed during reactions to scavenge and terminate free radicals. In biologic membranes, one such antioxidant is **vitamin E**, where scavenge of peroxylipid radicals may serve a cellular self preservation role. The existence of simple enzyme systems to catalytically detoxify oxygen radicals was first confirmed in 1969, when McCord and Fridovich purified **superoxide**

dismutase.[13,86,87] This enzyme is found in all aerobic organisms and is the prime cellular mechanism by which the enzymatic dismutation of superoxide radical to hydrogen peroxide is achieved. Hydrogen peroxide in the presence of another enzyme, **catalase**, then can be catalyzed to water. The presence of glutathione and **glutathione peroxidase**, can also facilitate the reaction of hydrogen peroxide to water. The **cytochrome system**, unique to aerobic cells, is a most efficient mechanism capable of detoxifying molecular oxygen by direct tetravalent reduction to water (table 4).[13] Bulkley has suggested that perhaps the cytochrome system of aerobic cells evolved primarily as a means to simply detoxify oxygen and was then secondarily exploited for its energy producing capacity.[13]

Table 4 **Detoxifying Molecular Oxygen**



Free Radicals in Reperfusion Injury

Direct quantitation of the biologic production of oxygen free radicals is technically difficult due to the high reactivity and short half-life of free radicals. Most studies have relied primarily on the ability to prevent injury by free radical scavengers or by indirect measures of free radical injury such as lipid peroxidation and malonyldialdehyde.[1,2,12] Nevertheless, a growing body of evidence exists to suggest that free radicals actively mediate the reperfusion injury following an ischemic insult. Scavengers such as superoxide dismutase, catalase, and even iron chelation by deferoxamine have been employed to demonstrate that oxygen derived free radicals mediate much of the reperfusion injury in a diversity of ischemic organs such as heart, skeletal muscle, skin, bone, kidney, and bowel.[2,12,25,33,44,55,81,120,136,139]

Biologic Sources of Free Radicals

The existence of oxygen free radicals, in wide association with reperfusion injury among different organs, suggests the presence of a common biologic source. However, controversy surrounds the proposed sites and mechanisms responsible for the majority of oxygen radicals produced during reperfusion. Five biologic systems have been implicated as possible sources (table 5). The enzyme systems most studied and with potential clinical relevance are xanthine oxidase[86,96] and nicotinamide adenine dinucleotide (NADPH) oxidase.[32,66,76,140] Though not fully investigated, univalent 'leaks' from the mitochondrial electron transport, arachidonic acid metabolism, and auto-oxidation of catecholamines are three other plausible sources of oxygen free radicals.[13,121,147]

Table 5.	**Potential Biologic Sources of Oxygen Free Radicals**
	1) Xanthine Oxidase
	2) NADPH Oxidase
	3) Univalent 'leaks' from the Mitochondrial Cytochrome
	4) Arachidonic Acid Metabolism
	5) Auto-oxidation of Catecholamines

Xanthine oxidase is located within parenchymal and endothelial cells and is the mechanism most ascribed to the reperfusion injury.[104] In theory, ischemia causes affected tissues to accumulate the enzyme xanthine oxidase and its substrate, hypoxanthine, a degradation product of ATP during ischemia. With reperfusion, the molecular oxygen in blood combines with accumulated hypoxanthine in a redox reaction catalyzed by xanthine oxidase to form the superoxide radical. In support of this mechanism, Linas has experimentally demonstrated in a rat model of renal ischemia, that the conversion of xanthine dehydrogenase to oxidase did indeed occur

during an ischemic insult.[74] In addition, Friedl has documented increased xanthine oxidase activity in the effluent blood of a human ischemic limb.[37]

However, studies attempting to clarify the significance of xanthine oxidase in the pathogenesis of post-ischemic flap or organ injury have yielded conflicting reports.[56,92,134] First, attempts to prevent reperfusion injury in skin flaps by inhibiting xanthine oxidase activity with allopurinol have proved inconsistent.[54,55,99] Second, allopurinol in models of ischemic heart have failed to yield conclusive evidence as regards to prevention of the reperfusion injury.[106,123] Furthermore, comparison of xanthine oxidase activity in skin of different species has shown human skin to contain minimal levels.[99] Thus, in the arena of clinical free flaps, doubt remains as to the role of xanthine oxidase in the mediation of ischemia and reperfusion injury.

Neutrophil NADPH oxidase is another biologic source of oxygen derived free radicals.[4,5] Under normal host conditions this enzyme is quiescent. However when activated, this enzyme can catalyze the production of massive quantities of superoxide radicals which may in turn generate a wide spectrum of toxic oxygen derivatives.[6] This endogenous source of toxic oxidants has traditionally been known to benefit the host by its ability to localize and eradicate microbial infection. However, evidence now exists to implicate toxic processes of the neutrophil as effectors of self-destruction in states of post-ischemic reperfusion injury, adult respiratory distress, and autoimmunity.[32,52,68,93] As this thesis focuses on the role of the neutrophil in the pathogenesis of reperfusion injury in globally ischemic flaps, a brief review of the neutrophil and the mechanisms by which it effects injury is due.

THE NEUTROPHIL

Historically, Paul Ehrlich at the turn of the century has been credited with the first description of the neutrophil. In a review by Weissmann,[141] he notes that Paul Ehrlich in a doctoral thesis identified and classified the blood neutrophil based on its characteristic staining with aniline dyes. Soon after, Metchnikoff demonstrated the functional purpose of white blood cells such as the neutrophil, were to localize and digest microbial 'invaders'. In 1908, the Nobel prize was awarded to Ehrlich and Metchnikoff for their individual works on immunity and host defence.[141] Our knowledge of neutrophil morphology, physiology, maturation, and pathophysiologic mechanisms have since steadily increased.

The green purulent discharge characteristic of infected wounds has been witnessed for centuries, however it was not until 1941 that its significance had only begun to be discovered. At that time, Agner purified a green enzyme, myeloperoxidase, from the purulent fluids of a tubercular empyema.[18] Metchnikoff had already demonstrated that phagocytic cells such as the neutrophil, were responsible for the suppurative processes necessary in localization and eradication of infecting microbes. It was not surprising that large amounts of myeloperoxidase were subsequently found in the neutrophil where it has been estimated to constitute up to 5% of the neutrophil's dry weight. More recently, the antimicrobial activity of neutrophils has been found to be closely integrated with the oxygen radical producing activity of a plasma membrane enzyme, NADPH oxidase.[4] We now know that myeloperoxidase in combination with NADPH oxidase system, comprises an integral part of the neutrophil's arsenal to generate a wide spectrum of oxygen dependant toxic species.[66] Furthermore, identification of toxic proteolytic enzymes secreted by the activated neutrophil has suggested that injury may indeed be mediated by an orchestrated attack of both oxygen dependant and independent mechanisms against noxious stimuli.[31,133]

Clearly, this destructive potential has been used to host advantage as witnessed in the suppurative response mounted by blood neutrophils to eradicate infection. However, nonspecific or uncontrolled activation of these neutrophil mediated destructive processes have since been implicated in the pathogenesis of complex syndromes such as adult respiratory distress syndrome, autoimmunity, and multiple organ systems failure.[11,32,52,68,80,93] This potential for neutrophil mediation of host destruction has most recently been explored in conditions of post-ischemic organ failure. Alterations in the microenvironment of organs complicated by an ischemic and reperfusion insult may activate the neutrophil to express its destructive potential and hence amplify the post-ischemic tissue injury. This reperfusion injury can be effected by any combination of two neutrophil processes; namely,

1) oxygen dependent system.[66] or 2) oxygen independent system.[31]

Oxygen Dependent Toxic Systems

NADPH oxidase has been localized to the cell membrane of the neutrophil.[6,20,66] The precise components governing the activity and structure of NADPH oxidase has not been fully elucidated. Evidence supports NADPH oxidase as a complex composed of elements integrally related to functional units within the electron transport chain of mitochondrial membranes, namely cytochrome b, flavoprotein, and ubiquinone.[23,39,66] Full activity of NADPH oxidase is expressed only on priming and activation of the neutrophil.[24,36] This results in a 'respiratory burst', characterised by increased oxygen and glucose consumption. Glucose is utilized through the hexose monophosphate shunt to regenerate NADPH from $NADP^+$.[4,65] Almost all of the oxygen consumed by an activated neutrophil during the **respiratory burst** is utilized in a redox reaction with NADPH, catalyzed by NADPH oxidase to generate superoxide radicals[4] (figure 1). Hydrogen peroxide, a product of superoxide radical dismutation, can serve as a substrate to propagate the production of other oxygen derived free radicals. In the presence of chloride, hydrogen peroxide can be

catalyzed by neutrophil myeloperoxidase to initiate the formation of an even wider spectrum of chlorinated oxidants.[140] These mechanisms of generating a diversity of oxygen free radicals are crucial to the neutrophil microbial killing capacity. However beneficial superoxide production may be to host defence, when released to the extracellular space where there is close cellular contact, the potential for host cell injury is high.[115]

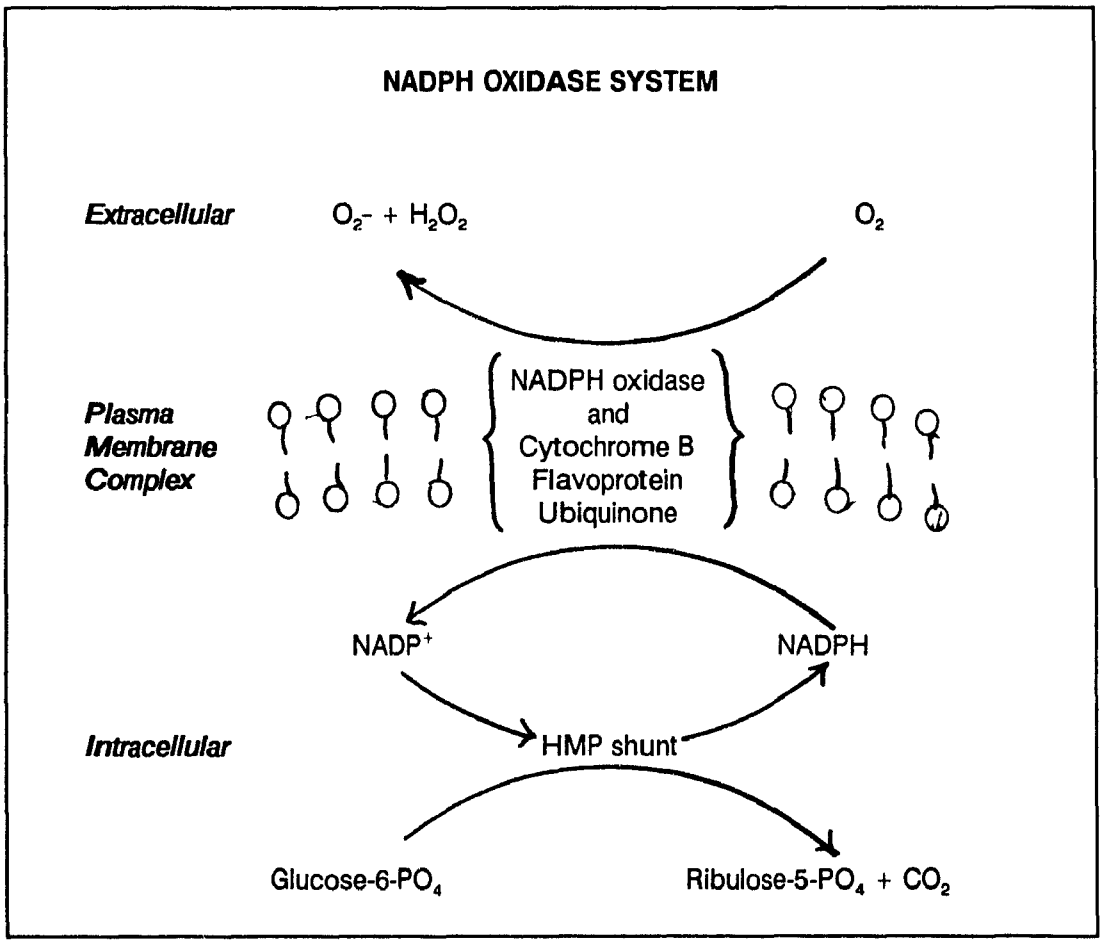


Figure 1. The mechanism of superoxide production from the plasma membrane bound NADPH oxidase enzyme of leukocytes.

Oxygen Independent Toxic Systems

Over 15 oxygen independent catalytic enzymes have been identified within cytoplasmic granules of the neutrophil.[11,31,140] For most of these enzymes, the exact

advantage that they impart to a neutrophil's antimicrobial response has not been fully delineated. However, the action of three proteolytic enzymes 1) elastase, 2) collagenase, and 3) gelatinase, have been proposed as potential self-destructive enzymes capable of mediating organ injury by dissolution of the extracellular matrix in tissue.[140] A complex mix of collagens, elastin, proteoglycans, and glycoproteins comprise the physical scaffold and physiologic microenvironment necessary for parenchymal cells of an organ to differentiate, grow, and function normally. Under most circumstances, these matrix components are protected by an anti-proteinase screen. However, deactivation of the anti-proteinase screen, as might occur with neutrophil mediated chlorinated oxidants, can initiate destruction of the extracellular matrix by unveiling the toxic potential of neutrophil proteolytic enzymes. Weiss has postulated that reperfusion injury in the post-ischemic organ may be mediated or potentiated by oxygen independent neutrophil proteolysis of the tissue extracellular matrix.[140]

Neutrophil Reperfusion Injury?

Experimental models of ischemic myocardium, skeletal muscle, and intestine have been studied in attempts to clarify the role of the neutrophil in the pathogenesis of reperfusion injury.

Neutrophil localization

Richard[108] and Romson[111] individually used indium-111 labelled neutrophils to determine neutrophil localization during reperfusion in a canine model of myocardial ischemia. Both confirmed significant neutrophil accumulation on reperfusion of the ischemic **myocardium.** Grisham[45,46] used myeloperoxidase to quantitate neutrophil accumulation in a feline model of **intestinal** ischemia. In this model, he demonstrated that reperfusion of ischemic intestine was accompanied by an estimated 18 fold increase in neutrophil accumulation. Furthermore, when modalities effective in salvage of ischemic bowel were employed, a concomitant

normalization of neutrophil accumulation occurred.

Neutrophil depletion

Neutropenia has been successfully employed in the salvage of ischemic canine **myocardium.**[89,110] Litt[75] and Westlin[143] employed leukocyte filters to clear neutrophils from the reperfusing blood of ischemic canine myocardia. Litt demonstrated that the extent of myocardial infarction was reduced with leukopenia. Westlin showed improved cardiac function and alleviation of myocardial stunning in hearts reperfused with leukopenic blood. Using radiation induced neutropenia in rats, Belkin found a significant post-ischemic **skeletal muscle** salvage.[7] In an ischemic canine skeletal muscle model, Korthuis used leukocyte filters to deplete leukocytes from reperfusing blood; muscle salvage was also significantly improved in this leukopenic model.[68]

Inhibition of neutrophil function

Antiinflammatory agents such as ibuprofen, iloprost, and BW755C have been reported to inhibit neutrophil function.[78,79,89,98,119] Ischemic canine myocardial studies employing these agents have demonstrated improved post-ischemic **myocardial** salvage.[89,109,111,119] Belkin has reported improved post-ischemic canine **skeletal muscle** salvage when iloprost was employed.[8]

PURPOSE

Cutaneous and myocutaneous flaps form the bulk of a reconstructive microsurgeon's armament. Flap movement by distant free transfer has proven to be an elegant solution in difficult cases of wound reconstruction. Post-ischemic injury resulting from thrombotic complications constitute the major threat jeopardizing the success of a transferred free flap. The purpose of this thesis was to determine if neutrophils mediate the injury resulting from global ischemia and reperfusion in cutaneous and myocutaneous flaps.

Animal Model

The sparse hair, pink color, and pattern of blood supply of swine skin has made this animal popular in the field of flap research. In our laboratory, a wealth of experience has been acquired by more than a decade of research employing a standard model of swine buttock cutaneous and latissimus dorsi myocutaneous island flaps (plate 1). Although the rat, rabbit, and dog are often used, the pig is strongly favoured because its cutaneous anatomy and hematological state are more similar to man's than any of the loose skin animals.[63] Furthermore, biochemical studies measuring xanthine oxidase activity in the ischemic skin of rats have yielded dramatically different profiles from that of both swine and humans, suggesting that the pig is a superior model to study ischemic tissue injury.[100] Global ischemia in island flaps by clamp application was employed to represent the clinical scenario of an ischemic free flap. This was preferred as it completely avoided any variability that would be inherent to global ischemia induced by performing microvascular anastomoses.

Plate 1. Standard buttock cutaneous and latissimus dorsi myocutaneous flaps in the pig.

Hypothesis

In ischemic organs such as heart, skeletal muscle, and intestine, the existing literature has largely been supportive of a neutrophil mediated reperfusion injury. Few studies have evaluated this possibility in flaps. Based on the literature reviewed, the following primary hypothesis was extrapolated.

1^0 hypothesis **The neutrophil is an etiologic factor in the pathogenesis of reperfusion injury in globally ischemic flaps.**

From this hypothesis, four corollaries were derived. In the following series of experiments, each of these corollaries were separately evaluated in a swine model of globally ischemic cutaneous and myocutaneous island flaps.

1) **Function**;** Neutrophil function must be augmented during reperfusion.
2) **Localization;** Neutrophils must localize to post-ischemic flaps.
3) **Depletion;** Neutrophil depletion should improve flap survival.
4) **Inhibition;** Inhibited neutrophil function should improve flap survival.

** Neutrophil function in the ischemic buttock cutaneous flap was not studied because of technical difficulties in acquiring venous effluent from this flap.

EXPERIMENT #1

Neutrophil Function

INTRODUCTION

Vascular reperfusion and tissue reoxygenation are absolute necessities to arrest the deleterious effects of an ischemic insult. During early reperfusion, the beneficial role of reoxygenation is often nullified by the paradoxical transformation of molecular oxygen into free radicals. In many post-ischemic organs, reperfusion injury has been shown to be mediated by oxygen derived radicals.[13,87,97] However, controversy exists as to the exact biologic mechanism responsible for these destructive oxygen metabolites.[100] One potential source for the formation of oxygen radicals is the neutrophil. Functional activation of the normally quiescent NADPH oxidase is an obligatory step in initiating neutrophil production of oxygen radicals.[24,36] The neutrophil has already been postulated as a significant source of oxygen free radicals in genesis of the reperfusion injury in ischemic organs such as myocardium,[75,89,110,143] skeletal muscle,[7,113] and intestine.[45,46,96] Yet no studies have directly assessed the functional state of the neutrophil after reperfusion of an ischemic organ.

Neutrophil Function

Using a swine model of flaps, we sought to evaluate the functional activity of NADPH oxidase in blood neutrophils reperfusing ischemic myocutaneous flaps. Neutrophils isolated from the venous blood of myocutaneous flaps were microassayed by an inhibitable superoxide ferricytochrome c reduction.[102] Kinetics of the neutrophil superoxide production after phorbol myristate acetate stimulation was used as a measure of NADPH oxidase activity.

Neutrophil Sequestration

Neutrophils have reportedly been sequestered by post-ischemic organs such as myocardium and intestine.[46,108,111,113] Arterial venous difference in flap blood neutrophil counts were computed to yield a crude estimate of the number neutrophils extracted from the reperfusing blood and sequestered by post-ischemic flaps.

Anatomic Distribution of Muscle Injury

In addition to an assessment of the neutrophil, close observation of the pattern of skin and muscle necrosis were made to enhance the interpretation of the neutrophil data. Despite a uniform insult sustained by all regions of a global ischemic myocutaneous flap, tissue injury and necrosis have been documented to occur in an asymmetric but consistent pattern.[101,150] Necrosis of the skin has been observed with greatest frequency in the most distal portions of a flap.[101,150] Based on such an observation one might expect a similar anatomic distribution of post-ischemic necrosis in the muscle portion of a myocutaneous flap. However, this has been controversial. Morris et al[88] in a similar swine model of ischemic myocutaneous flaps found a predominant distal latissimus dorsi muscle necrosis. However, Labbe et al[71,72] have reported a predominant proximal muscle necrosis in their canine model of ischemic gracilis muscle. To clarify the anatomic distribution and pattern of post-ischemic skeletal muscle necrosis, nitroblue tetrazolium was employed to determine the extent of muscle necrosis in myocutaneous flaps.

MATERIALS and METHODS

Anesthesia and Surgery

Seventeen female white Landrace pigs (22 ± 2 kg) were sedated with intramuscular injections of ketamine (20 mg/kg) and xylazine (2 mg/kg) and anesthetized with an intravenous injection of sodium pentothal (6 mg/kg). Following intubation and spontaneous respiration of room air, repeated doses of intravenous sodium pentobarbital were titrated to achieve and maintain surgical anesthesia. In each pig bilateral 10 by 10 cm latissimus dorsi myocutaneous island flaps were designed with one flap randomized to ischemia while the contralateral flap served as control. Six to 8 hours of global ischemia was created by application of Acland V2 clamps to the thoracodorsal artery and vein. Dermofluorometry (Fluoroscan, Santa Barbara Technology, 1155 Via Tranquila, Santa Barbara, Calif. 93110) was

used to confirm complete pedicle occlusion during clamping and reperfusion on unclamping.[130] The thoracodorsal vein was preserved while all other venous tributaries to the axillary vein were ligated. A short segment distal to the thoracodorsal vein was preserved for placement of a cannula. Pure flap venous effluent was collected passively from a heparinized axillary vein cannula (PE-190, Clay Adams) by temporarily occluding the proximal axillary vein (Acland V3). Sequential paired venous effluent samples were collected simultaneously from ischemic and control flaps immediately (17/17 pigs), 1 hour (11/17 pigs), and 4 hours (9/17 pigs) following reperfusion. To determine blood leukocyte counts, 12 of the 17 pigs had saphenous arterial, control flap venous, and ischemic flap venous bloods drawn simultaneously during the immediate reperfusion period. After completion of venous sampling, the cannulae were removed and the animal followed for 3 to 5 days.

Superoxide Microassay

Chemicals and media

Ferricytochrome c (type VI), superoxide dismutase (bovine erythrocyte, 3000 U/mg protein), phorbol myristate acetate (PMA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co., St. Louis, MO. Hank's buffered saline (HBSS), without phenol red was obtained from GIBCO, Grand Island, NY. PMA was dissolved in DMSO to a stock concentration of 20 μg/ml. Prior to use the stock PMA in DMSO was further diluted with HBSS, to yield final assay concentrations of PMA 100 ng/ml and DMSO less than 0.25%.

Cell harvest

Purified neutrophils were prepared employing standard techniques.[41] Heparinized flap venous effluent was diluted volume for volume with saline followed by a 1 hour dextran-70 sedimentation of red cells. The neutrophil rich supernatant was then layered onto a density medium (Ficoll-Paque, Pharmacia) and centrifuged

(700g x 25 mins) to remove mononuclear white cells. Final hypotonic lysis of red cells yielded isolated neutrophil suspensions. Neutrophil concentrations were determined with Turk stain and hemocytometer counts.

