

**THE ROLE OF THE XANTHINE OXIDASE ENZYMATIC
SYSTEM AND ALLOPURINOL IN THE ISCHEMIA
REPERFUSION INJURY OF EXPERIMENTAL SKIN AND
MYOCUTANEOUS FLAPS**

by

Luis A. Picard-Ami, Jr, MD

Division of Surgical Research

**Department of Surgery, McGill University,
Montreal, Quebec, Canada. March, 1991.**

**A Thesis submitted to Faculty of Graduate Studies and
Research in partial fulfilment of the requirements
for the degree of Master of Sciences.**

c. Luis A. Picard-Ami, Jr, MD, March, 1991.

ABSTRACT

Complications resulting in flap failure and tissue necrosis constitute a serious and frequent problem to the plastic surgeon. Oxygen derived free radicals have been implicated in a variety of pathological processes including the ischemia reperfusion injury (IRI) occurring in skin flaps. Previous work with experimental rat skin flaps has suggested that the xanthine oxidase (XO) enzymatic system may be the major source for these toxic radicals. Before the clinician and the patient can benefit from these experimental findings an animal model which closer resembles the clinical setting needs to be tested. In the laboratory I examined the role of XO and its potent inhibitor allopurinol in the IRI of skin and myocutaneous flaps. I have found negligible levels of XO enzyme in pig and human skin when compared to the rat. I have also found that no beneficial effect on survival could be observed by treating the flaps with several different dose regimens of allopurinol. Based on my results I conclude that it is unlikely that xanthine oxidase is of major importance in the IRI of skin flaps.

RÉSUMÉ

Les complications qui mènent aux échecs de lambeaux et ensuite à la nécrose de leurs tissus représentent un problème sérieux et assez fréquent pour le plasticien d'aujourd'hui. Les radicaux libre dérivés de l'oxygène ont été identifiés aux tissus moux suite à la reperfusion sanguine qui a lieu au niveaux de certains lambeaux cutanés. Ceci a déjà été suggéré au cours de certaines expériences antérieures, dont la groupe enzymatique du xanthine oxidase (XO) serait responsable, en grande partie, pour la production de ces radicaux toxiques. Cependant, avant que le médecin et son patient puissent profiter des trouvailles expérimentelles mentionnées ci-haut, je propose de faire l'examen clinique d'un modèle animal qui ressemble les conditions du patient. Mes expériences au laboratoire décrivent l'effet clinique qu'a l'allopurinol (inhibiteur connu du XO); en particulier j'ai examiné l'effet que ceux-ci ont aux dommages causés aux tissus moux lors de la reperfusion sanguine à laquelle sont sujets les lambeaux cutanés et myocutanés. J'ai trouvé de niveaux négligeable de XO dans les tissus humains ainsi que ceux du porc, lorsque comparé à ceux d'un rat. De même, J'ai aussi trouvé aucun effet thérapeutique démontré par l'Allopurinol à la survie de lambeaux divers qui avaient été traité avec celui-ci. D'après les résultats de mon expérience, je peux conclure que le XO n'occupe pas un rôle important au dommages

causés aux tissus moux lors de la reperfusion sanguine qui a lieu au niveau de certaines lambeaux cutanés.

ACKNOWLEDGEMENTS

I would like to thank everyone who in one way or another contributed to the successful completion of this work. To Dr. Carolyn L. Kerrigan, my thesis supervisor, a very special acknowledgement for introducing me to the world of research, for her invaluable help throughout the project, her endless availability, patient guidance, words of wisdom and most of all for her extraordinary characteristic of teaching excellence by example. To her I am forever grateful.

For their contribution to the experiments, I thank: Andrew MacKay, B.A., for his immense patience and invaluable technical assistance; Dr. and Mrs. Michael Im, Division of Plastic Surgery, Johns Hopkins University School of Medicine, for their altruism in sharing the xanthine oxidase and malonyldialdehyde assays; Peter Cernacek, Ph.D., Division of Biochemistry and Dr. E.W. Quillen, Ph.D. Department of Obstetrics and Gynaecology both at the Royal Victoria Hospital, McGill University, for their technical advice regarding biochemical methods and radioisotopes, respectively, and generosity with their equipment.

I wish to thank Gina Bravo., Ph.D., Department of Epidemiology and Biostatistics, McGill University for her constructive criticisms and recommendations in the statistical analysis of all experiments. I also like to thank Mrs. Genevieve Holding for her help with the typing of the manuscripts

from these experiments for publication. Last, but not least, I would like to thank my dear friends, Dr. and Mrs. Juan and Christel Segovia for their help in the translation of the Abstract of this work into the french.