Superoxide production measurements

Superoxide production was based on the inhibitable reduction of ferricytochrome c by superoxide radicals.[5,102,112] Specificity of the reduction was verified by its inhibition with superoxide dismutase. With all samples run in triplicate, 5 x 10^5 neutrophils were added to each well of flat bottomed microtiter plates (Microtest III, Falcon) containing 160 μM solution of ferricytochrome c with or without PMA 100 ng/ml. Blank wells contained superoxide dismutase 300 U/ml in addition to the above components of each well. Plates were incubated at 37°C in a humidified incubator with 5% CO_2 on a microshaker. Readings were taken at baseline, 5, 15, and 30 minutes incubation on a Titerek Multiskan Plus MKII reader (Flow Laboratories) at 550 nm. Optical densities were transformed into nmol using ΔE_{550nm} = 15.5 x$10^3 M^{-1}cm^{-1}$.[5,112] Results were expressed in nmol per 10^6 neutrophils. Under these conditions, intra-assay variability was less than 6%.

CBC and Differentials

Complete blood cell counts were measured on arterial and both venous samples using the Technicon H*1 system (Technicon Instruments Corporation, Tarrytown, New York 10591). Standard blood smears with Wright-Giemsa stain were used to determine the differential counts. To correct for hemoconcentration, leukocyte counts were expressed per gram of hemoglobin.

Survival

Flap survival was assessed after 3 to 5 days of reperfusion. Skin survival was determined by inspection in all animals. Skin survival tracings were recorded on transparent plastic with survival areas calculated using a digitizing tablet (Houston

Instrument, TG-1017) and computer software (SigmaScan, Jandel Scientific). Segmental muscle survival was assessed in 7 pigs by transversely dividing the myocutaneous portion of the flap into ten (1 cm) segments, and by dividing the proximal muscle extension into 2 segments. These 12 flap segments were stained by immersion in 0.05% nitroblue tetrazolium solution (in 0.2% Tris buffer, adjusted pH 7.4) for 20-30 minutes at room temperature.[16,71] Muscle survival was reported as the per cent width survival on the distal surface of each flap segment.

STATISTICAL ANALYSIS

All errors were reported as standard error of the mean. Paired one tailed Student's t-test ($p \leq .05$ significant) was used where applicable. The width of muscle injury was analyzed with blocked analysis of variance (blocked ANOVA, $p \leq .05$ significant), followed by Waller-Duncan K ratio test (K ratio ≥ 100 significant) where applicable.

RESULTS

Superoxide Microassay

Neutrophils isolated from control flap venous effluent ($n=17$) of the immediate reperfusion period showed superoxide production to be 0.7 ± 0.3, 3.8 ± 0.8, and 8.6 ± 1.1 nmol of superoxide radical/10^6 neutrophils after 5, 15, and 30 minutes of PMA stimulation respectively. Neutrophils isolated from ischemic flaps venous effluent ($n=17$) showed significant increases of $64 \pm 26\%$ ($p=.01$), and $15 \pm 7\%$ ($p=.02$) over contralateral control values at 5 and 15 minutes PMA stimulation (figure 2). No difference in PMA stimulated neutrophil superoxide production was detected in the neutrophils isolated from ischemic and contralateral control flaps at 1 and 4 hours reperfusion. Unstimulated neutrophils showed no difference between groups and any of the time periods tested.

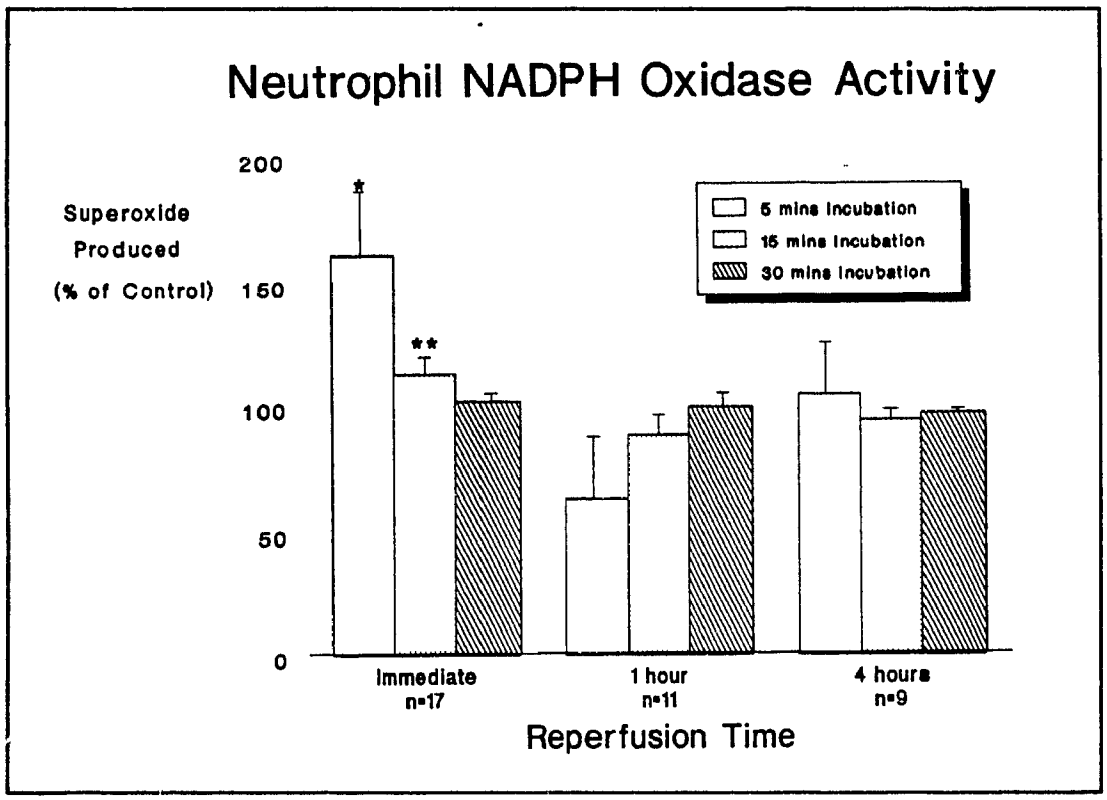


Figure 2. PMA stimulated superoxide production from neutrophils isolated from ischemic flaps; expressed as a percent increase over the contralateral control flaps. (Paired Student's t-test, *p=.01 and **p=.02)

When the difference in neutrophil superoxide production from the immediate reperfusion period was re-analyzed with regard to outcome of flap survival, the skin survival subgroup of (n=9) was no longer significant over contralateral controls. However, the skin necrosis subgroup of ischemic flaps (n=8) continued to show significant mean increases over contralateral controls at 5 and 15 minutes PMA stimulation.

CBC and Differentials

Arterial venous (A-V) leukocyte difference, as determined from complete and differential blood cell counts, was significantly higher in ischemic compared to control flaps, respectively 23.9 ± 3.5 versus 8.7 ± 5.3 $x10^6$ leukocytes/g hemoglobin

(p=.001). When differential counts were used to divide the leukocyte population into neutrophil and non-neutrophil subpopulations, the higher A-V leukocyte difference in ischemic flaps was attributable to the neutrophil subpopulation (22.2 ± 4.2 versus 5.3 ± 5.3 $x10^6$ neutrophils/g haemoglobin, p=.00006) while the non neutrophil subpopulation showed no difference (figure 3).

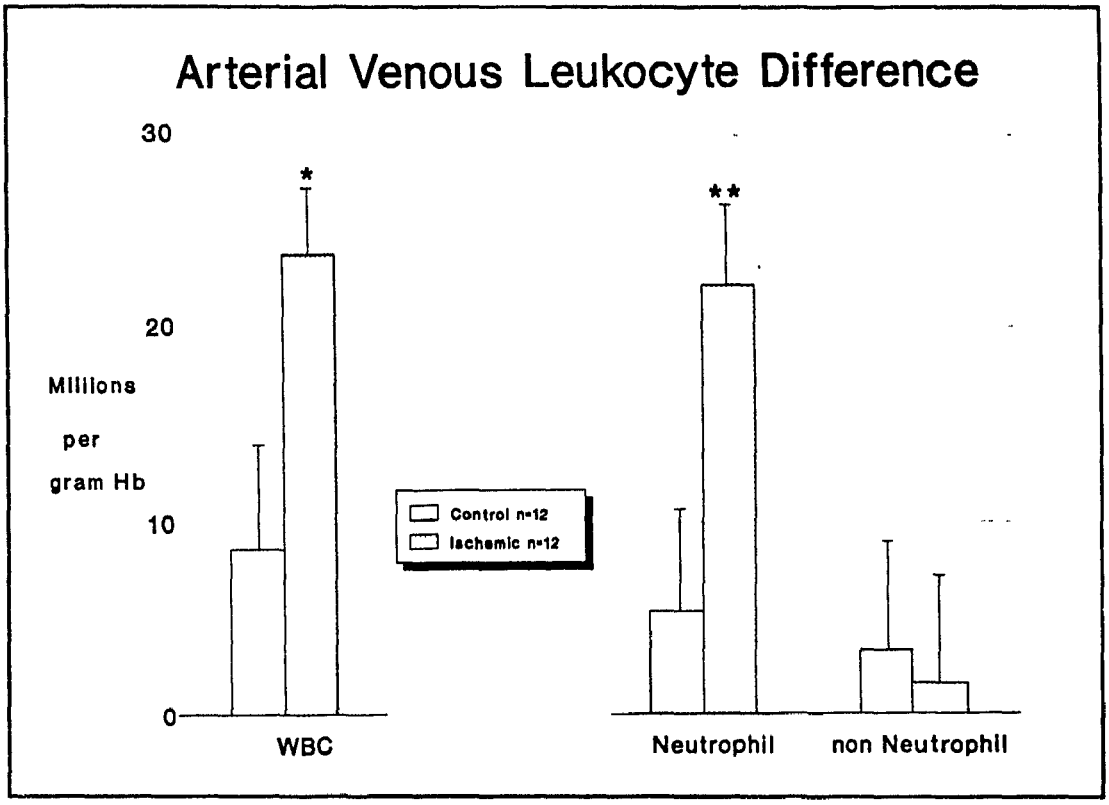


Figure 3. Arterial venous (A-V) differences in WBC, neutrophil, and non-neutrophil blood counts. (Paired Student t-test, *p=.001 and **p=.00006)

Survival

Ischemia significantly decreased skin survival area to 72 ± 10% (p=.005). Skin survival of the ischemic group was further divided into two subgroups, flaps that completely survived the ischemia (n=9) and flaps with partial skin necrosis (n=8). The subgroup of ischemic flaps with partial skin necrosis continued to show a statistically significant decreased survival area (40 ± 13%, p=.001) compared to contralateral controls (figure 4).

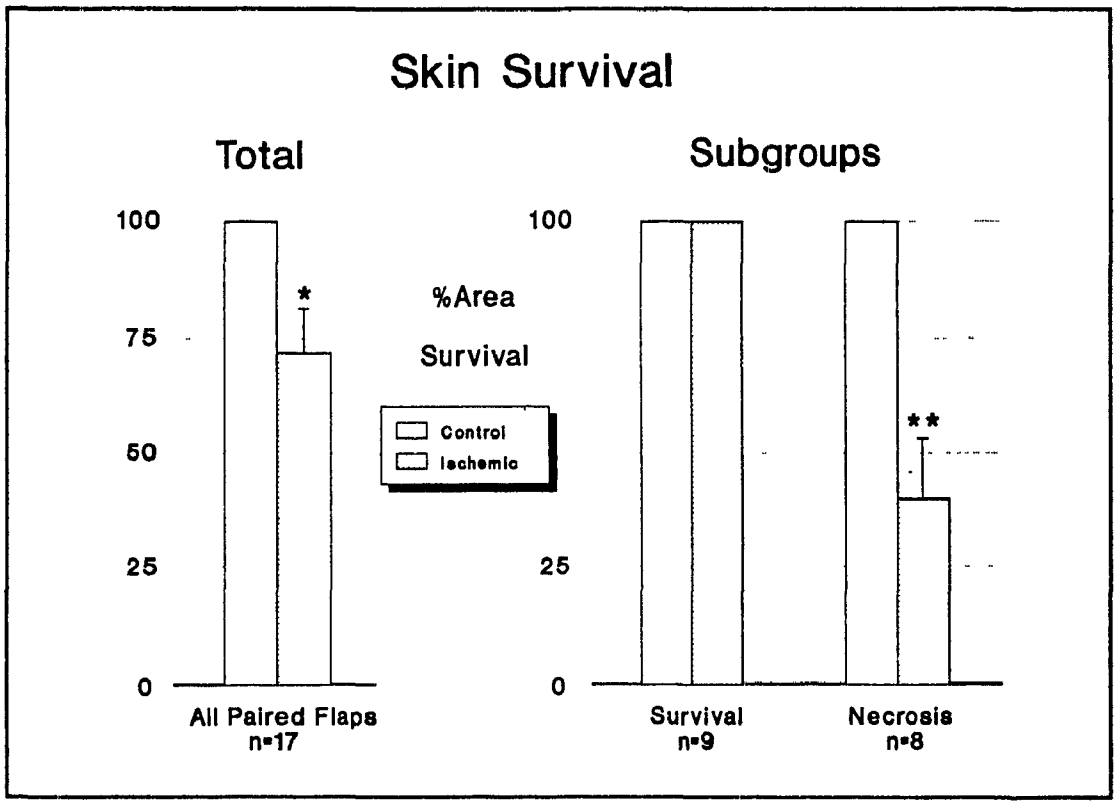


Figure 4. Percent of the area of skin surviving in myocutaneous flaps subjected to 6-8 hours of global ischemia. (Paired Student's t-test, *p=.005 and **p=.001)

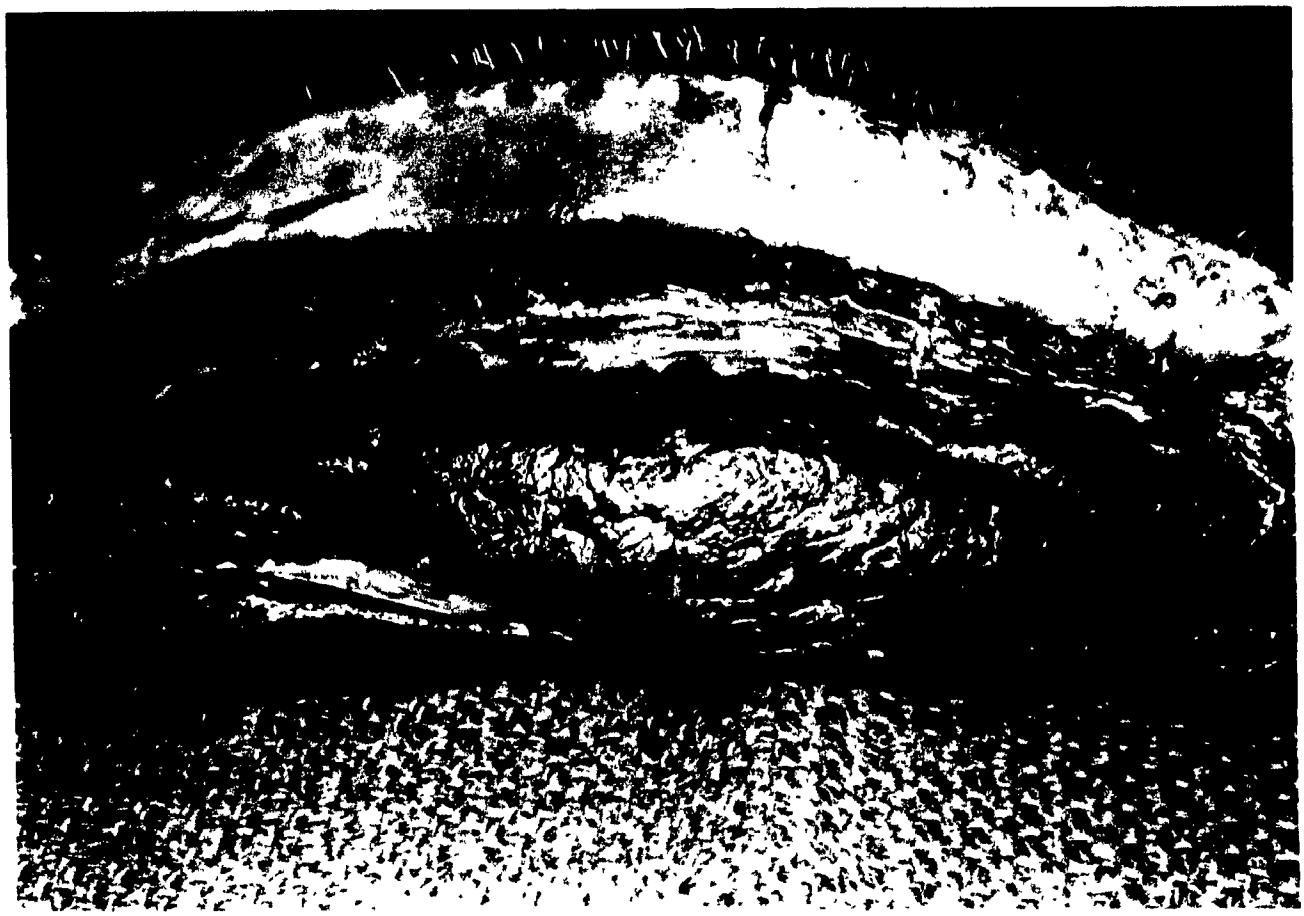


Plate 2. Necrosis was greatest in thick core of the muscle.

Latissimus dorsi muscle width survival was significantly decreased for all segments compared to contralateral controls (K ratio≥100). The distribution of the necrosis was surprising with the necrosis occurring in the thickest core of muscle (plate 2) and the per cent width necrosis highest in the proximal 3 muscle segments (figure 5). Survival of the panniculus carnosus muscle was distinctly different, with a distal necrosis which was in close concordance with the distal skin necrosis.

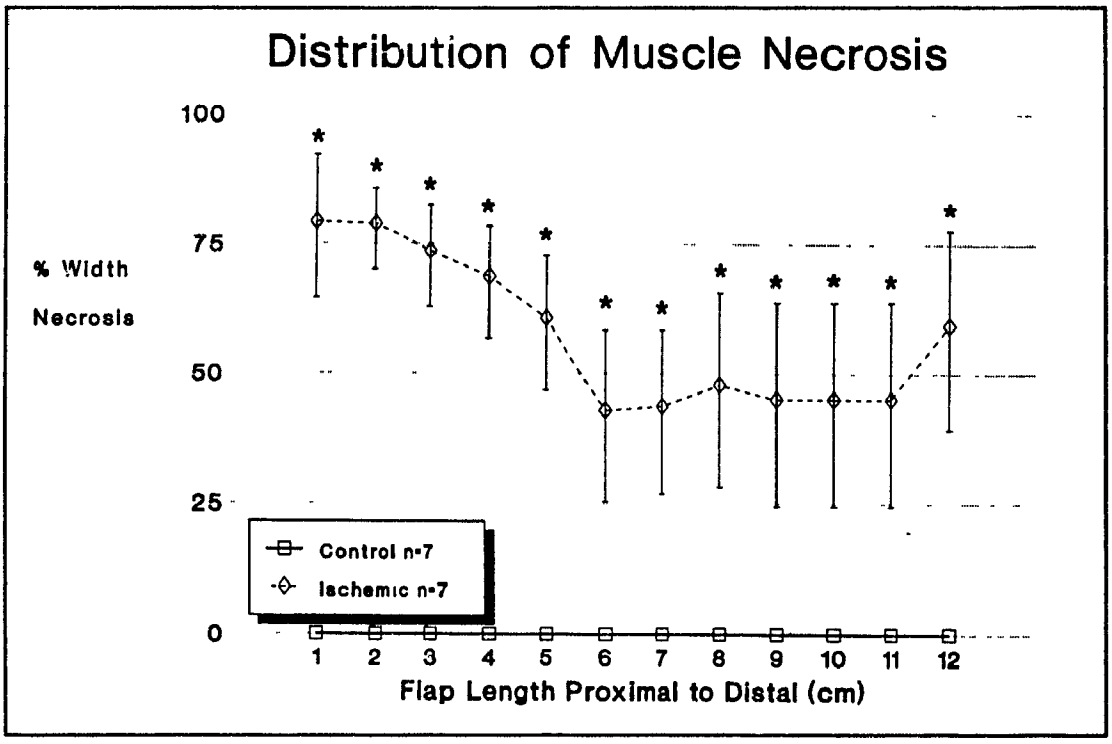


Figure 5. Percent width of the necrotic muscle in myocutaneous flaps subjected to 6-8 hours of global ischemia. (Blocked ANOVA significant with $p<.05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control muscle segments)

DISCUSSION

Neutrophil Function

Post-ischemic organ injury is in part generated by the destructive consequences of oxygen derived free radicals. It has been well established that oxygen radicals are formed during the early period of vascular reperfusion, however the precise biologic mechanisms giving rise to these toxic oxygen metabolites have yet to be clarified. Although the neutrophil is generally regarded for its favourable effect in eradicating infection, it has been postulated as a plausible source of deleterious oxygen free radicals potentially giving rise to the reperfusion injury in post-ischemic organs. NADPH oxidase of the neutrophil, when activated, is a potent biologic mechanism of generating superoxide radicals. Since NADPH oxidase is normally quiescent, factors formed within the milieu of an ischemic environment must prime and functionally activate this enzyme to unveil its destructive potential.

In this study, neutrophils isolated from the venous effluent of ischemic swine myocutaneous flaps did display enhanced NADPH oxidase activity. This was manifest by an accelerated release of superoxide radical from the neutrophil on PMA stimulation. Multiple factors are capable of priming the functional activity of neutrophil NADPH oxidase. In canine skeletal muscle, Rubin[114] has demonstrated that ischemia induced activation of the alternate pathway of complement, resulting in an accumulation of its by products. During reperfusion, these metabolic by products of complement have been proposed as activators of the neutrophil NADPH oxidase. Platelet activating factor (PAF), a phospholipid, released from damaged tissue has been documented to rise during intestinal ischemia in the dog.[35] Kubes[70] has suggested that PAF can partially mediate reperfusion injury in post-ischemic intestine by activating the neutrophil. Other known entities capable of priming the neutrophil include platelet products,[82] arachidonic acid metabolites, and endothelial cell vasoactive products.

The enhanced functional activity of NADPH oxidase was detected only in blood neutrophils isolated from the flap venous effluent retrieved at immediate reperfusion. This enhanced enzyme activity was absent in the neutrophils retrieved after 1 and 4 hours of reperfusion. The lack of increased neutrophil NADPH oxidase activity after 1 and 4 hours of reperfusion may have resulted from complete clearing of the ischemia accrued factors responsible for neutrophil priming, neutrophil down-regulation upon total receptor occupancy, or may reflect complete capture/sequestration of the primed neutrophil population within the flap, thus its absence in the venous effluent.

Neutrophil degranulation with extracellular release of granular enzymes and proteases usually transpires concomitantly with the activation of neutrophil NADPH oxidase. Although neutrophil granular enzymes are not generally regarded as the principle mechanism by which neutrophils effect injury, their presence has been postulated to amplify the tissue destruction from activated neutrophils. While these oxygen independent destructive processes of the neutrophil were not evaluated in this study, their relevance in intensifying the reperfusion injury of post-ischemic organs should not be ignored. One such granular enzyme, myeloperoxidase is capable of transforming oxygen derived radicals into another harmful class of chlorinated oxidants. Neutrophil proteases may further effect injury by lysing the extracellular matrix, thus destroying the architecture of tissue itself.

Neutrophil Sequestration

Flap neutrophil sequestration is dependant on total flap blood flow and on neutrophil extraction from the perfusing blood. Although blood flow was not measured in this study, Morris[88] in a similar swine model of ischemic latissimus dorsi myocutaneous flaps, has measured total flap blood flow using radioactive microspheres. He has demonstrated that total flap blood flow following 6 to 8 hours of global ischemia was not different from control non-ischemic flaps. Thus, a crude

and indirect measure of flap neutrophil sequestration can be estimated by simply computing the neutrophil extraction from the perfusing blood. If the above assumptions are accurate, then ischemic myocutaneous flaps significantly sequester more neutrophils than non-ischemic flaps. This was evident on immediate reperfusion as the A-V difference in blood neutrophil counts was significantly greater in ischemic flaps.

Anatomic Distribution of Muscle Injury

Consistent with the work reported by Labbe et al,[71,72] muscle necrosis in our ischemic swine model of latissimus dorsi myocutaneous flaps arose predominantly in the most proximal portion of the flap. The necrosis was continuous and developed in the thick central core of muscle. There was relative sparing of the distal thin muscle. Potential factors influencing the distribution of muscle injury include the rate of tissue cooling, microvascular architecture, and neutrophil localization. During flap elevation, the thinner and most peripheral portion of a flap more rapidly attains the cooler room temperatures and thus, is subject to a relative cool ischemia. Corrosion cast studies have demonstrated an intense network of microvascular channels surrounding skeletal muscle cells.[103] With edema formation and limited space at the core of the muscle, it is conceivable that the nutrient microvascular channels might be impaired by compressive forces. Another speculative explanation for this unexpected pattern of muscle necrosis, could be the rate and distribution of flap neutrophil accumulation after reperfusion.

In summary, the enhanced functional response exhibited by neutrophils reperfusing through an ischemic myocutaneous flap was compatible with its proposed vital role in the mediation of reperfusion injury. The A-V difference in blood neutrophil counts determined at immediate reperfusion provided indirect evidence for neutrophil sequestration by ischemic flaps. Further confirmation of ischemic flap neutrophil sequestration by direct measures is desirable. In addition, determination

of the neutrophil localization within the flap itself may yield information regarding the asymmetric distribution of post-ischemic muscle necrosis.

EXPERIMENT #2

Neutrophil Localization

INTRODUCTION

If the neutrophil is to be appraised as a credible mediator of reperfusion injury in ischemic flaps, then events necessary for neutrophil mediated killing must be substantiated. In ischemic flaps, we have already demonstrated enhanced function in the neutrophils reperfusing through post-ischemic tissues. This process is necessary to activate the neutrophil to release oxygen metabolites and to effect cell killing.[140] However, oxygen metabolites are effective only at close cell to cell distances, thus blood neutrophils must localize to the target to effect injury.[115] In states of infection this is accomplished by vessel adherence, diapedis across the vascular wall, and movement along a chemotaxis gradient to localize at an invading microbe. Using vital microscopy, Roth[113] has directly observed a remarkable similarity in the action of blood neutrophils reperfusing post-ischemic tissue. Previous investigators have already demonstrated a significant neutrophil sequestration in post-ischemic organs such as myocardium,[111] skeletal muscle,[114] and intestinal mucosa.[46] An association between the magnitude of neutrophil accumulation and the distribution of post-ischemic tissue injury has been inferred, however, this has never been directly examined.[46,111]

In experiment #1, increased neutrophil extraction from the blood reperfusing an ischemic myocutaneous flap was found. From this singular finding, we circuitously inferred that neutrophils preferentially localized to ischemic tissue. In this study, our primary goal was to validate this deduction. Radiolabelled neutrophils were used to directly estimate neutrophil localization within post-ischemic cutaneous and myocutaneous flaps. As a secondary interest, the relationship of post-ischemic neutrophil sequestration to the pattern of tissue injury was examined to determine if any correlation existed. Injury was documented by tissue water content (used as an indirect measure of edema) and by directly measuring flap survival.

MATERIALS and METHODS

Anesthesia and Surgery

Six female first generation Landrace cross Yorkshire white pigs (21 ± 1 kg) were sedated with intramuscular injections of ketamine (20 mg/kg) and xylazine (2 mg/kg) and anesthetized with an intravenous injection of sodium pentothal (6 mg/kg). Following intubation and spontaneous respiration of room air, repeated doses of intravenous sodium pentobarbital were titrated to achieve and maintain surgical anesthesia. Vascular access was secured by open cut down of the external jugular vein. Through the external jugular vein, a polyethylene catheter (PE-190, Clay Adams) was inserted proximally into the superior vena cava. This simplified blood letting and facilitated central infusion of the radiolabelled neutrophils. In each pig, bilateral 10 by 20 cm latissimus dorsi myocutaneous and 10 by 18 cm buttock cutaneous island flaps were devised. Flaps on one side of each animal were randomized to ischemia while the contralateral side served as nonischemic controls. Six hours of global ischemia was created by application of Acland V2 clamps to the thoracodorsal vessels and Acland V3 clamps to the branch of the circumflex iliac vessels supplying the buttock flap. Circulating ^{111}Indium labelled blood neutrophils have been reported to peak 30 minutes following intravenous infusion.[138] Consequently, radiolabelled neutrophils were autotransfused 30 minutes prior to vascular unclamping. Dermofluorometry (Fluoroscan, Santa Barbara Technology, 1155 Via Tranquila, Santa Barbara, Calif. 93110) was used to confirm complete pedicle occlusion during clamping and reperfusion on unclamping.[130] All animals were sacrificed after 24 hours of reperfusion. Flap survival, tissue water content, and radioactivity were determined.

Flap Neutrophil Localization

Neutrophil Radiolabelling

In conjunction with a commercial laboratory (Mallinckrodt Canada Inc., Diagnostic Imaging Services, 7500 Trans Canada, Pointe Claire, Quebec, H9R 5H8),

neutrophil cell isolation and ^{111}Indium labelling was performed using standard methods.[85]

At operation, 50-80 ml of heparinized blood was drawn from the central venous cannula of each animal for neutrophil purification and ^{111}Indium radiolabelling. Hydroxyethyl starch (6% Volex, American Critical Care, McGraw Park, IL) was added one part to five parts of heparinized blood and allowed to stand at room temperature for 1 hour. This was used to facilitate red cell sedimentation. The leukocyte rich supernatant was recovered and layered onto a double density gradient of Ficoll-Hypaque (specific gravity 1.077 and 1.114). The gradient tubes were centrifuged at 700 x g for 15 minutes to yield isolated neutrophil suspensions. With this technique of harvesting neutrophils, yields of approximately 10^8 have been reported.[85,138]

The isolated neutrophils were resuspended in 0.5 ml of plasma. Radiolabelling was achieved by a 25 minute incubation with ^{111}Indium oxine (500 μCi in 0.1 ml of Tris buffer, pH 7). Following this, neutrophils were washed and resuspended in 2 ml of plasma. Radiolabelling efficiency was determined prior to autotransfusion.

Radioactivity

Excised whole flap scintillation images were obtained by nuclear scan. Flaps were scanned under a diverging medium energy collimator. Windows of the scintillation camera (PhoGamma V, Siemens Electrical Co.) were set to include both the 173 and 247 keV peaks of ^{111}Indium. Images were obtained with a preset scanning time of 600 seconds.

Following this, quantitative tissue gamma counts were determined. Flaps were transversely divided into 2 cm segments; in the latissimus dorsi flap segments, skin

and muscle were separated into their respective tissues. Individual tissue segments were then placed into scintillation vials and the emitted gamma radiation measured. Radiation from vials were counted for 1 minute in a gamma counter (Beckman Gamma 8000, Beckman Instruments Inc.) with windows set to include both the 173 and 247 keV peaks of ^{111}Indium. Whole flap radioactivities were estimated from the sum of the individual tissue segments. Tissue gamma counts were expressed as radioactive counts per minute per gram of dry tissue weight.

Survival

Skin

Skin survival determinations were aided by intravenous fluorescein injection (15mg/kg) and Wood's lamp illumination. Skin survival tracings were recorded on transparent plastic with survival areas calculated using a digitizing tablet (Houston Instrument, TG-1017) and computer software (SigmaScan, Jandel Scientific). Anatomical flap length survival was estimated by averaging three equally spaced longitudinal measurements of the surviving skin.

Muscle

Latissimus dorsi muscle survival was assessed by a previously described method of planimetry.[71,72] The muscle was transversely divided into ten (2 cm) segments. Surviving muscle was stained by immersion in 0.05% nitroblue tetrazolium solution (in 0.2% Tris buffer, adjusted pH 7.4) for 20-30 minutes at room temperature. Survival on the cut surface of each muscle segment was traced on transparent plastic; in each segment survival was estimated by averaging the per cent area survival on the two cut surfaces of each muscle segment.

Tissue Water Content

Both the buttock and latissimus dorsi flaps were divided into 2 cm segments. Wet tissue weights were determined for skin and muscle tissue of each flap segment.

Following completion of radioactive counts, dry tissue weights were determined by oven drying (200°C) until a steady dry weight was reached (8-12 hours).

STATISTICAL ANALYSIS

All errors were reported as standard error of the mean. Paired one tailed Student's t-test ($p \leq .05$ significant) was used where applicable. All multiple comparisons were analyzed with blocked analysis of variance (blocked ANOVA, $p \leq .05$ significant), followed by Waller-Duncan K ratio test (K ratio ≥ 100 significant) where applicable.

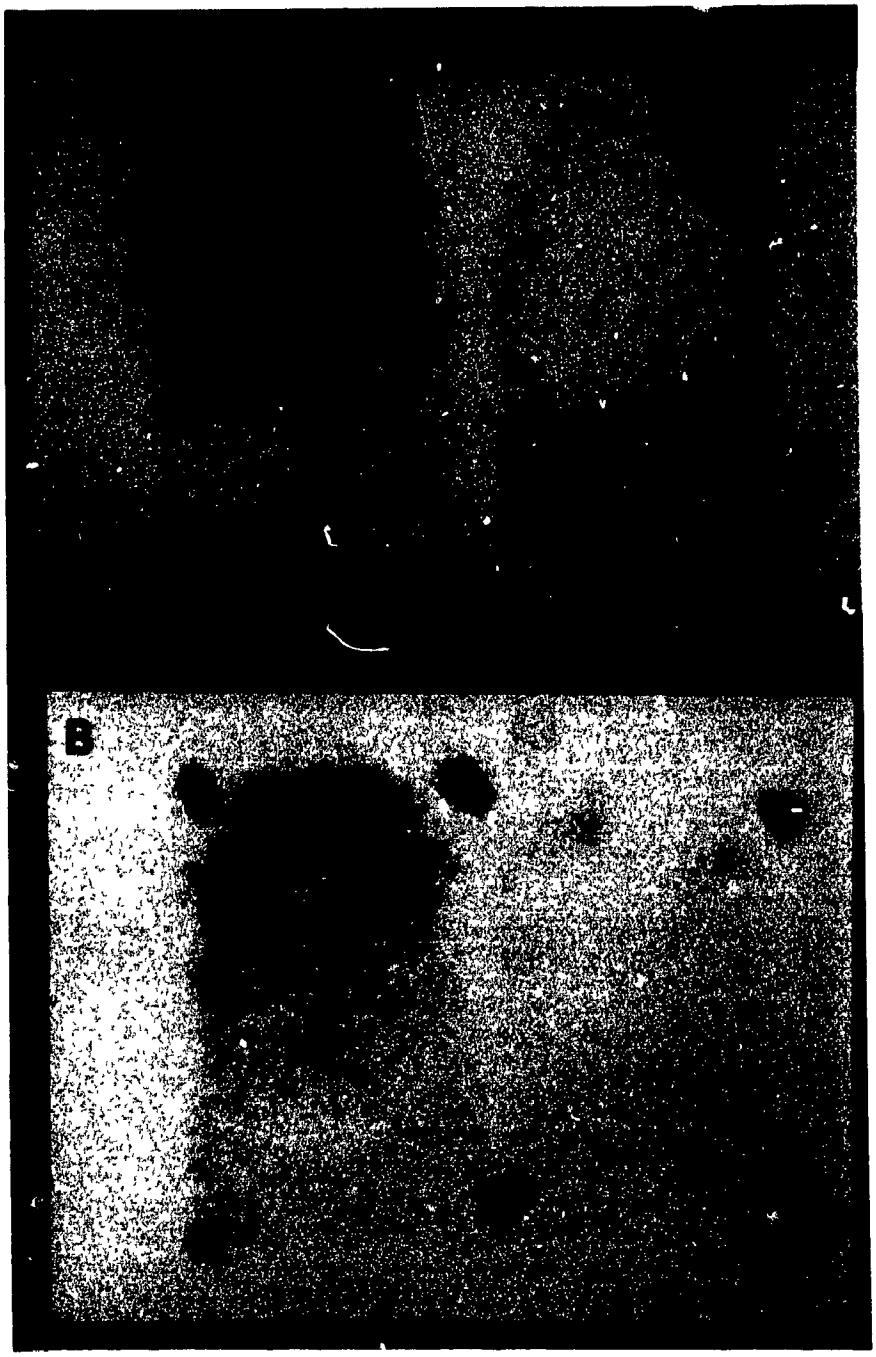
RESULTS

Radioactivity

Efficiency of ^{111}Indium uptake by the neutrophil was 93.7 ± 1.1%. Whole flap scintillation images grossly demonstrated increased radioactive uptake in all post-ischemic flaps (buttock n=6 pairs and latissimus dorsi n=6 pairs) when compared to contralateral controls (plate 3). The most distal portion of ischemic buttock and latissimus dorsi flaps demonstrated least radioactivity.

Quantitative whole flap radioactive counts showed the post-ischemic flaps to significantly accumulate more radioactivity (figure 6). Next, radioactivity was corrected for the dry tissue weight. Post-ischemic buttock skin showed significantly increased radioactivity. Post-ischemic latissimus dorsi muscle showed significantly increased radioactivity. Post-ischemic latissimus dorsi skin was not statistically different from control (figure 7).

PROXIMAL



Ischemic Flaps **Control Flaps**

DISTAL

Plate 3. All ischemic flaps demonstrated increased radioactivity when compared to contralateral controls (A) Latissimus dorsi flaps n = 6 pairs (B) Buttock flaps n = 6 pairs

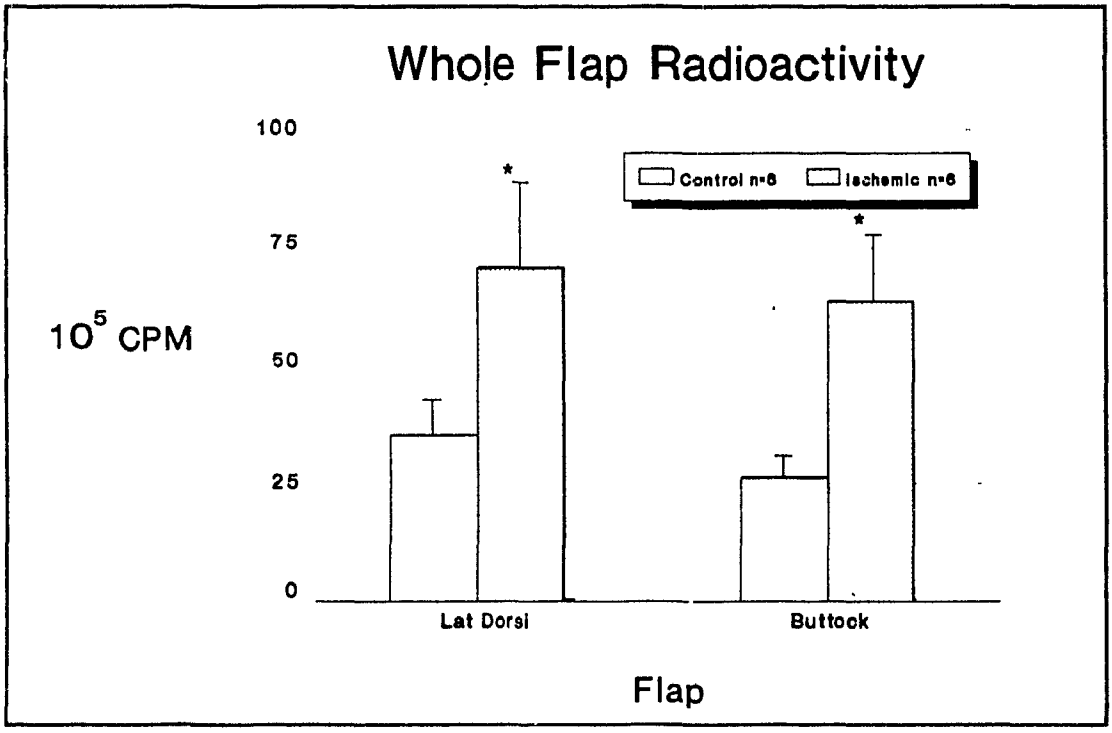


Figure 6. Whole flap radioactivity, expressed as radioactive counts per minute per flap. (Paired Student's t-test; *p<.01)

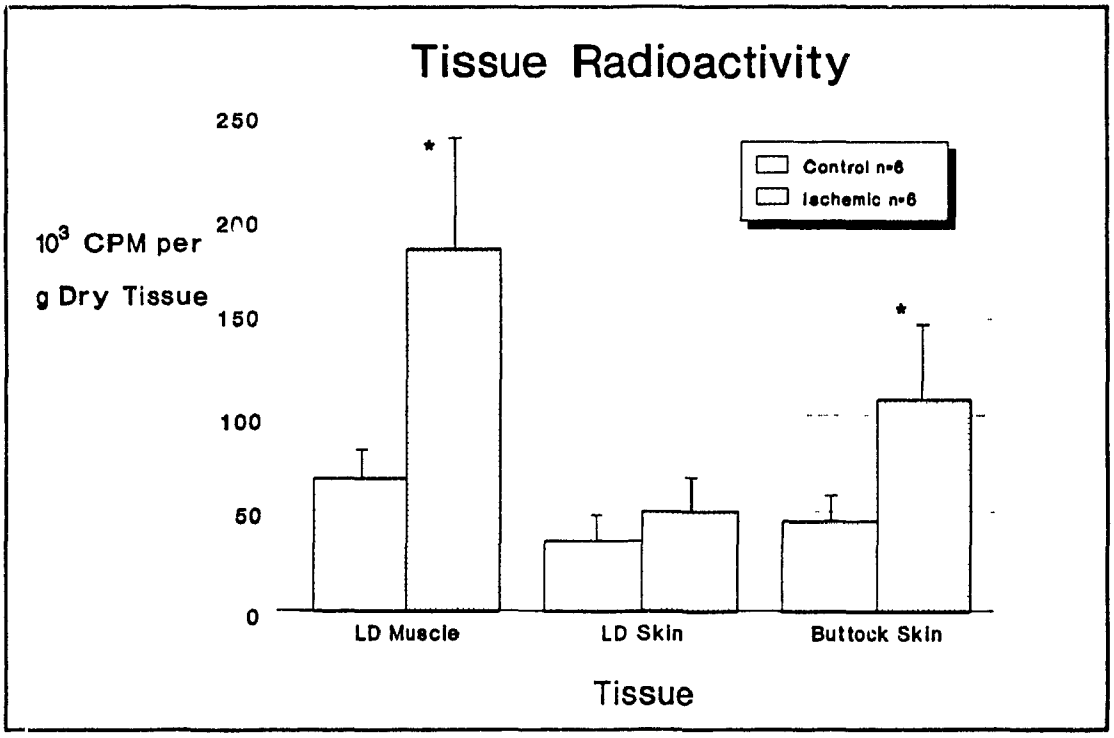


Figure 7. Tissue radioactivity, expressed as radioactive counts per minute per gram of dry tissue weight. (Blocked ANOVA significant with p<.05, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

Skin radioactivity was highest in the most proximal portion of ischemic buttock flaps (figure 8), yet this area always survived the global ischemic insult. In contrast, radioactivity in the skin of latissimus dorsi flaps (figure 9) was low throughout the length of the flap. Muscle radioactivity was uniformly high (figure 10).