Special thanks to my wife, Lita, for proofreading and assembling the separate manuscripts, as well as for the heavy task of typing this thesis. To her, and my daughter Alexandra, I am forever indebted; without their infinite love, understanding and constant support, I could have never finished.

TABLE OF CONTENTS

ABSTRACT	i
RÉSUMÉ	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vi
PREFACE	1
INTRODUCTION	3
THE PROBLEM	4
HISTORICAL BACKGROUND	4
CHAPTER I	
CRITICAL ISCHEMIA TIMES AND SURVIVAL PATTERNS OF EXPERIMENTAL SKIN FLAPS	11
ABSTRACT	12
BACKGROUND AND PURPOSE	12
MATERIALS AND METHODS	14
Experiment #1 (Critical Ischemia Time)	14
Experiment #2 (Survival Patterns Following Ischemia)	15
Statistical Analysis	16
RESULTS	16
Critical Ischemia Time	18
Survival Patterns	18
DISCUSSION AND CONCLUSIONS	20

CHAPTER II
PATHOPHYSIOLOGY OF ISCHEMIC SKIN FLAPS: DIFFERENCES
IN XANTHINE OXIDASE LEVELS BETWEEN RAT, PIG AND HUMAN
SKIN

	23
ABSTRACT	24
BACKGROUND AND PURPOSE	25
MATERIALS AND METHODS	26
Rats	26
Pigs	27
Humans	28
Xanthine Oxidase Determination	28
Statistical Analysis	29
RESULTS	30
DISCUSSION AND CONCLUSIONS	32

CHAPTER III
EFFECT OF ALLOPURINOL ON THE SURVIVAL OF PIG AND
RAT EXPERIMENTAL FLAPS

	37
ABSTRACT	38
BACKGROUND AND PURPOSE	38
EXPERIMENT #1	40
Materials and Methods	40
Skin Flap Survival	41
Malonyldialdehyde (MDA) Determination	42
MDA Assay	43
Blood Flow	44
Results	45
Skin Survival	45
MDA	46
Blood Flow	48
EXPERIMENT #2	50
Materials and Methods	50
Results	51
EXPERIMENT #3	53
Materials and Methods	53
Results	54
DISCUSSION AND CONCLUSIONS	56

CONCLUSIONS

viii

REFERENCES

62

66

PREFACE

This research project was carried out at the Microsurgical Research Laboratories, Division of Plastic Surgery, Royal Victoria Hospital and McGill University, Montreal, Canada and was supported by the Medical Research Council of Canada # MA 7240.

Chapters 1 (Critical Ischemia Times and Survival Patterns of Experimental Flaps) has been published and Chapter 2 (Pathophysiology of Ischemic Skin Flaps: Differences in Xanthine Oxidase Levels Between Rat, Pig and Man), has been accepted for publication by the Journal of Plastic and Reconstructive Surgery, Williams and Wilkins, Baltimore, Maryland 21202. Chapter 3 has been submitted in part to the same journal for consideration of publication. Materials from chapters 1, 2 and 3 have been presented in Part at one of the following meetings: Annual Meeting of the Association of Plastic Surgeons of Quebec, February 1989, L'Esterel, Quebec, Canada; 43rd Annual Meeting of the Canadian Society of Plastic Surgeons: Groupe pour l'avancement de la Microchirurgie (GAM CANADA) May 31, 1989, Edmonton, Alberta, Canada; 34th Annual Meeting of the Plastic Surgery Research Council, May 21, 1989, Atlanta, Georgia, USA; 5th Annual Meeting of the American Society of Reconstructive Microsurgery and the 44th Annual Meeting of the American Society for Surgery of the Hand, September 12 and 13-16, 1989, respectively, Seattle, Washington, USA; 45th

Annual Session of the Forum on Fundamental Surgical Problems, Surgical Forum Plastic Surgery Section, October 17, 1989, Atlanta, Georgia, USA.

J. Grant Thomson, MD contributed to chapter 1 by performing the operations in experiment # 1; Andrew MacKay, BA assisted to all experiments as a laboratory technician. Dr. and Mrs. Michael Im, Division of Plastic Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA described and taught me the xanthine oxidase and malonyldialdehyde biochemical assays used in Chapters 2 and 3 respectively. Peter Cernacek, Ph.D., Division of Biochemistry, Dr. C. Gagnon, M.D., Division of Urology and Dr. E.W. Quillen, Ph.D. Department of Obstetrics and Gynaecology, Royal Victoria Hospital, McGill University, provided technical advice and equipment for the biochemical assays and measurements of the radioactive labelled tissue samples in chapters 2 and 3. Gina Bravo, Ph.D., Department of Epidemiology and Biostatistics, McGill University provided advice in statistical matters for all three chapters. Mr. R. Ippersiel, Wellcome Burroughs, Montreal, Canada, supplied the allopurinol powder for experiment #2 in the third chapter.