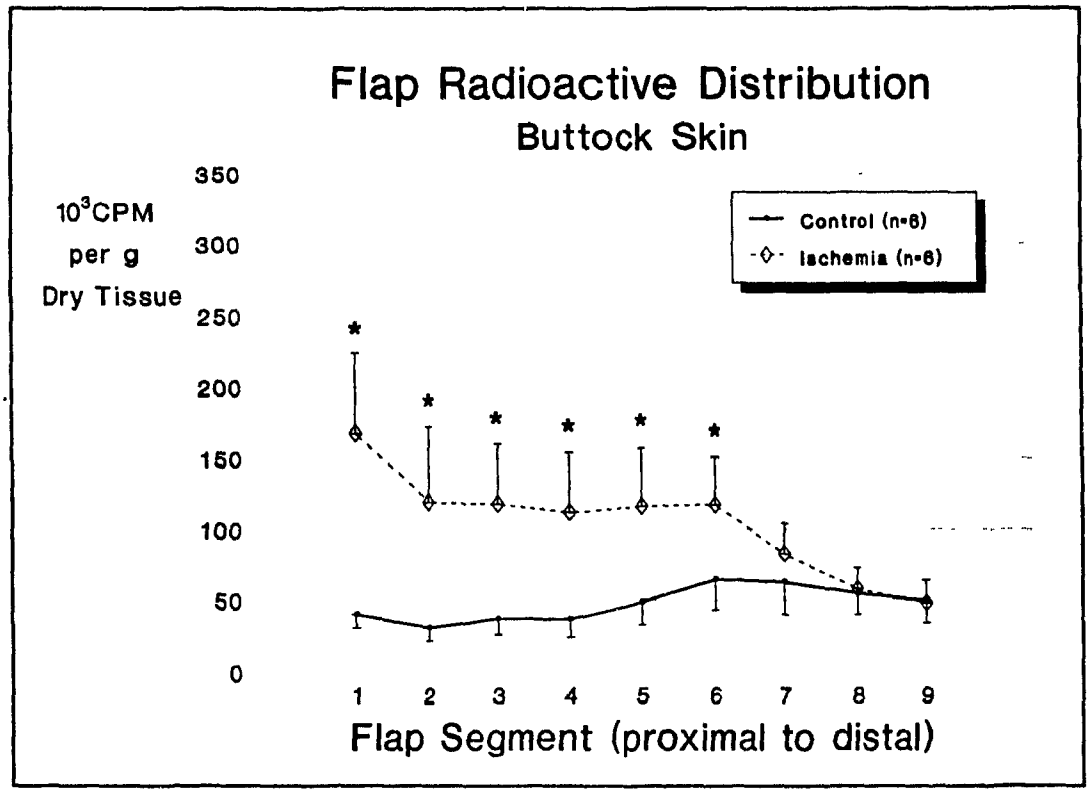


Figure 8. Segmental distribution of buttock skin radioactivity. (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

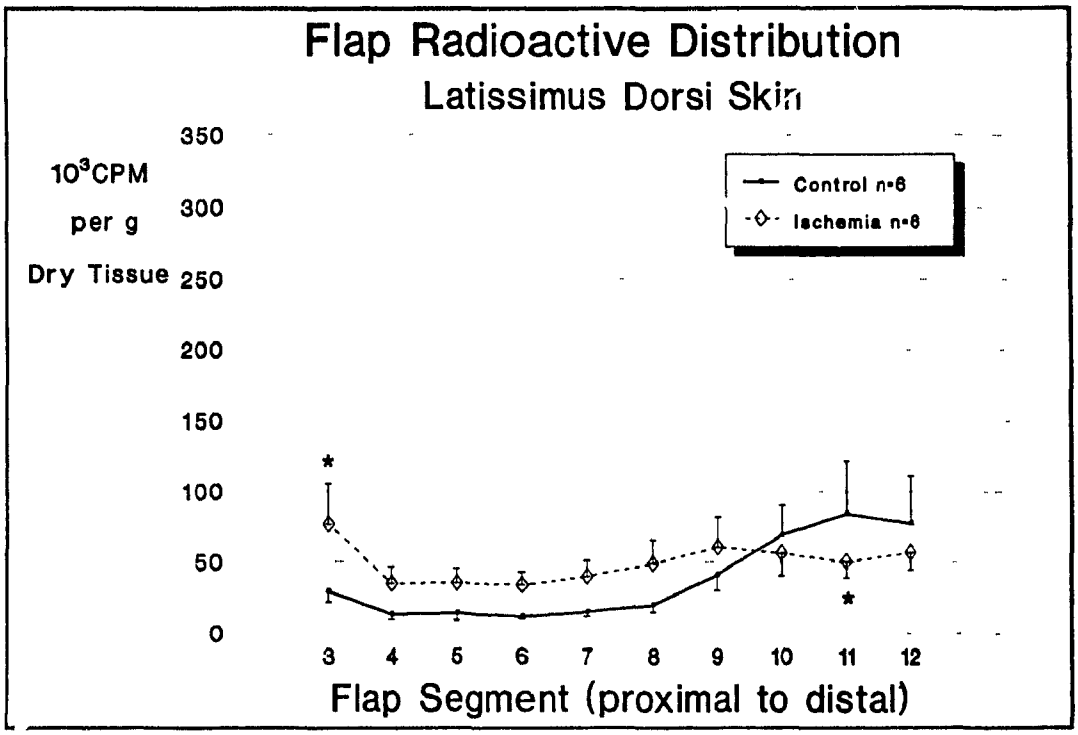


Figure 9. Segmental distribution of latissimus dorsi skin radioactivity. (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

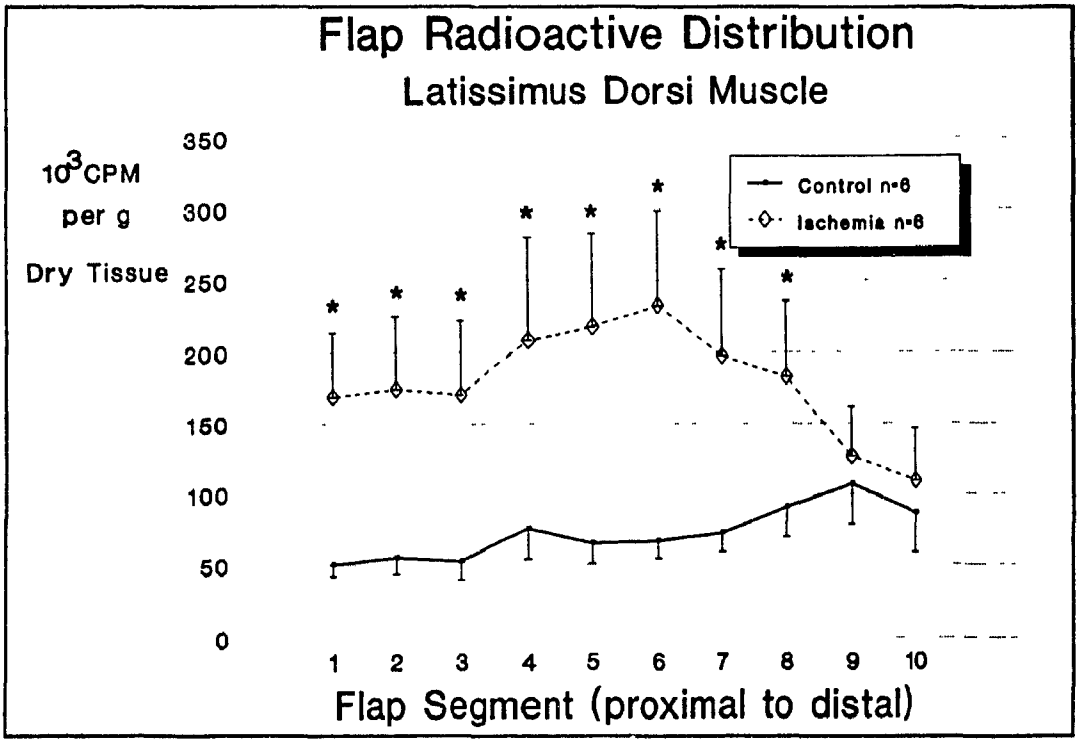


Figure 10. Segmental distribution of latissimus dorsi muscle radioactivity. (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

Survival

The anatomical length (figure 11) and area (figure 12) of skin survival in the buttock and latissimus dorsi flaps were significantly decreased following 6 hours of global ischemia. Skin necrosis took place in the most distal portion of all flaps (plate 4).

Muscle survival could not be reliably determined in the most distal 10^{th} muscle segment because of latissimus dorsi muscle insertion into the lumbar fascia. Thus, the 10^{th} muscle segment was omitted from survival analysis. Control muscle showed a negligible amount of muscle mass necrosis (figure 13). Muscle injured by global ischemia and reperfusion demonstrated a predominate proximal muscle mass necrosis (figure 14). Compared to control, post-ischemic muscle necrosis was significantly higher in all but the 6^{th} and 7^{th} muscle segments (figure 15). On visual inspection necrosis arose in proximal thick central core of the latissimus dorsi muscle (plate 5).

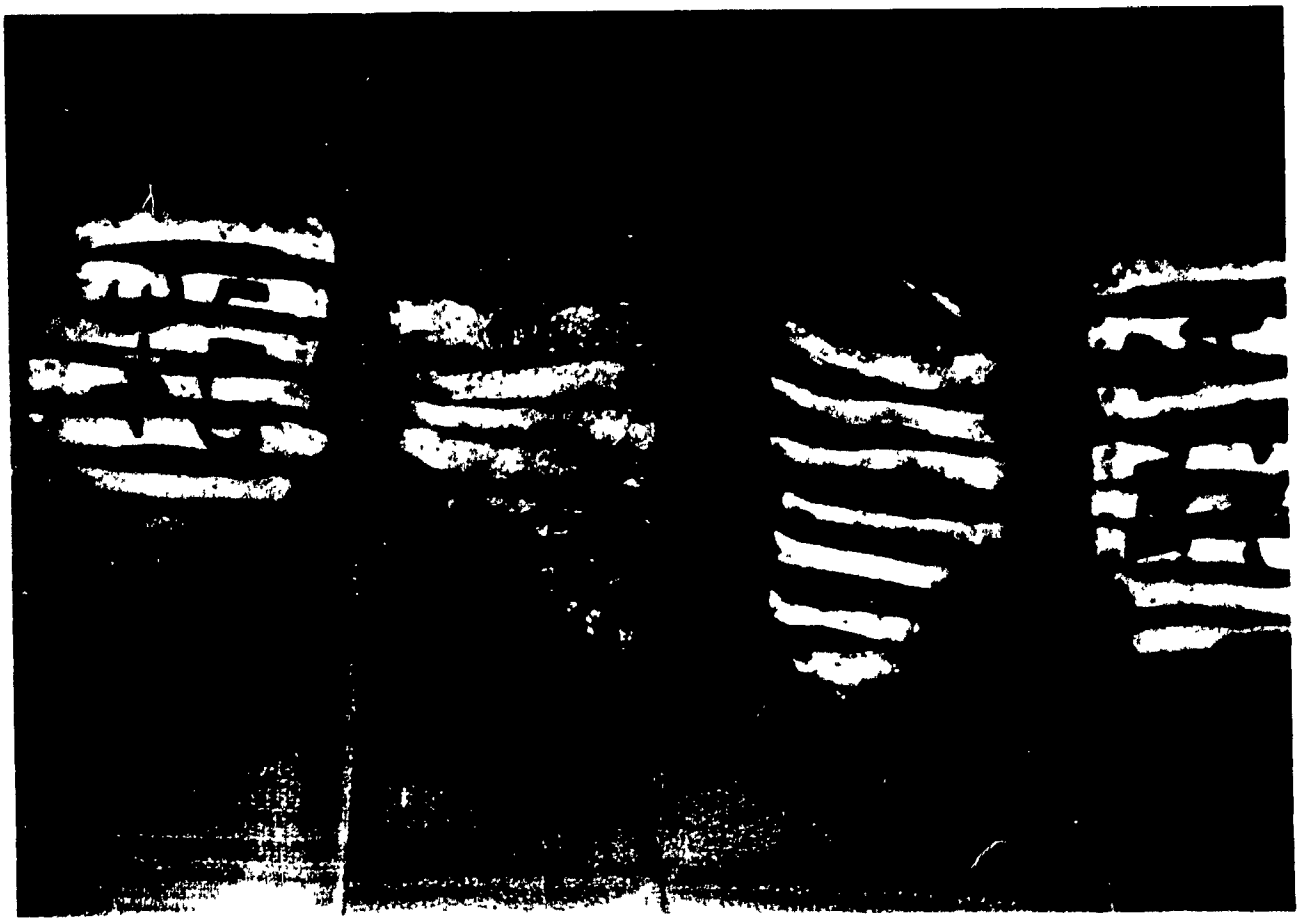


Plate 4 Skin necrosis always occurred in the distal portions of flaps.

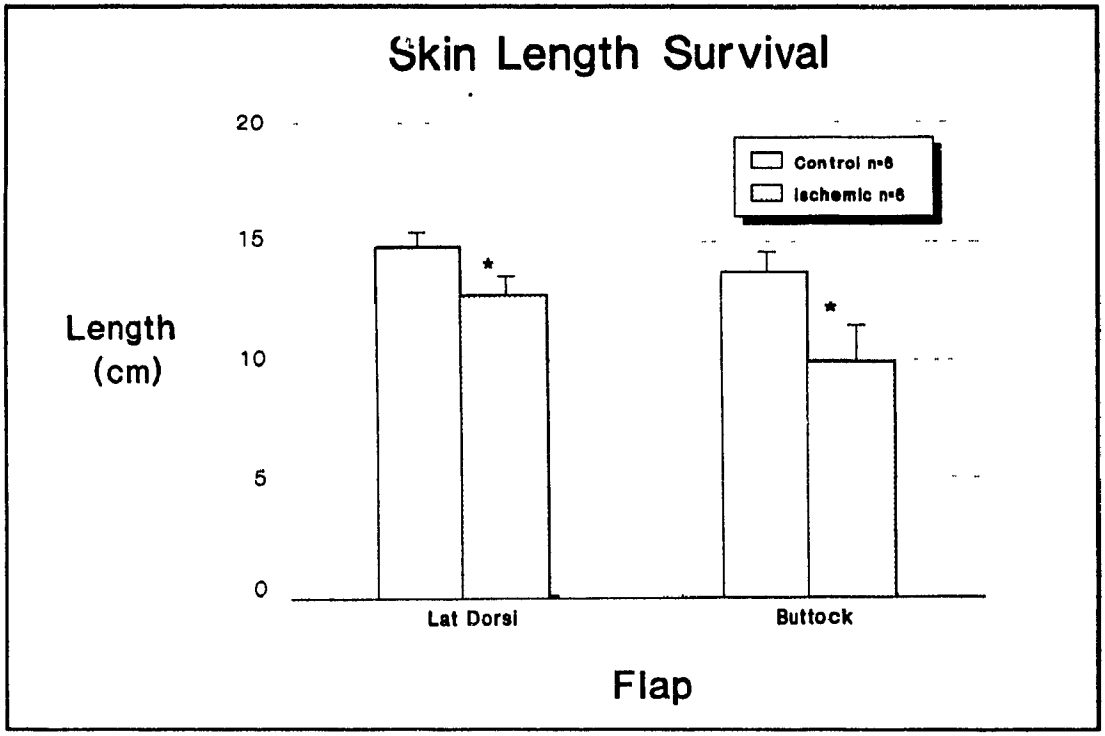


Figure 11. Anatomical skin length survival in buttock cutaneous and latissimus dorsi myocutaneous flaps. (Paired Student t-test; *p<.01)

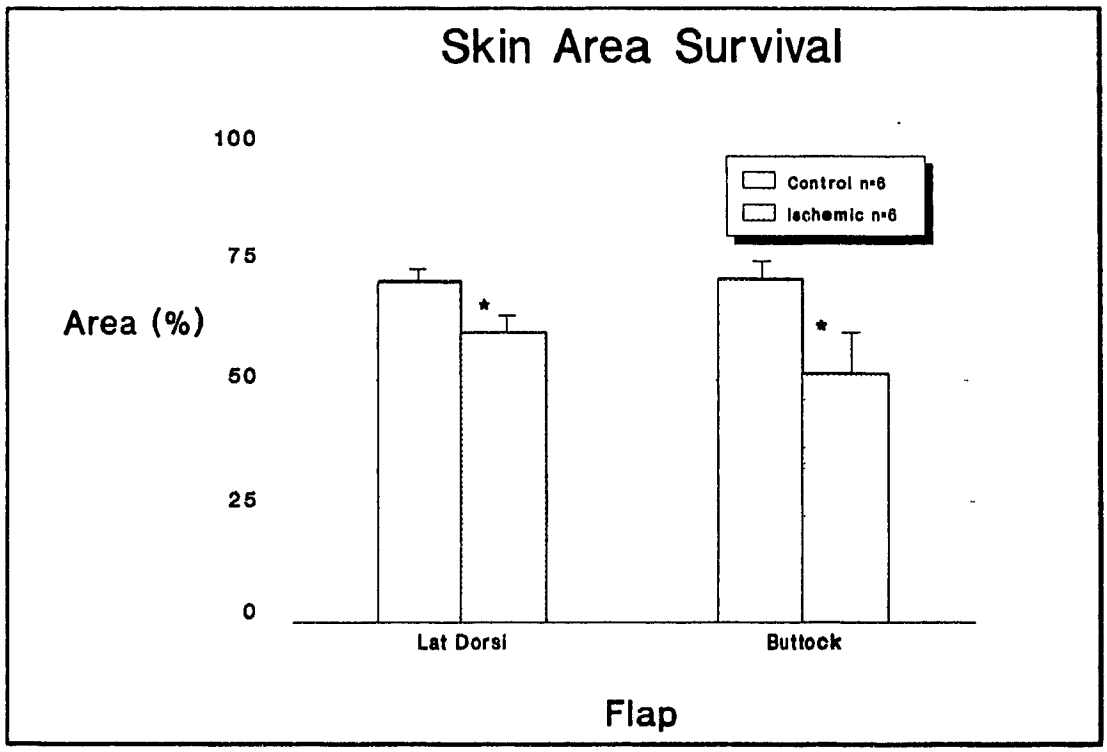


Figure 12. Area skin survival in buttock cutaneous and latissimus dorsi myocutaneous flaps. (Paired Student t-test; *p<.01)

PROXIMAL

DISTAL

Plate 5. Necrosis occurred predominantly in the thick proximal portion of muscle.

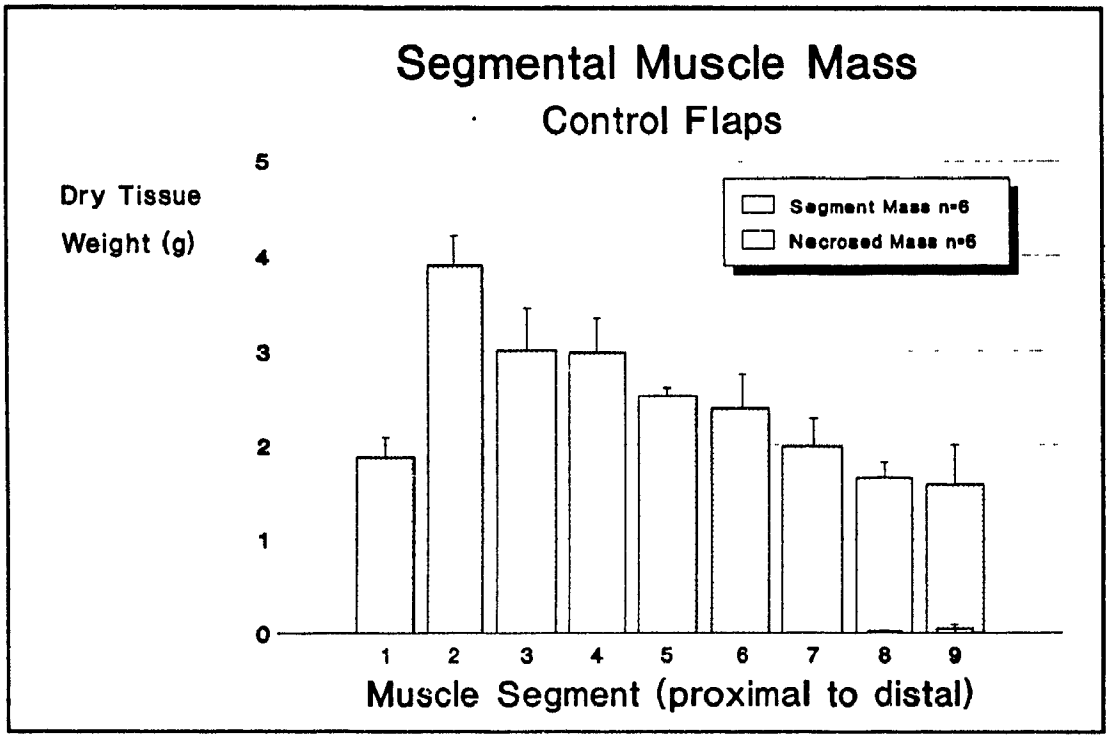


Figure 13. Proximal to distal lengthwise distribution of nonischemic latissimus dorsi dry muscle mass Segment 10 was omitted from analysis Muscle necrosis was minimal in these nonischemic control flaps.

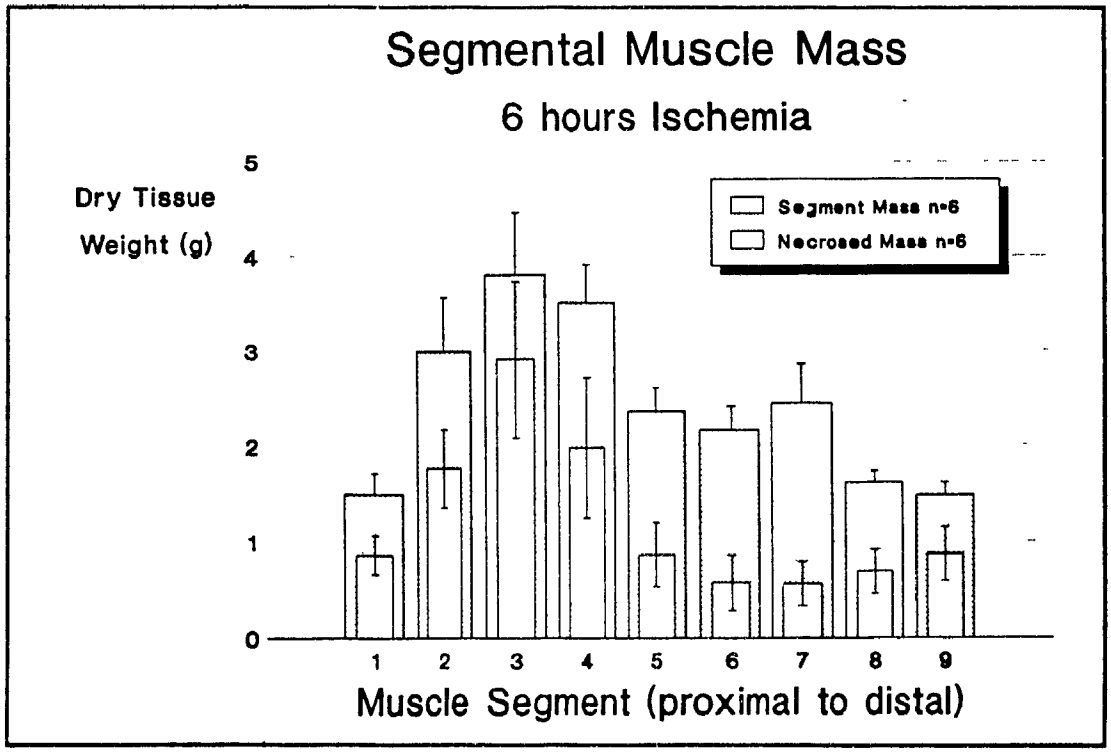


Figure 14. Proximal to distal lengthwise distribution of ischemic latissimus dorsi dry muscle mass Segment 10 was omitted from analysis After 6 hours of ischemia and 24 hours of reperfusion, muscle necrosis occurred predominantly in the proximal muscle

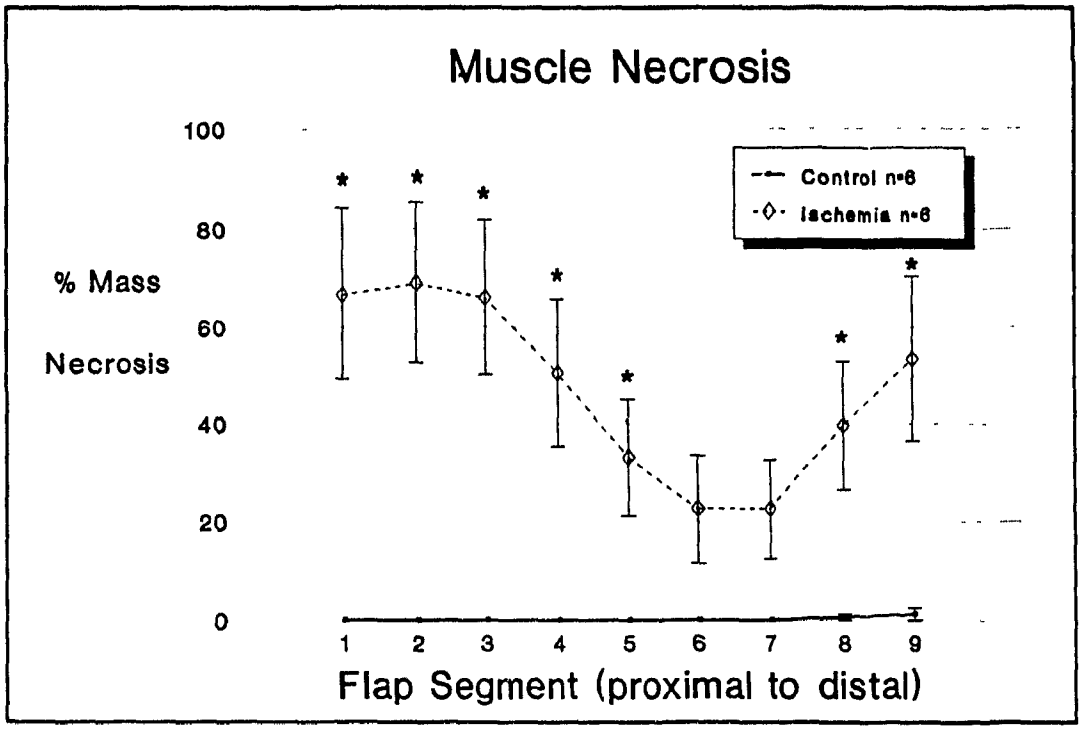


Figure 15. Segmental distribution of %muscle mass necrosis in control and ischemic myocutaneous flaps. Segment 10 was omitted from analysis (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control muscle segments)

Tissue Water Content

The skin water content was distributed uniformly even along the length of the control buttock cutaneous flap. The post-ischemic injured buttock flaps demonstrated a different trend; a high proximal water content was found where the post-ischemic flap was destined to survive, yet in the necrotic distal flap, a low water content was found (figure 16).

A uniform rise in distal skin water content was found in the control latissimus dorsi flaps. The proximal portions of post-ischemic latissimus dorsi flaps demonstrated higher proximal skin water content than the contralateral controls (figure 17).

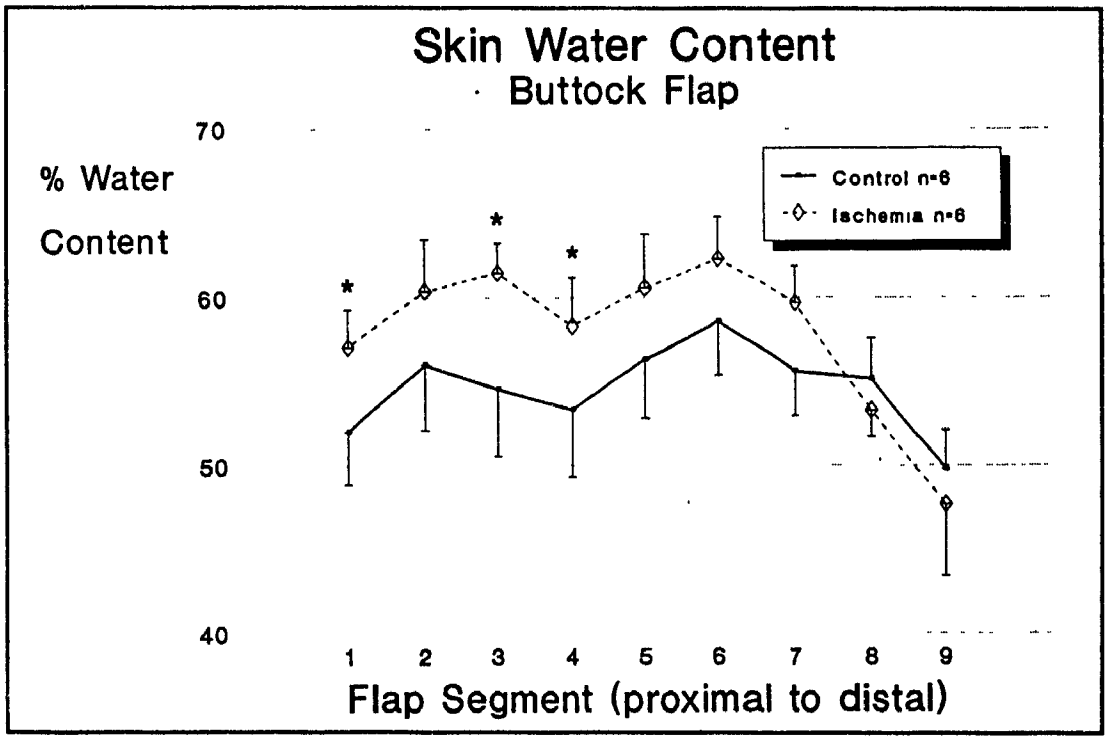


Figure 16. Segmental distribution of buttock skin flap water content. (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

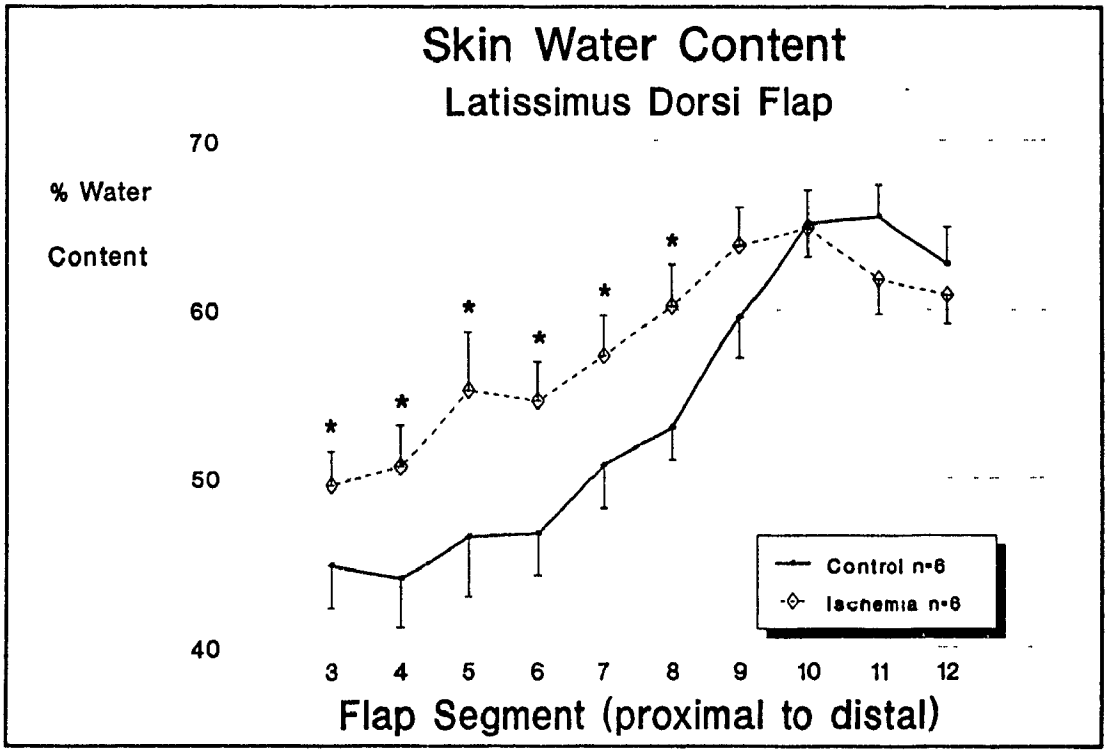


Figure 17. Segmental distribution of latissimus dorsi skin flap water content. (Blocked ANOVA significant with $p < .05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

The post-ischemic latissimus dorsi muscle uniformly showed higher water content than contralateral control muscle (figure 18).

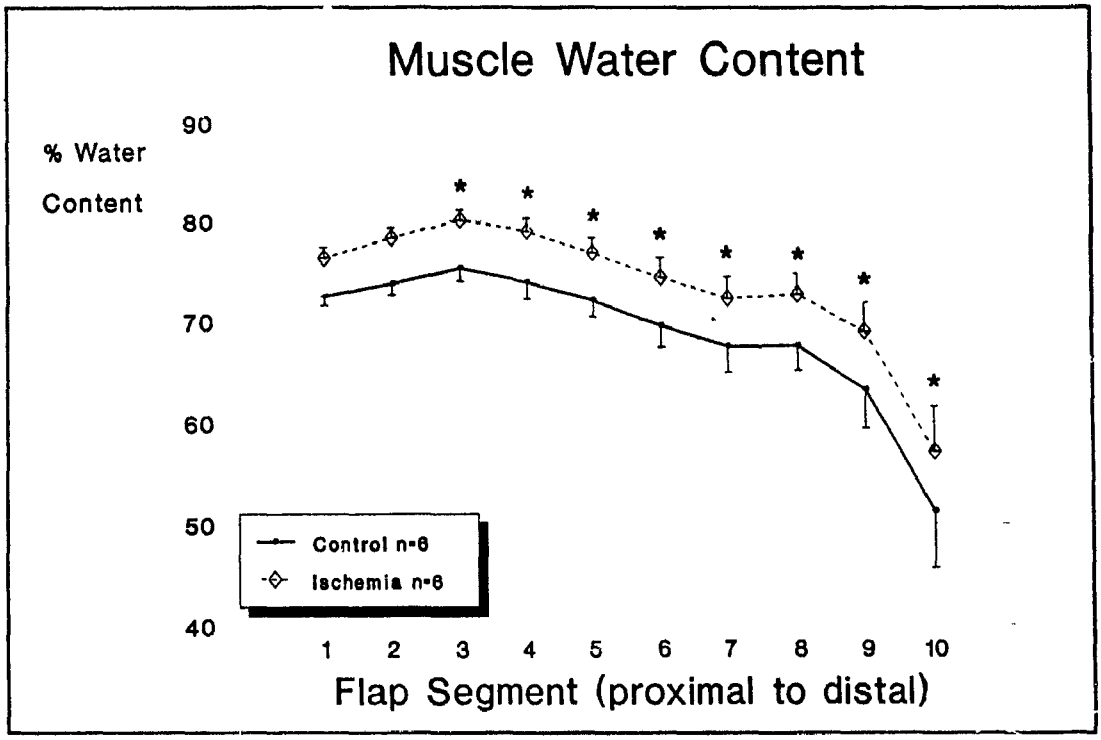


Figure 18. Segmental distribution of latissimus dorsi muscle flap water content (Blocked ANOVA significant with $p<.05$, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral control)

DISCUSSION

Using radiolabelled neutrophils, this study directly confirmed increased neutrophil localization to ischemic cutaneous and myocutaneous flaps (figure 6). When the myocutaneous flap was analyzed with regard to cutaneous and muscular components, the muscle was found to be responsible for the increased neutrophil localization following an ischemic insult (figure 7). In contrast, the skin from ischemic myocutaneous flaps did not significantly accumulate neutrophils. This discrepancy in affinity for neutrophil accumulation in the skin of ischemic cutaneous

and myocutaneous flaps was surprising. The absence of neutrophil sequestration by the skin of ischemic myocutaneous flaps perhaps can be explained by:

1) the muscle avidly extracting activated blood neutrophils, thus resulting in its depletion in the blood supplied to the overlying skin.

2) a reduced surgical trauma compared to the cutaneous flap; in myocutaneous flaps the plane of dissection is deep to the muscle. In cutaneous flaps, a traumatic plane is created to elevate the flap from the muscular bed.

Tissue water content was found to be higher in cutaneous flaps than in the skin of myocutaneous flaps. This observed difference was most readily explained by the obligatory surgical trauma necessary to elevate the cutaneous flap from its muscular bed. No correlation between neutrophil accumulation and tissue water content was detected in cutaneous flaps.

Skin necrosis consistently occurred in the most distal ischemic portions of cutaneous flaps. Paradoxically, neutrophil accumulation was least in these necrotic segments of flap. Thus, skin injury in post-ischemic cutaneous flaps failed to demonstrate any correlation with the distribution of neutrophil accumulation. This finding perhaps was related to the design of the cutaneous flaps. In order to have an accurate assessment of survival, the cutaneous buttock flap was intentionally created 'too large' for its blood supply. Hence, the distal segments destined to die were not truly subjected to reperfusion upon clamp removal after the 6 hours of global ischemia. This suggests that the low neutrophil counts distally may have resulted simply because of an absence of blood flow in the distal flap rather than a failure of the neutrophil to mediate the reperfusion injury. Nevertheless, the lack of correlation to skin survival even in the proximal flap segments raises some doubt as to the importance of the neutrophil in the pathogenesis of post-ischemic injury in cutaneous flaps. Insignificant neutrophil accumulation in the skin of myocutaneous

flaps precluded any significant correlation with the distribution of skin injury resulting from a global ischemic insult.

Post-ischemic muscle edema, as measured by tissue water content, was uniformly increased over the entire length of the myocutaneous flap. However, muscle necrosis was predominantly proximal, occurring in the thickest core of muscle. This survival pattern of muscle confirms previous reports.[71,72] Perhaps with edema formation, nutrient microvascular channels are more impaired by compressive forces in the proximal muscle as the limited space at the core of the thick proximal portion of muscle is less forgiving than the distal more distensible thin part of muscle. Thus, it is conceivable that injury as measured by edema may remain uniform while muscle survival is variable along the length of the flap. Neutrophil sequestration was uniformly increased along most of the ischemic muscle length. Although the pattern of neutrophil accumulation does not perfectly correlate with the pattern of muscle injury, enough similarity exists such that it is still feasible that the neutrophil mediates the reperfusion injury in ischemic muscle. However, the absence of perfect correlation with tissue injury implies that the neutrophil may only partially mediate the injury in post-ischemic muscle of myocutaneous flaps.

In summary, radiolabelled neutrophils were used to determine neutrophil accumulation in cutaneous and myocutaneous flaps after being subjected to 6 hours of global ischemia and 24 hours of reperfusion. In the buttock cutaneous flap, neutrophil accumulation did occur during reperfusion following a global ischemic insult. The anatomical distribution of injury, however, failed to correlate with the pattern of neutrophil accumulation. Although this can be partially explained by the design of the flap, the lack of correlation of skin survival to neutrophil accumulation does cast doubt as to the significance of the neutrophil in the pathogenesis of the post-ischemic injury in cutaneous flaps. In the latissimus dorsi myocutaneous flap, increased neutrophil sequestration was attributable to the ischemic muscle. Since the pattern of muscle edema, did to some degree, follow the pattern of muscle neutrophil

sequestration, this similarity can be considered consistent with a neutrophil mediated injury. However, the absence of perfect correlation with the pattern of ischemic muscle necrosis suggests that the neutrophil may only partly mediate the reperfusion injury in globally ischemic myocutaneous flaps.

Neutrophil activation and localization to post-ischemic flaps are consistent with a neutrophil mediated reperfusion injury yet still do not establish a causal role. Experiment #3 was designed to determine if the neutrophil functions to mediate post-ischemic injury in cutaneous and myocutaneous flaps.

EXPERIMENT #3

Neutrophil Depletion

INTRODUCTION

The microenvironment of ischemic skin flaps have demonstrated the ability to sequester neutrophils from reperfusing blood and to prime the reperfusing neutrophils, as evidenced by enhanced neutrophil function (experiment #1). These findings are consistent with neutrophil mediation of the reperfusion injury in skin flaps, however do not establish a causal relationship. This study evaluated the leukocyte as a potential etiologic factor in the pathogenesis of the post-ischemic injury in skin flaps. Cyclophosphamide, a chemotherapeutic alkylating agent, was used to deplete blood neutrophils. In swine, cyclophosphamide has been shown to effectively reduce blood leukocytes, with a preferential depletion of the neutrophil subpopulation.[77] In a blinded and randomized trial, animals were rendered neutropenic to determine if survival could be improved in cutaneous and myocutaneous flaps subjected to a global ischemic insult.

MATERIALS and METHODS

24 white Landrace female pigs (20.9 ± 0.6) were randomized to receive either placebo (n=12) or cyclophosphamide (n=12). Randomization was achieved by sealed envelope and drug dispensed by code such that the experimenter remained blind to treatment throughout the duration of the experiment.

Drug Treatment

The treatment regime was modified from a previous report showing effective swine neutropenia with low morbidity when using cyclophosphamide in divided doses over 5 days.[77] All animals were treated preoperatively with 3 repeated doses of placebo (0.9% NaCl) or cyclophosphamide (each dose, 30mg/kg) given by intraperitoneal injection on day 1, 3, & 5. After day 3 of drug treatment, all animals

were started on antibiotic prophylaxis with daily intramuscular injections of procaine Pen G (600000 U). At the time of surgery a single preoperative dose of crystalline Pen G (1 million U, intravenous) and streptomycin (1 g, intramuscular) was given.

Bloods were drawn on day 1 & 5 by percutaneous puncture of the superior vena cava. Complete blood cell counts were measured using the Technicon H*1 system (Technicon Instruments Corporation, Tarrytown, New York 10591). Standard blood smears with Wright-Giemsa stain were used to determine the differential counts. Data from the complete blood cell counts were not reviewed until completion of the study, thus maintaining a blinded and unbiased execution of the experiment.

Anesthesia and Surgery

After 5 days of treatment, animals were sedated with intramuscular injections of ketamine (20 mg/kg) and xylazine (2 mg/kg) and anesthetized with an intravenous injection of sodium pentothal (6 mg/kg). Following intubation and spontaneous respiration of room air, repeated doses of intravenous sodium pentobarbital were titrated to achieve and maintain surgical anesthesia. In each pig bilateral 10 by 18 cm buttock cutaneous and 10 by 20 cm latissimus dorsi myocutaneous island flaps were created. On one side of each animal, flaps remained nonischemic to define the flap area at risk for a global ischemic injury. Flaps on the contralateral side were subjected to six hours of global ischemia by the application of Acland V3 clamps to the division of the circumflex iliac vessels supplying the buttock cutaneous flap and Acland V2 clamps to the thoracodorsal vessels supplying the latissimus dorsi myocutaneous flap. Dermofluorometry (Fluoroscan, Santa Barbara Technology, 1155 Via Tranquila, Santa Barbara, Calif. 93110) was used to confirm complete pedicle occlusion during clamping and reperfusion on unclamping.[130] Animals were observed for 24 hours following reperfusion; flap survival was then determined.

Survival

Skin survival determinations were aided by intravenous fluorescein injection (15mg/kg) and Wood's lamp illumination. Skin survival tracings were recorded on transparent plastic with survival area calculated using a digitizing tablet (Houston Instrument, TG-1017) and computer software (SigmaScan, Jandel Scientific). In each animal, skin survival ratios were reported for the buttock cutaneous and latissimus dorsi myocutaneous flaps. The skin survival ratio, calculated from the survival flap area of the ischemic to contralateral nonischemic side of each pair of pig buttock and latissimus dorsi flaps, was used to represent the portion of flap that directly survived the global ischemic insult.

Latissimus dorsi muscle survival was assessed by a previously described method of planimetry.[71,72] The muscle was transversely divided into ten (2 cm) segments and the weight of each segment recorded. Surviving muscle was stained by immersion in 0.05% nitroblue tetrazolium solution (in 0.2% Tris buffer, adjusted pH 7.4) for 20-30 minutes at room temperature. Survival on the cut surface of each muscle segment was traced on transparent plastic; in each segment survival was estimated by averaging the per cent area survival on the two cut surfaces of each muscle segment. Survival mass was calculated by multiplying the weight with the estimated per cent survival of each muscle segment. Individual survival masses were summed to yield cumulative survival data for the whole muscle.

STATISTICAL ANALYSIS

All errors were reported as standard error of the mean. A one-tailed paired Student's t-test ($p \leq .05$ significant) was used to analyze the complete blood cell counts. A one-tailed unpaired Student's t-test was used to analyze the survival data ($p \leq .05$ significant).

RESULTS

Drug Treatment

Blood neutrophils were dramatically depleted in animals treated by cyclophosphamide compared to placebo treated animals, 52 ± 26 versus 7780 ± 710 (neutrophils/mm^3 blood, $p < 10^{-9}$), respectively. To a lesser extent, the non-neutrophil subpopulation of leukocytes was decreased by cyclophosphamide treatment (figure 19). Cyclophosphamide modestly decreased the platelet count. No significant change in hemoglobin was detected (figure 20).

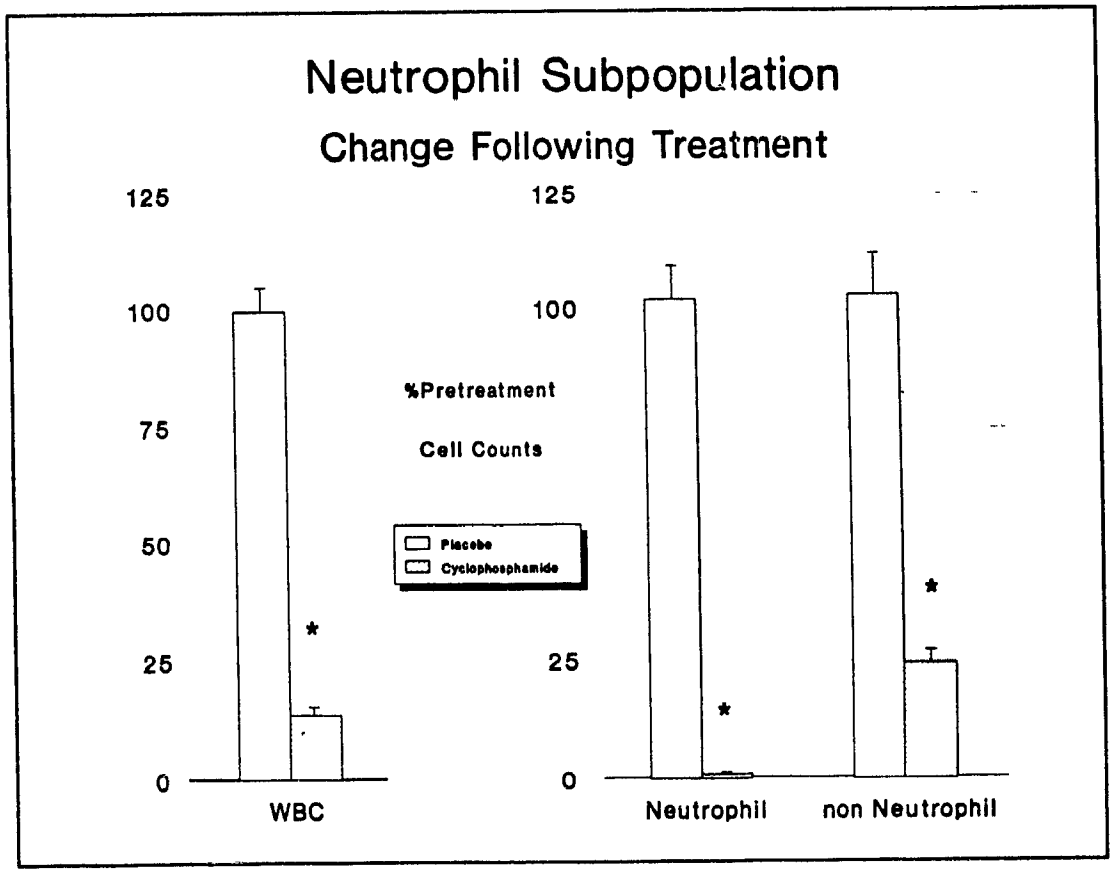


Figure 19. Leukocyte cell counts following 5 days of cyclophosphamide or placebo treatment. Severe neutropenia. Modest reduction in non-neutrophil subpopulation. Expressed as % of the pretreatment cell counts. (Student t-test; *p<.005)

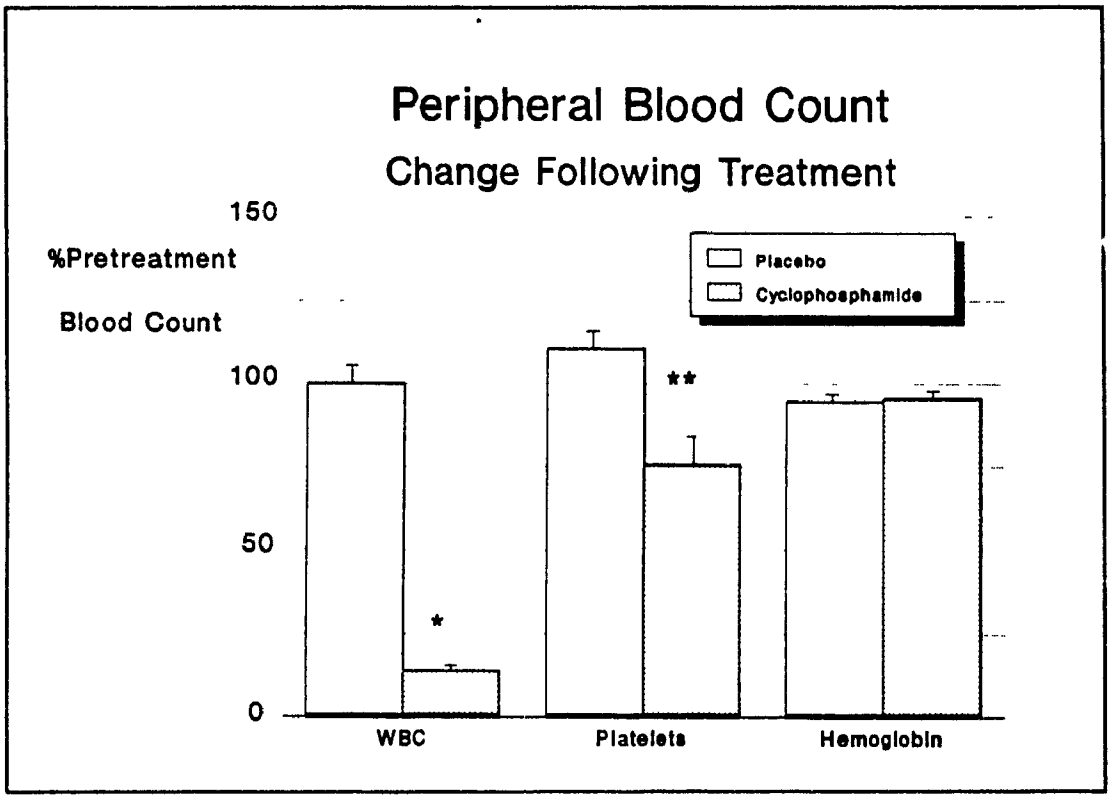


Figure 20. Complete blood cell counts following 5 days of cyclophosphamide or placebo treatment. Severe leukopenia. Modest reduction in platelet counts. Expressed as % of the pretreatment cell counts (Student t-test; *$p < .0001$, **$p < .005$)

Survival

Buttock skin survival was not improved by cyclophosphamide treatment (figure 21). Latissimus dorsi ischemic skin area survival and skin survival ratio was significantly improved by cyclophosphamide treatment (figure 22). The observed improvement in ischemic latissimus dorsi muscle survival was not significant on statistical testing (figure 23). Skeletal muscle necrosis predominantly occurred in the proximal portion of the ischemic myocutaneous flap (figure 24).

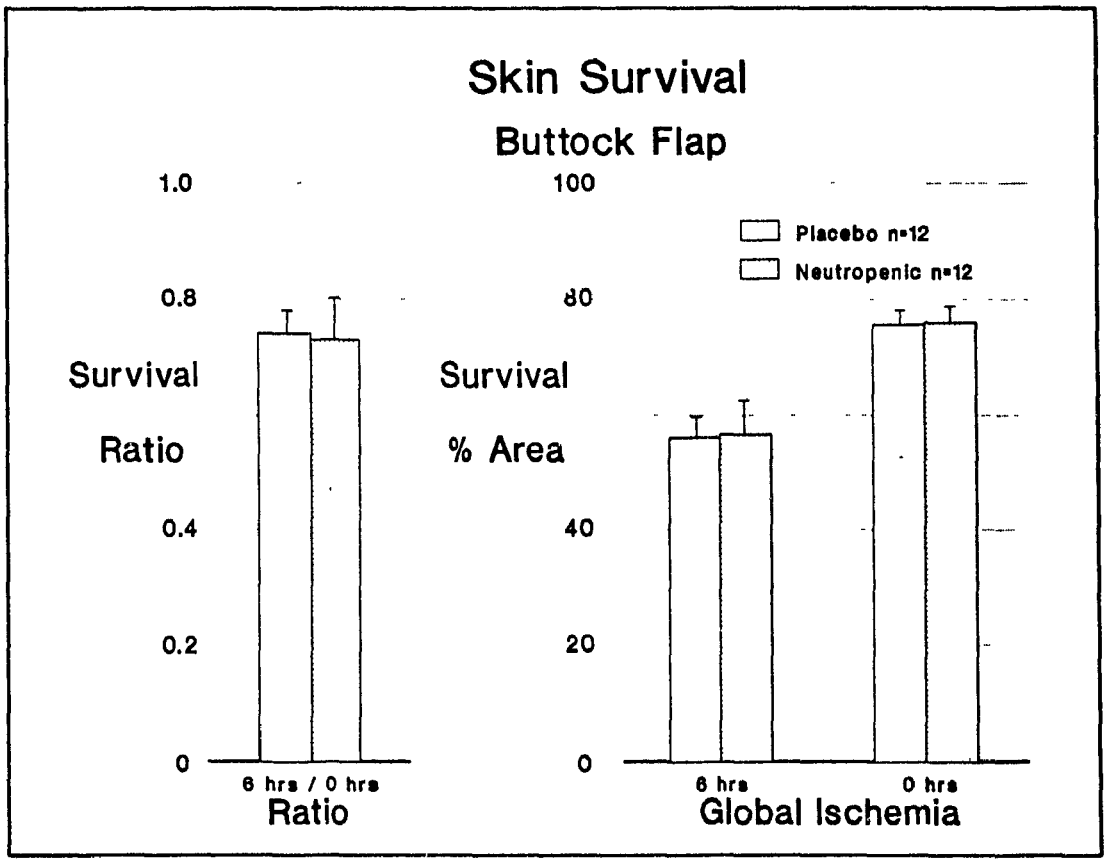


Figure 21. Buttock skin survival. Ratio of ischemic (6 hours) to nonischemic (0 hours) area survival represents the fraction of skin directly surviving the global ischemic insult. Buttock cutaneous flap survival not improved by neutropenia. (Student t-test)

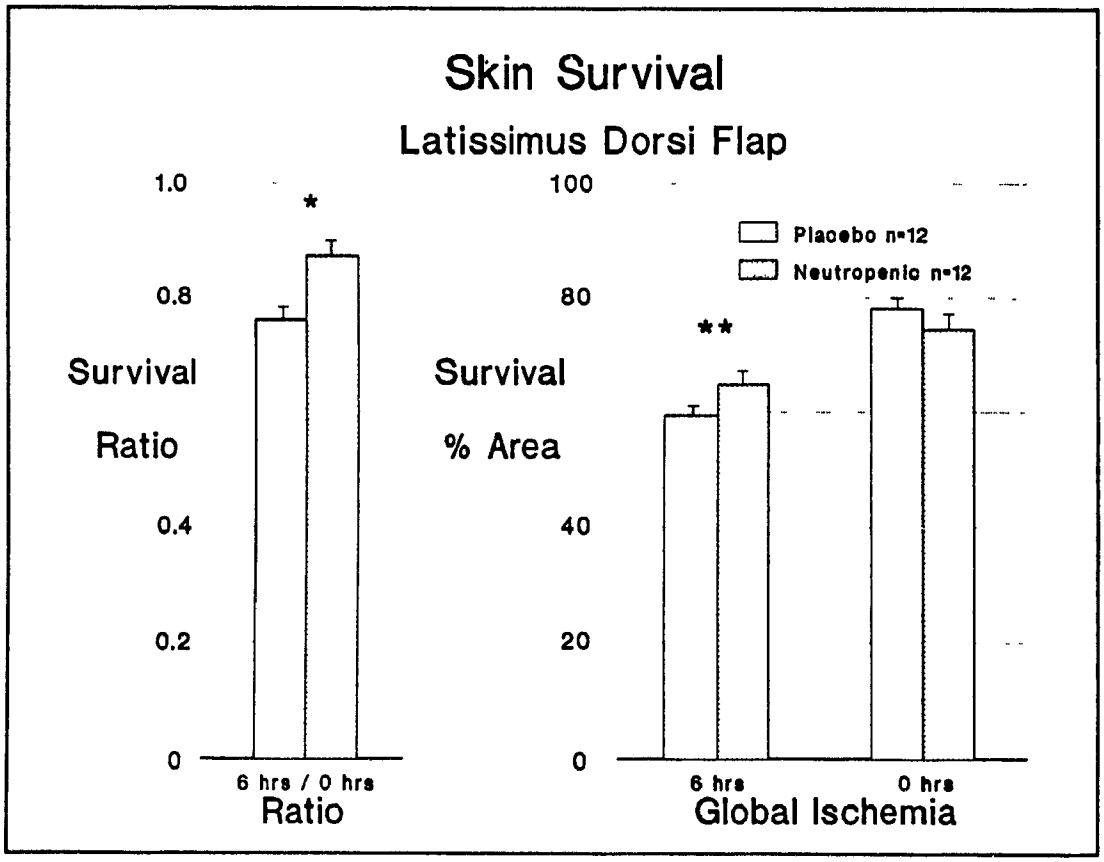


Figure 22. Latissimus dorsi skin survival. Ratio of ischemic (6 hours) to nonischemic (0 hours) area survival represents the fraction of skin directly surviving the global ischemic insult. Skin survival of post-ischemic latissimus dorsi myocutaneous flap improved by neutropenia. (Student t-test;*$p < .002$,**$p < .05$)

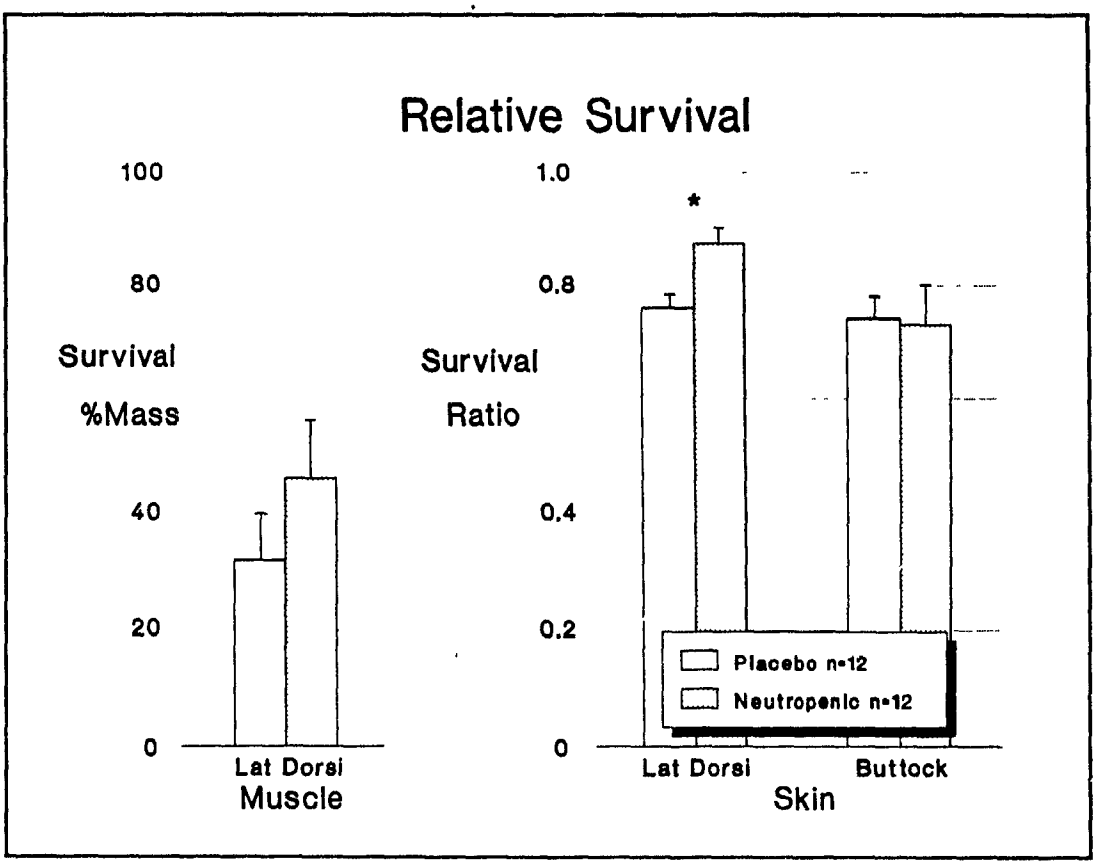


Figure 23. Ratio of ischemic (6 hours) to nonischemic (0 hours) area of skin survival represents the fraction of skin directly surviving the global ischemic insult. The improved %muscle mass survival seen was not significant on statistical analysis. (Student t-test, *p < .002)

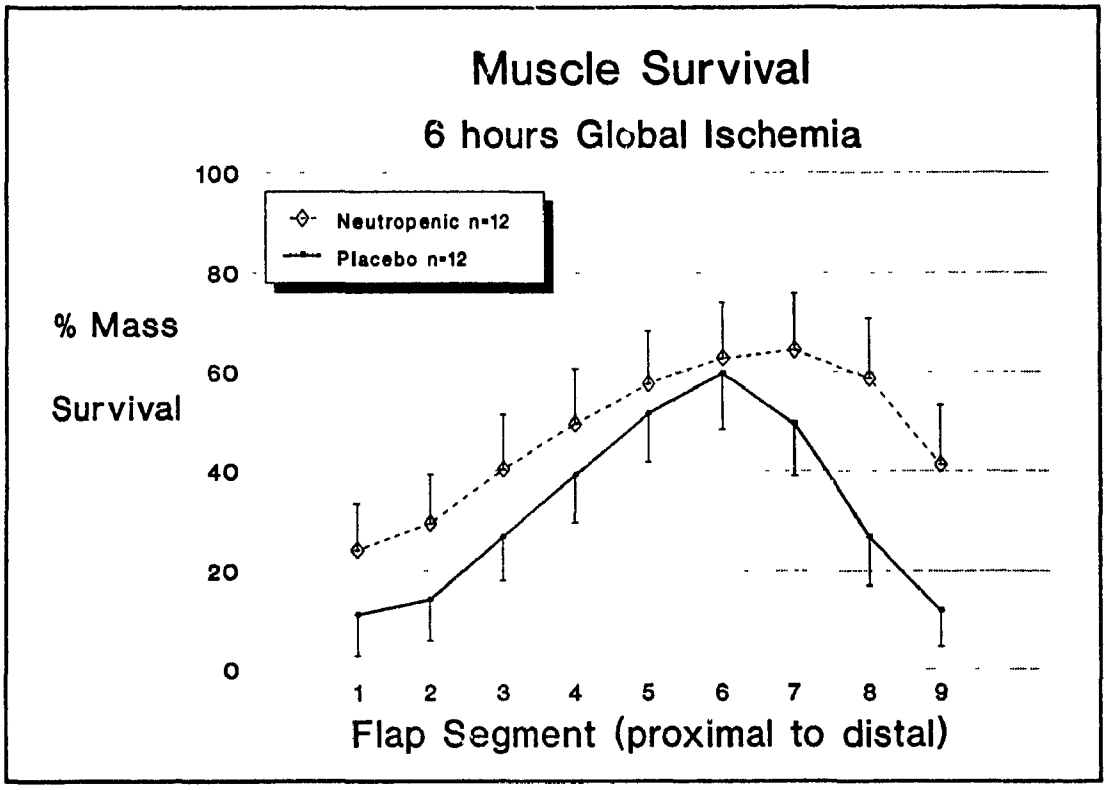


Figure 24. Proximal to distal anatomical distribution of the % surviving muscle following 6 hours of global ischemia.

DISCUSSION

Neutropenia, as achieved by leukocyte filters,[68,75,143] antibodies,[110] radiation,[7] and chemotherapeutic agents,[89] has been reported to be successful in the salvage of ischemic myocardium and skeletal muscle. In this study, cyclophosphamide was employed to induce neutropenia. Under the regime used, dramatic leukopenia was evident and near complete destruction of the neutrophil subpopulation was demonstrated. Cyclophosphamide treated animals were indistinguishable from placebo treated animals, thus maintaining 'blind' conditions throughout the duration of the experiment. Myocardial ischemia studies examining platelet kinetics in ischemic tissue, have suggested platelet accumulation as an unlikely factor influencing

reperfusion injury.[111] Thus, the minor decrease in platelet count with cyclophosphamide treatment was not considered a significant factor in the evaluation of neutropenic ischemic skin flap salvage.

In experiment #2, neutrophils were localized to ischemic flaps to determine if there existed any trends that correlated with the observed anatomical pattern of tissue injury. Although post-ischemic cutaneous flaps demonstrated increased neutrophil sequestration, the cutaneous flap segments that progressed to necrosis did not demonstrate increased neutrophil accumulation. This casted doubt as to the existence of a neutrophil mediated post-ischemic injury in cutaneous flaps. In this study, neutropenia failed to improve post-ischemic survival in cutaneous flaps. Thus, the neutrophil does not significantly mediate post-ischemic injury in cutaneous flaps.

In contrast, skin survival was significantly improved in neutropenic post-ischemic myocutaneous flaps. The disparity between post-ischemic skin survival of cutaneous and myocutaneous flaps is difficult to explain. The skin of post-ischemic myocutaneous flaps has already been shown not to significantly sequester neutrophils (experiment #2). Thus, it is highly unlikely that neutrophil depletion would directly salvage the skin of a post-ischemic myocutaneous flap. Perhaps skin of the myocutaneous flap was privileged secondarily by neutropenic post-ischemic salvage of muscle and/or preservation of the musculocutaneous perforators supplying the overlying skin.

Primary muscle salvage by neutropenia would be a plausible explanation for the improved skin survival of post-ischemic myocutaneous flaps. This speculative explanation would be concordant with the findings of Belkin and Korthuis.[7,68] Using radiation induced neutropenia in rats, Belkin found a significant post-ischemic skeletal muscle salvage.[7] Korthuis demonstrated significant post-ischemic canine skeletal muscle salvage, by using leukocyte filters to deplete cells from the

reperfusing blood.[68] However, the improved post-ischemic muscle survival observed in the myocutaneous flaps of neutropenic swine in this study, was not significant on statistical analysis. This may be the case of a type 2 error (accepting a null hypothesis when the alternate hypothesis is true) because of an insufficient sample size for the relatively high animal to animal muscle survival variability. In future studies, one solution to reduce this 'statistical noise' would be to employ experimental designs that would allow each animal to function as its own control.

Although neutropenia was partly effective in salvaging the post-ischemic myocutaneous flap, its clinical application is nullified by the morbid risks of systemic infection. However, antiinflammatory agents such as ibuprofen, iloprost, and BW755C have been reported to reversibly inhibit neutrophil function.[78,79,89,98,119] Ischemic canine myocardial studies employing some of these agents have demonstrated improved post-ischemic myocardial salvage.[89,109,111,119] Belkin has reported improved post-ischemic canine skeletal muscle salvage when iloprost was employed.[8] Pharmacologic inhibition of neutrophils may become a feasible clinical option to attenuate the post-ischemic injury resulting from the neutrophil.

In summary, the anatomical distribution of injury in the post-ischemic cutaneous flap has previously (in experiment #2) failed to demonstrate any correlation with neutrophil sequestration. In this study, neutropenia failed to improve post-ischemic survival in cutaneous flaps. Thus, the neutrophil does not significantly mediate post-ischemic injury in cutaneous flaps.

In post-ischemic myocutaneous flaps, neutropenia significantly improved the skin survival. This finding in combination with previous reports of altered neutrophil function induced by reperfusion of an ischemic myocutaneous flap and neutrophil localization to the muscle of ischemic myocutaneous flaps provide strong evidence to suggest that the neutrophil in part plays a causal role in the pathogenesis of the reperfusion injury in ischemic myocutaneous flaps.

EXPERIMENT #4

Neutrophil Inhibition

INTRODUCTION

Microsurgery complicated by acute arterial impairment often precipitates free flap and replant failure. Ideal treatment demands early detection and rapid revascularization to minimize the duration and injury caused by ischemia and reperfusion. In clinical practice delays in detection or difficult microsurgical repairs are common circumstances that may preclude rapid reversal of an ischemic insult. Despite surgical revascularization, the increased ischemia time increases the risk of tissue injury.[101] Adjunctive nonsurgical modalities designed to improve tissue tolerance to ischemia are few. Systemic pharmacotherapy is an attractive therapeutic modality that remains to be proven.[62]

The pathogenesis of the injury sustained by a skin flap complicated by ischemia and reperfusion is in part explained by neutrophil activation and localization. Although neutropenia has been demonstrated to significantly attenuate the post-ischemic injury, the morbid risk of systemic infection nullifies its clinical applicability.[7,89,110] Attenuation of neutrophil mediated reperfusion injury without the morbid risks of neutropenia might reasonably be achieved by temporary pharmacologic inhibition of specific neutrophil functional and localizing responses.

In vitro and in vivo effects of nonsteroidal antiinflammatory drugs such as ibuprofen, include inhibition of neutrophil activation and reduced neutrophil localization to ischemic tissue.[8,78,79,91,98,135,137] Furthermore, ibuprofen has been reported to improve post-ischemic canine myocardial and rat skin flap salvage.[29,109] This provided the stimulus to examine the efficacy of ibuprofen treatment to salvage ischemic cutaneous and myocutaneous flaps.

MATERIALS and METHODS

Ibuprofen was provided by the Upjohn Company, Kalamazoo, MI. An intravenous preparation (25mg/ml) was used for the initial single intraoperative dose to immediately achieve high serum drug levels. Subsequent doses were given enterally by sedating the animal and dosing by oral-gastric gavage. Crystalline ibuprofen was prepared for enteral use by dissolution in 0.2 M Na_2CO_3 (25mg/ml) with pH adjusted to 7.5-8.0 using 1.0 N HCl.[109] An ibuprofen dose of 25 mg per kg of body weight, given at 8 hourly intervals, was used in this study.

Sedation and Anesthesia

Sedation for minor procedures in animals was achieved with intramuscular injections of ketamine (10-20 mg/kg) and xylazine (2 mg/kg). Surgical procedures requiring anesthesia were accomplished by an intravenous injection of sodium pentothal (6 mg/kg) followed by intubation and spontaneous respiration of room air; repeated intravenous doses of sodium pentobarbital were titrated to maintain surgical anesthesia.

Serum Ibuprofen Measurements

A crude pharmacokinetic profile was constructed by dosing two female white Landrace pigs (20-25 kg) with ibuprofen. In the anesthetized animal, the external jugular vein was cannulated with a polyethylene catheter (PE-190, Clay Adams) to provide access for repeated blood sampling. An intravenous ibuprofen dose of 12.5 mg/kg and enteral gavage doses of 6.25, 12.5, and 25 mg/kg were each dosed once. Blood was drawn 0, 1, 2, 4, 6, and 8 hours after each different drug dose to determine peak serum levels and estimate ibuprofen serum half life. High performance liquid chromatography was used to measure serum ibuprofen levels. This technique has been previously described.[3,117]

Experimental Design and Surgery

10 white Landrace pigs (20.9 ± 0.9) were subjected to two operations. At both operations unilateral 10 by 20 cm latissimus dorsi(LD) myocutaneous island flaps and 10 by 18 cm buttock cutaneous island flaps were elevated. Fifteen minutes prior to clamp application (Acland 2V/3V) animals were treated with one intravenous dose of ibuprofen (25mg/kg) or placebo (0.9 NaCl). Following clamp application, all flaps were subjected to 6 hours of global ischemia. One hour prior to unclamping the vascular pedicle, animals were dosed enterally by oral-gastric gavage to ensure peak serum drug levels at immediate reperfusion. Animals were then lightened from anesthesia, extubated, and placed back in the housing pens. Thereafter, enteral drug administration was achieved by sedation and oral-gastric gavage at 8 hourly intervals for 48 hours.

After the first operation, a 24 hour 'drug free period' ensued to allow clearing of residual drug. The alternate treatment was then instituted for the second operation. Half of the animals (n=5) received placebo treatment first and ibuprofen second, while the other half received ibuprofen first.

Survival

Skin survival was determined after 48 hours of reperfusion in all flaps. Skin survival determinations were aided by intravenous fluorescein injection (15mg/kg) and Wood's lamp illumination. Skin survival tracings were recorded on transparent plastic. Anatomical flap length survival was estimated by averaging three equally spaced longitudinal measures of the surviving skin.

Latissimus dorsi muscle survival was determined at the time of sacrifice (5 days after the first surgery). The muscle was transversely divided into 1 cm segments. Surviving muscle was stained by immersion in 0.05% nitroblue tetrazolium solution (in 0.2% Tris buffer, adjusted pH 7.4) for 20-30 minutes at room temperature.

Muscle width and surviving muscle width were recorded for each muscle segment. Using these width measurements, a two dimensional surface map of the muscle along with an estimated tracing of the survival-necrosis interface was created on a paper grid. This facilitated the calculation of the surviving muscle surface area.

All survival areas were calculated using a digitizing tablet (Houston Instrument, TG-1017) and computer software (SigmaScan, Jandel Scientific).

STATISTICAL ANALYSIS

All errors were reported as standard error of the mean. One-tailed paired Student's t-test ($p \leq .05$ significant) was used where applicable. Segmental muscle width necrosis was analyzed with blocked analysis of variance (blocked ANOVA, $p \leq .05$ significant), followed by Waller-Duncan K ratio test (K ratio ≥ 100 significant) where applicable.

RESULTS

Serum ibuprofen measurements showed that peak serum drug levels occurred within 1 hour of an enteral dose. The estimated serum half life was approximately 2 hours (figure 25).

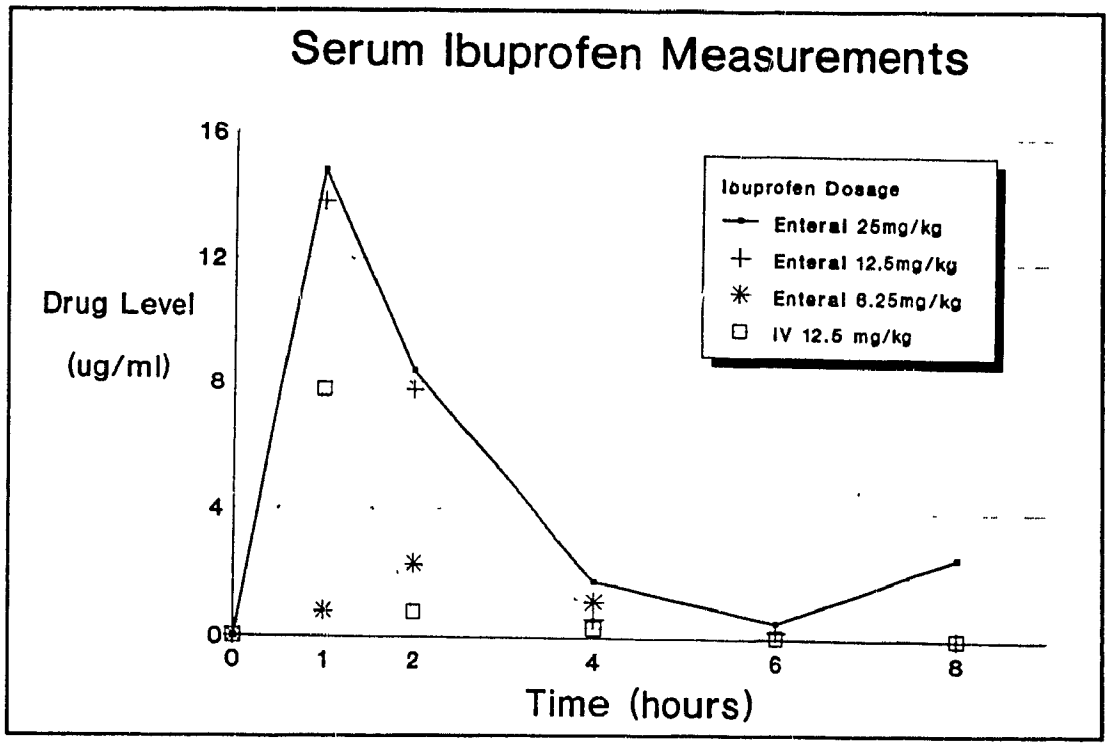


Figure 25. A crude pharmacokinetic profile of ibuprofen in swine. Serum ibuprofen levels were determined by high performance liquid chromatography.

Skin length survival was not improved in the buttock flap. Skin length survival was modestly improved in the ibuprofen treated latissimus dorsi flaps (figure 26).

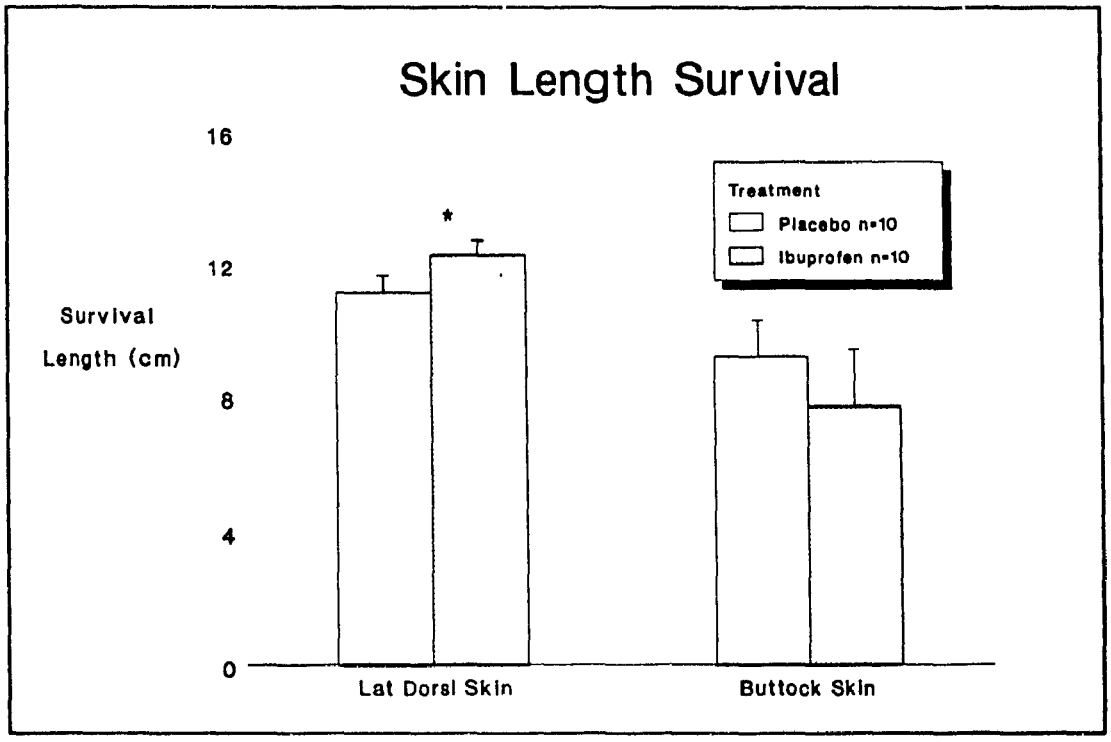


Figure 26 Length of skin survival in placebo and ibuprofen treated skin flaps following 6 hours of ischemia (Paired Student t-test,*p < 05)

Ibuprofen treatment dramatically improved the area of LD muscle survival by 30.4% ± 9.3 (p=.005), whereas LD skin was only modestly improved with a 6.0% ± 3.2 (p=.046) increase in area survival. The area of buttock skin survival was not improved (figure 27). Survival was unaffected by treatment order.

The distribution of muscle necrosis occurred predominantly in the proximal muscle segments (plate 6). Ibuprofen uniformly improved survival through the length of the flap, however the trend of proximal muscle necrosis remained unchanged (figure 28).

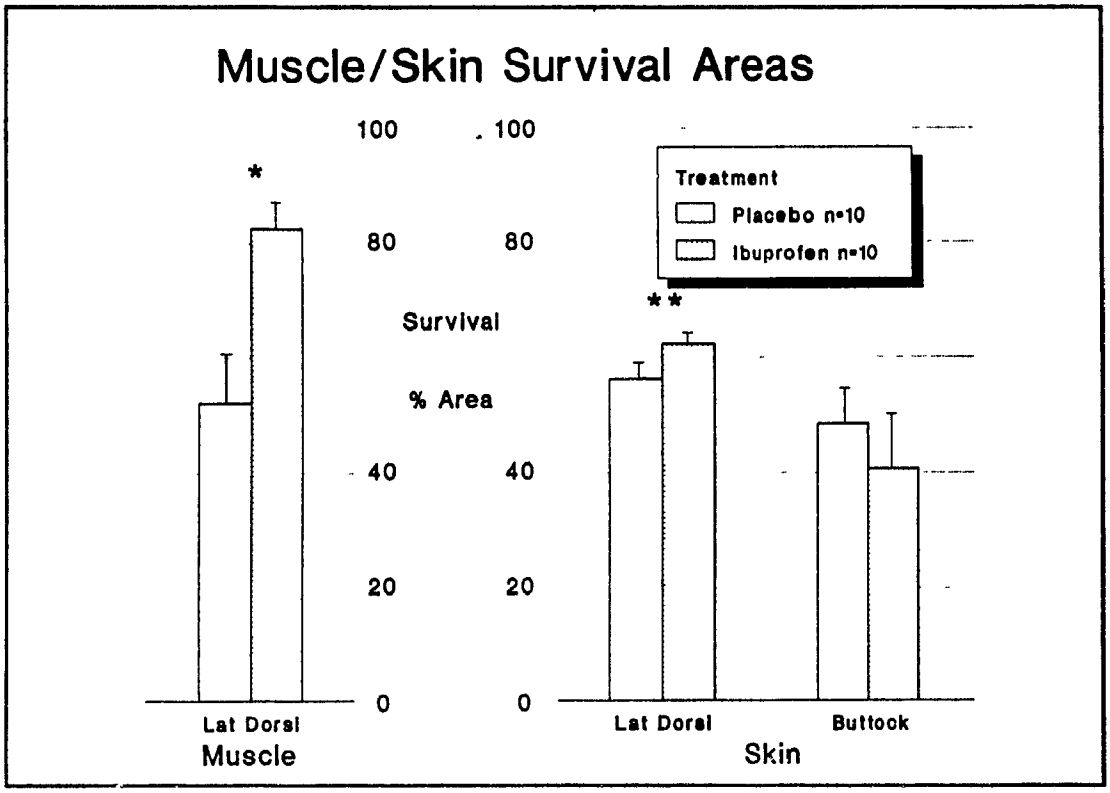


Figure 27. Area of skin and muscle survival in placebo and ibuprofen treated flaps following 6 hours of ischemia. (Paired Student t-test;*p < .005,**p < .05)

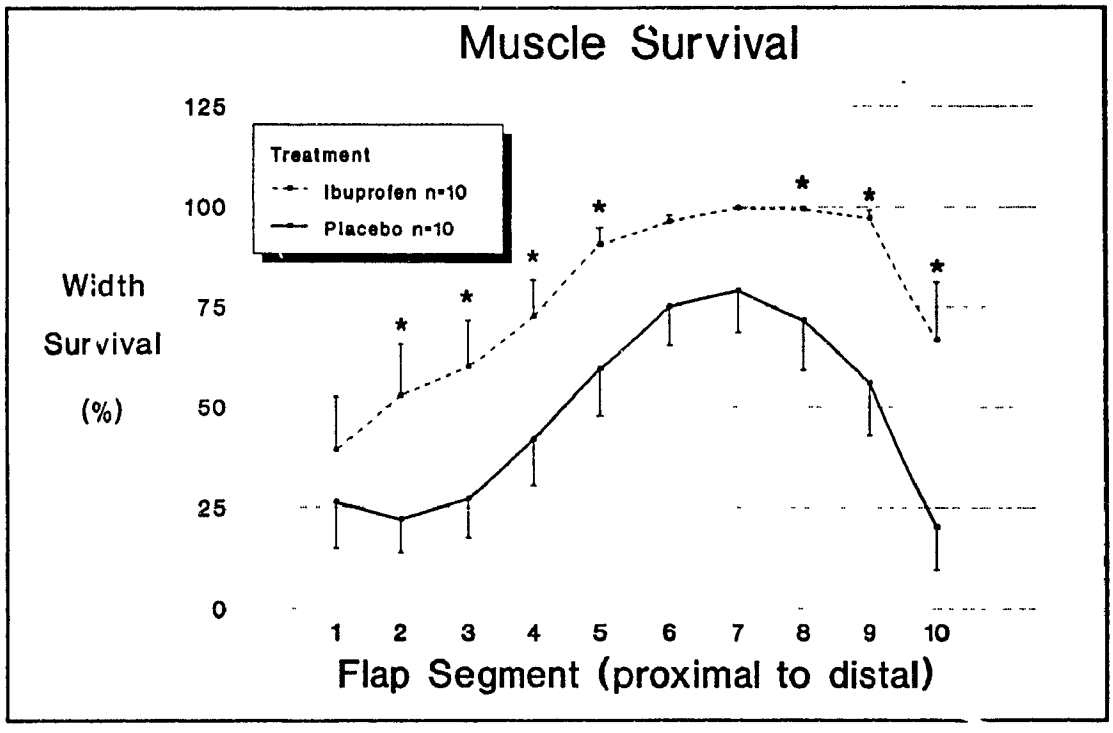


Figure 28. Proximal to distal anatomical distribution of the surviving muscle width following 6 hours of global ischemia (Blocked ANOVA significant with p< 05, followed by Waller-Duncan K ratio, where *K ratio≥100 was significant when compared to contralateral placebo treated flap)

Plate 6. Ibuprofen treatment resulted in improved survival of muscle which was easily detected on gross inspection after staining with nitroblue tetrazolium

DISCUSSION

Muscle necrosis was highest in the most proximal and thickest central core of muscle confirming previous findings. In experiment #3, the effect of neutrophil depletion on ischemic flap survival was examined in a blinded fashion. In that study, there was speculation that the improved muscle survival in neutropenic animals may not have attained significance on statistical testing because of high animal to animal muscle survival variability. The experimental design in this study used each animal as its own control to minimize the animal to animal variability. The dramatically improved post-ischemic skeletal muscle survival by ibuprofen treatment was highly significant on statistical testing supporting a primary ischemic muscle salvage.

Skin survival was not improved in ibuprofen treated buttock flaps and only modestly improved in latissimus dorsi flaps. Because the buttock cutaneous flap failed to demonstrate improved survival, ibuprofen was unlikely to effect a primary skin salvage. Rather, the pattern of differential skin survival in cutaneous and myocutaneous flaps can be better explained by a primary ibuprofen ischemic muscle salvage with preservation of patency to the vascular perforators. Hence, resulting in a secondary benefit to the overlying myocutaneous skin. This discrepancy in cutaneous and myocutaneous flap survival after a global ischemic insult was remarkably similar and consistent with that found with neutropenic animals in experiment #3.

The mechanism for ibuprofen ischemic muscle salvage was not directly assessed in this study. However, elements to consider in determining the mechanism of ischemic skeletal muscle salvage include the effect of ibuprofen on neutrophil function, distribution of blood flow during reperfusion, muscle metabolic rate, prostaglandin and thromboxane synthesis. Neutrophil function has previously been shown to be altered by ibuprofen treatment. Ibuprofen treated neutrophils have

demonstrated inhibited free radical production.[78,91,98,137] Chemotaxis and adherence are necessary steps for neutrophil localization to target cells; this too has been demonstrated to be impaired by ibuprofen.[78,79,135] The similar flap survivals achieved with neutropenia, along with previous studies documenting impaired neutrophil function with ibuprofen, suggests that the mechanism of ibuprofen ischemic muscle salvage is by inhibition of the neutrophil mediated reperfusion injury.

Blood flow on reperfusion and the metabolic demand of ischemic tissues have not been documented to be altered by ibuprofen treatment.[109] Disturbances in the ratio of thromboxane to prostacyclins have also been suggested as a potential cause of post-ischemic injury.[34] On speculation, cyclooxygenase inhibition by ibuprofen might afford protection against ischemic injury by providing a more favourable prostaglandin environment in the microcirculation.

In summary, ibuprofen treatment dramatically improved skeletal muscle survival in the post-ischemic myocutaneous flap. Failure to improve buttock cutaneous flap survival suggests that the improved skin survival found in ibuprofen treated post-ischemic latissimus dorsi myocutaneous flaps resulted because of a secondary benefit from the improved muscle survival. Ibuprofen treatment is a clinically feasible nonsurgical adjunct in the prevention of skeletal muscle injury resulting from ischemia and reperfusion.

CONCLUSIONS

In ischemic organs such as heart, skeletal muscle, and intestine, the existing literature has largely been supportive of a neutrophil mediated reperfusion injury. Few studies have evaluated this possibility in flaps. In a series of four experiments, this thesis evaluated neutrophil function, localization, depletion, and inhibition in ischemic cutaneous and myocutaneous flaps. The results and hence, conclusions were surprisingly different for cutaneous and myocutaneous flaps.

CUTANEOUS FLAP

Although neutrophil function was not assessed in cutaneous flaps, neutrophil localization did occur on reperfusion of ischemic cutaneous flaps. However, the failure to improve cutaneous flap survival by either depletion or pharmacologic inhibition of the neutrophil provided strong evidence arguing against a neutrophil mediated post-ischemic injury in cutaneous flaps. Thus, the injury in cutaneous flaps precipitated by global ischemia and reperfusion is not mediated by the neutrophil.

MYOCUTANEOUS FLAP

Enhanced function was detected in neutrophils reperfusing ischemic myocutaneous flaps. Neutrophil localization was also demonstrated in post-ischemic myocutaneous flaps. Ischemic myocutaneous flap survival was improved by neutrophil depletion. Pharmacologic inhibition of the neutrophil also improved ischemic myocutaneous flap survival. Cumulatively, the evidence presented in this thesis substantiates the primary hypothesis in myocutaneous flaps. Namely, the neutrophil is an etiologic factor in the pathogenesis of reperfusion injury in globally ischemic myocutaneous flaps.

REFERENCES

1. Angel, M. F., Narayanan, K., Swartz, W. M., et al. The etiologic role of free radicals in hematoma-induced flap necrosis. *Plast. Reconstr. Surg.* 77: 795, 1986.
2. Angel, M. F., Narayanan, K., Swartz, W. M., et al. Deferoxamine increases skin flap survival: additional evidence of free radical involvement in ischaemic flap surgery. *Brit. J. Plast. Surg.* 39: 469, 1986.
3. Avgerinos, A., and Hutt, A. J. High-performance liquid chromatographic determination of ibuprofen in human plasma and urine by direct injection. *J. Chromatogr.* 380: 468, 1986.
4. Babior, B. M. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* 298: 659, 1978.
5. Babior, M. B., Kipnes, R. S., and Curnutte, J. T. Biological defense mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* 52: 741, 1973.
6. Babior, G. L., Rosin, R. E., McMurrich, B. J., et al. Arrangement of the respiratory burst oxidase in the plasma membrane of the neutrophil. *J. Clin. Invest.* 67: 1724, 1981.
7. Belkin, M., LaMorte, W. L., Wright, G., et al. The role of leukocytes in the pathophysiology of skeletal muscle ischemic injury. *J. Vasc. Surg.* 10: 14,1989.
8. Belkin, M., Wright, G., and Hobson, R. W. Iloprost infusion decreases skeletal muscle ischemia-reperfusion injury. *J. Vasc. Surg.* 11: 77,1990.
9. Belzer, F. O., and Southard, J. H. Organ preservation and transplantation. In *Transplantation: Approaches to Graft Rejection*. Alan R. Liss, Inc., 1986, p. 291-303.
10. Belzer, F. O., and Southard, J. H. Principles of solid-organ

preservation by cold storage. *Transplantation* 45: 673, 1988.

11. Boxer, L. A., and Morganroth, M. L. Neutrophil function disorders. *Disease-a-Month* 33: 684, 1987.
12. Brown, J. M., Grosso, M. A., Whitman, G. L., et al. The coincidence of myocardial reperfusion injury and hydrogen peroxide production in the isolated rat heart. *Surgery* 105: 496, 1989.
13. Bulkley, G. B. The role of oxygen free radicals in human disease processes. *Surgery* 94: 47, 1983.
14. Buncke, H. J., and Schulz, W. P. Total ear reimplantation in the rabbit utilising microminiature vascular anastomoses. *Brit. J. Plast. Surg.* 19: 15, 1966.
15. Buser, P. T., Wagner, S., Wu, S. T., et al. Verapamil preserves myocardial performance and energy metabolism in left ventricular hypertrophy following ischemia and reperfusion. *Circulation* 80: 1837, 1989.
16. Chachques, J. C., Fabiani, J. N., Perier, P., et al. Reversibility of muscular ischemia: A histochemical quantification by nitroblue tetrazolium (NTB) test. *Angiology* 36: 493, 1985.
17. Chang, N., and Mathes, S. J. Comparison of the effect of bacterial inoculation in myocutaneous and random-pattern flaps. *Plast. Reconstr. Surg.* 70: 1, 1982.
18. Cheung, J. Y., Bonventre, J. V., Malis, C. D., et al. Calcium and ischemic injury. *N. Engl. J. Med.* 314: 1670, 1986.
19. Clarkson, P. M., MacArthur, B. A., Barratt-Boyes, B. G., et al. Developmental progress after cardiac surgery in infancy using hypothermia and cardiac arrest. *Circulation* 62: 855, 1980.
20. Cohen, H. J., Chovaniec, M. E., and Davies, W. A. Activation of the guinea pig granulocyte NAD(P)H-dependent superoxide generating enzyme: localization in a plasma membrane enriched particle and

kinetics of activation. *Blood* 55: 355, 1980.

21. Colen, S. R., Baker, D. C., and Shaw, W. W. Microvascular flap reconstruction of the head and neck: an . *Clin. Plast. Surg.* 10: 73, 1983.

22. Cotran, R. S., Kumar V., and Robbins, S. L. Cellular injury and adaptation (Ch. 1). In R. S. Cotran, et al (Eds.), *Robbins Pathologic Basis of Disease*, 4th Ed. Philadelphia, W. B. Saunders Company, 1989, p. 1.

23. Crawford, D. R., and Schneider, D. L. Ubiquinone content and respiratory burst activity of latex-filled phagolysosomes isolated from human neutrophils and evidence for the probable involvement of a third granule. *J. Biol. Chem.* 258: 5363, 1983.

24. Dahinden, C. A., Fehr, J., and Hugli, T. E. Role of cell surface contact in the kinetics of superoxide production by granulocytes. *J. Clin. Invest.* 72: 113, 1983.

25. Dalsing, M. C., Grosfeld, J. L., Shiffler, M. A., et al. Superoxide dismutase: a cellular protective enzyme in bowel ischemia. *J. Surg. Res.* 34: 589, 1983.

26. Daniel, R. K., and Kerrigan, C. L. Principles and physiology of skin flap surgery (Ch. 9). In J. G. McCarthy (Ed.), *Plastic and Reconstructive Surgery*, 3rd Ed. Philadelphia, W. B. Saunders Company, 1990, p. 275.

27. Daniel, R. K., and Taylor, G. I. Distant transfer of an island flap by microvascular anastomoses. *Plast. Reconstr. Surg.* 52: 111, 1973.

28. Dickson, W. A., Dickson, M. G., and Roberts, A. H. N. The complications of fasciocutaneous flaps. *Ann. Plast. Surg.* 19: 234, 1987.

29. Douglas, B., Weinbert, W., Song, Y., et al. Beneficial effects of ibuprofen on experimental microvascular free flaps: pharmacologic alteration of the no-reflow phenomenon. *Plast. Reconstr. Surg.* 79: 366,

1987.

30. Dunn, S. P., Gross, K. R., Dalsing, M., et al. Superoxide: a critical oxygen-free radical in ischemic bowel injury. *J. Pediatr. Surg.* 19: 740, 1984.

31. Elsbach, P., and Weiss, J. Phagocytic cells: oxygen-independent antimicrobial systems (Ch. 24). In J. I. Gallin, et al (Eds.), *Inflammation: Basic Principles and Clinical Correlates*. New York, Raven Press, 1988, p. 445.

32. Engler, R. Granulocytes and oxidative injury in myocardial ischemia and reperfusion. *Fed. Proc.* 46: 2395, 1987.

33. Feller, A. M., Roth, A. C., Russel, R. C., et al. Experimental evaluation of oxygen free radical scavengers in the prevention of reperfusion injury to skeletal muscle. *Ann. Plast. Surg.* 22: 321, 1989.

34. Feng, L. J., Berger, B. E., Lysz, T. W., et al. Vasoactive prostaglandins in the impending no-reflow state: evidence for a primary disturbance in microvascular tone. *Plast. Reconstr. Surg.* 81: 755, 1988.

35. Filep, J., Herman, F., Braquet, P., et al. Increased levels of platelet-activating factor in blood following intestinal ischemia in the dog. *Biochem. Biophys. Res. Commun.* 158: 353, 1989.

36. Fletcher, M. P., Seligmann, B. E., and Gallin, J. I. Correlation of human neutrophil secretion, chemoattractant receptor mobilization, and enhanced functional capacity. *J. Immunol.* 128: 941, 1982.

37. Friedl, H. P., Smith, D. J., Thomson, P. D., et al. Ischemia-reperfusion in humans: appearance of xanthine oxidase activity. *Am. J. Pathol.* 136: 491, 1990.

38. Friedl, H. P., Till, G. O., Trentz, O., et al. Roles of histamine, complement and xanthine oxidase in thermal injury of the skin. *Am. J. Pathol.* 135: 203, 1989.

39. Gabig, T. G., and Lefker, B. A. Catalytic properties of the resolved

flavoprotein and cytochrome B components of the NADPH dependent superoxide radical generating oxidase from human neutrophils. *Biochem. Biophys. Res. Commun.* 118: 430, 1984.

40. Gingrass, R. P., Culf, N. K., Garrett, W. S., et al. Complications with the deltopectoral flap. *Plast. Reconstrc. Surg.* 49: 501, 1972.
41. Goldstein, I. M., Roos, D., Kaplan, H. B., et al. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* 56: 1155, 1975.
42. Granger, D. N., Hollwoarth, M. E., and Parks, D. A. Ischemia-reperfusion injury: role of oxygen-derived free radicals. *Acta Physiol. Scand. Suppl.* 548: 47, 1986.
43. Granger, D. N., McCord, J. M., Parks, D. A., et al. Xanthine oxidase inhibitors attenuate ischemia-induced vascular permeability changes in the cat intestine. *Gastroenterology* 90: 80, 1986.
44. Granger, D. N., Rutili, G., and McCord, J. M. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* 81: 22, 1981.
45. Grisham, M. B., and Granger, D. N. Neutrophil-mediated mucosal injury: role of reactive oxygen metabolites. *Digest. Dis. Sci. Suppl.* 33: 6S, 1988.
46. Grisham, M. B., Hernandez, L. A., and Granger, D. N. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. *Am. J. Physiol.* 251: G567, 1986.
47. Harashina, T., and Buncke, H. J. Study of washout solutions for microvascular replantation and transplantation. *Plast. Reconstr. Surg.* 56: 542, 1975.
48. Harrison, D. H., Girling, M., and Mott, G. Methods of assessing the viability of free flap transfer during the postoperative period. *Clin. Plast. Surg.* 10: 21, 1983.
49. Hayes, G., Liauw, S., Romaschin, A., et al. Separation of reperfusion

injury from ischemia-induced necrosis. *Surg. Forum* 39: 306, 1988.

50. Hayhurst, J. W., O'Brien, B. M., Ishida, H., et al. Experimental digital replantation after prolonged cooling. *Hand* 6: 134, 1974.

51. Hearse, D. J., Garlick, P. B., and Humphrey, S. M. Ischemic contracture of the myocardium: mechanisms and prevention. *Am. J. Cardiol.* 39: 986, 1977.

52. Heflin, A. C., and Brigham, K. L. Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. *J. Clin. Invest.* 68: 1253, 1981.

53. Hoshino, T., Maley, W. R., Bulkley, G., et al. Ablation of free radical-mediated reperfusion injury for salvage of kidneys from non-heart beating donors. *Transplantation* 45: 284, 1988.

54. Im, M. J., Hoopes, J. E., Yoshimura, Y., et al. Xanthine: acceptor oxidoreductase activities in ischemic rat skin flaps. *J. Surg. Res.* 46: 230, 1989.

55. Im, M. J., Manson, P. N., Bulkley, G. B., et al. Effects of superoxide dismutase and allopurinol on the survival of acute island skin flaps. *Ann. Surg.* 201: 357. 1985.

56. Im, M. J., Shen, W. H., Pak, C. J., et al. Effect of allopurinol on the survival of hyperemic island skin flaps. *Plast. Reconstr. Surg.* 73: 276, 1984.

57. Jacobson, J. H., and Suarez, E. L. Microsurgery in anastomosis of small vessels. *Surg. Forum* 11: 243, 1960.

58. Jonsson, K., Hunt, T. K., Brennan, S. S., et al. Tissue oxygen measurements in delayed skin flaps: a reconsideration of the mechanisms of the delay phenomenon. *Plast. Reconstr. Surg.* 82: 328, 1988.

59. Kempczinski, R. F., and Rutherford, R. B. Fundamental techniques in vascular surgery (Ch. 23). In R. B. Rutherford (Ed.), *Vascular*

Surgery, 3rd Ed. Philadelphia, W. B. Saunders Company, 1989, p. 341.

60. Kerrigan, C. L. Skin flap failure: pathophysiology. *Plast. Reconstr. Surg.* 72: 766, 1983.

61. Kerrigan, C. L., and Daniel, R. K. Critical ischemia time and the failing skin flap. *Plast. Reconstr. Surg.* 69: 986, 1982.

62. Kerrigan, C. L., and Daniel, R. K. Pharmacologic treatment of the failing skin flap. *Plast. Reconstr. Surg.* 70: 541, 1982.

63. Kerrigan, C. L., Zelt, R. G., Thomson, J. G., et al. The pig as an experimental animal in plastic surgery research for the study of skin flaps, myocutaneous flaps and fasciocutaneous flaps. *Lab. Animal Sci.* 36: 408, 1986.

64. Kirklin, J. K., and Kirklin, J. W. Cardiopulmonary bypass for cardiac surgery (Ch. 32). In D. C. Sabiston, et al. (Eds.), *Surgery of the Chest*, 5th Ed. Philadelphia, W. B. Saunders Company, 1990, p. 1107.

65. Klebanoff, S. J. Oxygen metabolism and the toxic properties of phagocytes. *Ann. Int. Med.* 93: 480, 1980.

66. Klebanoff, S. J. Phagocytic cells: products of oxygen metabolism (Ch. 23). In J. I. Gallin, et al (Eds.), *Inflammation: Basic Principles and Clinical Correlates*. New York, Raven Press, 1988, p. 391.

67. Komatsu, S., and Tamai, S. Successful replantation of a completely cut off thumb. *Plast. Reconstr. Surg.* 42: 374, 1968.

68. Korthuis, R. J., Grisham, M. B., and Granger, D. N. Leukocyte depletion attenuates vascular injury in post-ischemic skeletal muscle. *Am. J. Physiol.* 254: H823, 1988.

69. Krizek, T. J., and Robson, M. C. Potential pitfalls in the use of the deltopectoral flap. *Plast. Reconstr. Surg.* 50: 326, 1972.

70. Kubes, P., Suzuki, M., and Granger, D. N. Platelet-activating factor-induced microvascular dysfunction: role of adherent leukocyte. *Am. J. Physiol.* 258: G158, 1990.

71. Labbe, R., Lindsay, T., Gatley, R., et al. Quantitation of post-ischemic muscle necrosis: Histochemical and radioisotope techniques. *J. Surg. Res.* 44: 45, 1988.

72. Labbe, R., Lindsay, T., and Walker, P. M. The extent and distribution of skeletal muscle necrosis after graded periods of complete ischemia. *J. Vasc. Surg.* 6: 152, 1987.

73. Lidman, D., and Daniel, R. K. Evaluation of clinical microvascular anastomosis - reasons for failure. *Ann. Plast. Surg.* 6: 215, 1981.

74. Linas, S. T., Whittenburg, D., and Repine J. E. Role of xanthine oxidase in ischemia/reperfusion injury. *Am. J. Physiol.* 258: F711, 1990.

75. Litt, M. R., Jeremy, R. W., Weisman, H. F., et al. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. *Circulation* 80: 1816, 1989.

76. Lucchesi, B. R., and Mullane, K. M. Leukocytes and ischemia-induced myocardial injury. *Ann. Rev. Pharmacol. Toxicol.* 26: 201, 1986.

77. Mackie, E. J. Immunosuppressive effects of cyclophosphamide in pigs. *Am. J. Vet. Res.* 42: 189, 1981.

78. Maderazo, E. G., Breaux, S. P., and Woronick, C. L. Inhibition of human polymorphonuclear leukocyte cell responses by ibuprofen. *J. Pharm. Sci.* 73: 1403, 1984.

79. Magliulo, E., Benzi-Cipelli, R., Lago, A., et al. Inhibiting effect of non-steroidal anti-inflammatory drugs on the chemotaxis of human leukocytes in vitro. *Int. J. Clin. Pharmacol.* 15: 417, 1977.

80. Malech, H. L., and Gallin, J. I. Neutrophils in human diseases. *N. Engl. J. Med.* 318: 687, 1987.

81. Manson, P. N., Anthenelli, R. M., Im, M. J., et al. The role of oxygen-free radicals in ischemic tissue injury in island skin flaps. *Ann. Surg.* 198: 87, 1983.

82. Maschio, A. D., Evangelista, V., Rajtar, G., et al. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am. J. Physiol.* 258: H870, 1990.

83. May, J. W., Chait, L. A., O'Brien, B. M., et al. The no-reflow phenomenon in experimental free flaps. *Plast. Reconstr. Surg.* 61: 256, 1978.

84. May, J. W., Gallico, G. G., Jupiter, J., et al. Free latissimus dorsi muscle flap with skin graft for treatment of traumatic chronic bony wounds. *Plast. Reconstr. Surg.* 73: 641, 1984.

85. McAfee, J. G., Subramanian, G., and Gagne, G. Technique of leukocyte harvesting and labelling: problems and perspectives. *Sem. Nucl. Med.* 14: 83, 1984.

86. McCord, J. M. Oxygen derived free radicals in post-ischemic tissue injury. *N. Engl. J. Med.* 312: 159, 1985.

87. McCord, J. M. The superoxide free radical: its biochemistry and pathophysiology. *Surgery* 94: 412, 1983.

88. Morris, S. F., Pang, C. Y., Lindsay, W. K., et al. Pathogenesis of ischemia-induced tissue damage in latissimus dorsi myocutaneous flaps in pigs. *Surg. Forum* 38: 612, 1987.

89. Mullane, K. M., Read, N., Salmon, J. A., et al. Role of leukocytes in acute myocardial infarction in anesthetized dogs: Relationship to myocardial salvage by anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* 228: 510, 1984.

90. Nayler, W. G., Ferrari, R., and Williams, A. Protective effect of pretreatment with verapamil, nifedipine and propranolol on mitochondrial function in the ischemic and reperfused myocardium. *Am. J. Cardiol.* 46: 242, 1980.

91. Neal, T. M., Vissers, M. C. M., and Winterbourn, C. C. Inhibition by nonsteroidal anti-inflammatory drugs of superoxide production and

granule enzyme release by polymorphonuclear leukocytes stimulated with immune complexes or formyl-methionyl-leucyl-phenylalanine. *Biochem. Pharmac.* 36: 2511, 1987.

92. Nordstrom, G., Seeman, T., and Hasselgren, P. O. Beneficial effect of allopurinol in liver ischemia. *Surgery* 97: 679, 1985.

93. Oldham, K. T., Guice, K. S., Ward, P. A., et al. The role of oxygen radicals in immune complex injury. *Free Rad. Biol. Med.* 4: 387, 1988.

94. Pang, C. Y., Forrest, C. R., and Morris, S. F. Pharmacological augmentation of skin flap viability: a hypothesis to mimic the surgical delay phenomenon or a wishful thought. *Ann. Plast. Surg.* 22: 293, 1989.

95. Pang, C. Y., Forrest, C. R., Neligan, P. C., et al. Augmentation of blood flow in delayed random skin flaps in the pig: effect of length of delay period and angiogenesis. *Plast. Reconstr. Surg.* 78: 68, 1986.

96. Park, P. O., Haglund, U., Bulkley, G. B., et al. The sequence of development of intestinal tissue injury after strangulation ischemia and reperfusion. *Surgery* 107: 574, 1990.

97. Parks, D. A., Bulkley, G. B., and Granger, D. N. Role of oxygen free radicals in shock, ischemia, and organ preservation. *Surgery* 94: 428, 1983.

98. Perianin, A. Giroud, J. P., and Hakim, J. Differential in vivo effects of indomethacin, ibuprofen, and flurbiprofen on oxygen-dependent killing activities of neutrophils elicited by acute nonimmune inflammation in the rat. *Inflammation* 12: 181, 1988.

99. Picard-Ami, L. A., MacKay, A., and Kerrigan, C. L. Effect of allopurinol on the survival of pig and rat experimental flap. Presented at the *43rd annual meeting of the Canadian Society of Plastic Surgery of Canada (GAM)*, 1989.

100. Picard-Ami, L. A., MacKay, A., and Kerrigan, C. L. Pathophysiology

of ischemic skin flaps: differences in xanthine oxidase activity levels between rat, pig, and man. *Plast. Reconstr. Surg.* In press.

101. Picard-Ami, L. A., Thomson, J. G., and Kerrigan, C. L. Critical ischemia times and survival patterns of experimental pig flaps. *Plast. Reconstr. Surg.* In press.

102. Pick, E., and Mizel, D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automated enzyme immunoassay reader. *J. Immun. Methods* 46: 211, 1981.

103. Potter, R. F., and Groom, A. C. Capillary diameter and geometry in cardiac and skeletal muscle studied by means of corrosion casts. *Microvasc. Res.* 25: 68, 1983.

104. Ratych, R. E., Chuknyiska, R. S., and Bulkley, G. B. The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery* 102: 122, 1987.

105. Razaboni, R., and Shaw, W. W. Preservation of tissue for transplantation and replantation. *Clin. Plast. Surg.* 10: 211, 1983.

106. Reimer, K. A., and Jennings, R. B. Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. *Circulation* 71: 1069, 1985.

107. Reimer, K. A., Lowe, J. E., and Jennings. R. B. Effect of the calcium antagonist verapamil on necrosis following temporary coronary artery occlusion in dogs. *Circulation* 55: 581, 1977.

108. Richard, V. J., Brooks, S. E., Jennings, R. B., et al. Effect of a critical coronary stenosis on myocardial neutrophil accumulation during ischemia and early reperfusion in dogs. *Circulation* 80: 1805, 1989.

109. Romson, J. L., Bush, L. R., Jolly. S. R., et al. Cardioprotective effects of ibuprofen in experimental and regional and global myocardial ischemia. *J. Cardiovac. Pharm.* 4: 187, 1982.

110. Romson, J. L., Hook, B. G., Kunkel, S. L., et al. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 67: 1016, 1983.

111. Romson, J. L., Hook, B. G., Rigot, V. H., et al. The effect of ibuprofen on accumulation of indium-111-labelled platelets and leukocytes in experimental myocardial infarction. *Circulation* 66: 1002, 1982.

112. Root, R. K., and Metcalf, J. A. H_2O_2 release from human granulocytes during phagocytosis: Relationship to superoxide anion formation and cellular catabolism of H_2O_2: Studies with normal and cytochalasin B-treated cells. *J. Clin. Invest.* 60: 1266, 1977.

113. Roth, A. C., Russell, R. C., Suchy, H., et al. Reperfusion injury in the microcirculation of skeletal muscle after ischemia: the movie. Presented at the *annual meeting of the Plastic Surgery Research Council*, San Francisco, May 1988.

114. Rubin, B., Smith, A., Liauw, S., et al. Skeletal muscle ischemia stimulates an immune system-mediated injury. *Surg. Forum* 40: 297, 1989.

115. Schraufstatter, I., Hyslop, P. A., Jackson, J. H., et al. Oxidant-induced DNA damage of target cells. *J. Clin. Invest.* 82: 1040, 1988.

116. Serafin, D., Rios, A. V., and Georgiade, N. Fourteen free groin flap transfers. *Plast. Reconstr. Surg.* 57: 707, 1976.

117. Shah, A., and Jung, D. Improved high-performance liquid chromatographic assay of ibuprofen in plasma. *J. Chromatogr.* 344: 408, 1985.

118. Shaw, W. W. Microvascular free flaps: the first decade. *Clin. Plast. Surg.* 10: 3, 1983.

119. Simpson, P. J., Mickelson, J., Fantone, J. C., et al. Iloprost inhibits neutrophil function in vitro and in vivo and limits experimental infarct

size in canine heart. *Circ. Res.* 60: 666, 1987.

120. Simpson, P. J., Mickelson, J., and Lucchesi, B. R. Free radical scavengers in myocardial ischemia. *Fed. Proc.* 46: 2413, 1987.

121. Southorn, P. A., and Powis, G. Free radicals in medicine I. *Mayo Clin. Proc.* 63: 381, 1988.

122. Southorn, P. A., and Powis, G. Free radicals in medicine II. *Mayo Clin. Proc.* 63: 390, 1988.

123. Stewart, J. R., Crute, S. L., Loughlin, et al. Prevention of free radical-induced myocardial reperfusion injury with allopurinol. *J. Thorac. Cardiovasc. Surg.* 90: 68, 1985.

124. Stranc, M. F., Labandter, H., and Roy, A. A review of 196 tubed pedicles. *Brit. J. Plast. Surg.* 28: 54, 1975.

125. Suzuki, S., Isshiki, N., Ohtsuka, M., et al. Experimental study on delay phenomenon in relation to flap width and ischaemia. *Brit. J. Plast. Surg.* 41: 389, 1988.

126. Takami, H., Matsuda, H., Hirose, H., et al. Myocardial energy metabolism in preserved heart: comparison of simple storage and hypothermic perfusion. *J. Heart. Transplant.* 7: 205, 1988.

127. Tamai, S. Digit replantation: analysis of 163 replantations in an 11 year period. *Clin. Plast. Surg.* 5: 195, 1978.

128. Taylor, G. I. The current status of free vascularized bone grafts. *Clin. Plast. Surg.* 10: 185, 1983.

129. Taylor, A. E., Martin, D., and Parker, J. C. The effects of oxygen radicals on pulmonary edema formation. *Surgery* 94: 433, 1983.

130. Thomson, J. G., and Kerrigan, C. L. Dermofluorometry: Thresholds for predicting flap survival. *Plast. Reconstr. Surg.* 83: 859, 1989

131. Till, G. O., Guilds, L. S., Mahrougui, M., et al. Role of xanthine oxidase in thermal injury of skin. *Am. J. Pathol.* 135: 195, 1989.

132. Uraizee, A., Reimer, K. A., Murry, C. E., et al. Failure of superoxide

dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs. *Circulation* 75: 1237, 1987.

133. Varani, J., Ginsberg, I., Schuger, L., et al. Endothelial cell killing by neutrophils: synergistic interaction of oxygen products and proteases. *Am. J. Pathol.* 135: 435, 1989.

134. Vasko, K. A., DeWall, R. A., and Riley, A. M. Effect of allopurinol in renal ischemia. *Surgery* 71: 787, 1972.

135. Venezio, F. R., DiVincenzo, C., Pearlman, F., et al. Effects of the newer nonsteroidal anti-inflammatory agents, ibuprofen, fenoprofen, and sulindac, on neutrophil adherence. *J. Infect. Dis.* 152: 690, 1985.

136. Walker, P. M., Lindsay, T. F., Labbe, R., et al. Salvage of skeletal muscle with free radical scavengers. *J. Vasc. Surg.* 5: 68, 1987.

137. Ward, P. A., Sulavik, M. C., and Johnson, K. J. Rat neutrophil activation and effects of lypoxygenase and cyclooxygenase inhibitors. *Am. J. Pathol.* 116: 223, 1984.

138. Weiblen, B. J., Forstrom, L., and McCullough, J. Studies of the kinetics of indium-111-labelled granulocytes. *J. Lab. Clin. Med.* 94: 246, 1979.

139. Weiss, A. P., Moore, J. R., Randolph, M. A. Preventing oxygen free-radical injury in ischemic revascularized bone grafts. *Plast. Reconstr. Surg.* 82: 486, 1988.

140. Weiss, S. J. Tissue destruction by neutrophils. *N. Engl. J. Med.* 320: 365, 1989.

141. Weissmann, G. Inflammation: historical perspective (Ch. 1). In J. I. Gallin, et al (Eds.), *Inflammation: Basic Principles and Clinical Correlates*. New York, Raven Press, 1988, p. 5.

142. Werns, S. W., and Lucchesi, B. R. Leukocytes, oxygen radicals, and myocardial injury due to ischemia and reperfusion. *Free Rad. Biol.*

Med. 4: 31, 1988.

143. Westlin, W., and Mullane, K. M. Alleviation of myocardial stunning by leukocyte and platelet depletion. *Circulation* 80: 1828, 1989.

144. Wicomb, W. N. Comparison of cardioplegic and UW solutions for short-term rabbit heart preservation. *Transplantation* 47: 733, 1989.

145. Wicomb, W. N., and Collins, G. M. 24-hour rabbit heart storage with UW solution. *Transplantation* 48: 6, 1989.

146. Wicomb, W. N., Collins, G. M., Wood, J., et al. Improved cardioplegia using new perfusates. *Transplant. Proc.* 21: 1357, 1989.

147. Wolfe, L. S. Eicosanoids: Prostaglandins, thromboxanes, leukotrienes, and other derivatives of carbon-20 unsaturated fatty acids. *J. Neurochem.* 38: 1, 1982.

148. Wright, J. G., Kerr, J. C., Valeri, C. R., et al. Regional hypothermia protects against ischemia-reperfusion injury in canine gracilis muscle. *J. Trauma* 28: 1026, 1988.

149. Yoon, J. O., Im, M. J., Manson, P. N., et al. The role of metal ions in ischemia/reperfusion injury in skin flaps. *J. Surg. Res.* 46: 163, 1989.

150. Zelt, R. G., Olding, M., Kerrigan, C. L., et al. Primary and secondary critical ischemia times of myocutaneous flaps. *Plast. Reconstr. Surg.* 78: 498, 1986.