INTRODUCTION

THE PROBLEM

Flap coverage of difficult soft tissue defects secondary to congenital, traumatic, post surgical or radiation therapy has revolutionized wound management. However, flap surgery is not devoid of complications. Complications resulting in total or partial flap necrosis continue to plague the surgeon with a disturbing frequency. The incidence of minor necrosis may approach 30% and major necrosis ranges in the order of 10-15%. While total flap necrosis results in complete failure of the reconstructive procedure, partial flap necrosis occurs almost invariably in that portion of the flap that we depend on the most for coverage resulting in increased patient morbidity with the subsequent delay in his/her return to a useful life in society. Added to this human cost is the financial cost of prolonged utilization of the health resources as well as work days lost. Despite many advances in flap surgery the clinician has no reliable way to salvage a failing flap. A better understanding of the pathophysiological mechanisms leading to tissue injury and flap necrosis is needed.

HISTORICAL BACKGROUND

Since several choices often exist for management of an open wound, it is the job of the surgeon to select the optimal method, that is, the one that

can close the defect with the least risk of morbidity or mortality for the patient in the most expedient manner. In the organized thinking of the reconstructive surgeon a hierarchy of closure techniques based on operative complexity is utilized. Escalating the reconstructive ladder the surgeon can close the wound primarily, allow it to heal through secondary intention or close it using either skin grafts or flaps. A skin flap by definition consists of skin and subcutaneous tissue that can be moved from one part of the body to another with a vascular pedicle or attachment to the body being maintained for its nourishment.

Until the late 1960's, to avoid flap necrosis, the design of skin flaps was governed by poorly understood rules of set length-to-width ratios varying from 5:1 in the face to 1:1 in the lower extremities. These rules evolved over many years of clever and often painful clinical observations. Through the early years of reconstructive surgery they became dogma for the plastic surgeon. It was not until the 1970's with the work of investigators such as Milton,⁴² Daniel¹¹⁻¹³ and Daniel and Williams¹⁶ that some important principles of flap surgery became established. Their work showed that increasing the width of the flap did not improve the surviving length but, rather, it was the the size of the blood vessels incorporated into it that dictated the surviving length of a particular flap design.

In order that the surgeon could fully liberate himself from the constraints of set ratios, a better knowledge of the vascular anatomy and

territories was necessary. Daniel and Williams^{11-13,16} defined the blood supply of the skin as being of two types, either musculocutaneous or septocutaneous, previously defined as direct cutaneous. Musculocutaneous arteries are branches off the major vessels that supply the muscle and then continue into the overlying skin in a perpendicular fashion; musculocutaneous and random flaps are examples of this type of flap. Septocutaneous arteries arise from either segmental or muscular vessels passing through the fascial septa between muscles to supply both the enveloping fascia and the overlying skin, providing numerous parallel side branches in a Christmas tree fashion; the fasciocutaneous and arterial flaps are examples of this type of flaps. Flaps designed on septocutaneous vessels are of greater surgical importance compared to ones designed on musculocutaneous because of their larger sizes, more dependable blood supply and greater freedom in their method of movement. With this knowledge on hand, the number and variety of available flaps grew quickly and the list continues to increase every day, with descriptions of flaps including skin, skin and fascia, and skin fascia and muscle. As well, osteocutaneous and sensory flaps and other highly specialized flaps are being created for closure of complex defects.

Flap surgery was revolutionized again in 1973 with Daniel and Taylor's¹⁵ report of the first successful transfer of a free flap by microvascular anastomosis. Flaps can now be classified by their type of

blood supply, composition and method of movement. Certainly a better knowledge of vascular anatomy has increased the surgeon's options for successful coverage of difficult defects.

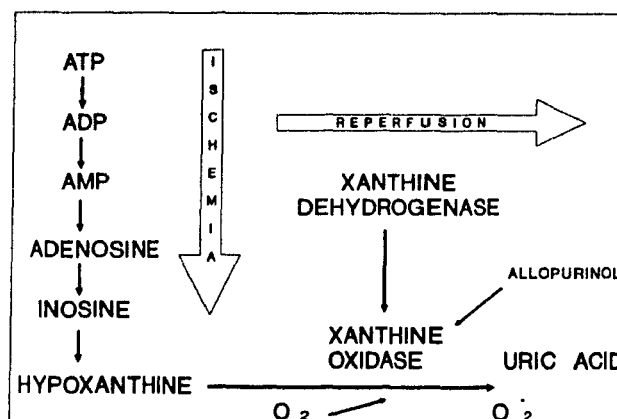
Despite great advances in the understanding of the vascular anatomy of flaps little is known about the events leading to flap failure. Although numerous methods have been attempted to improve flap survival, including some with anecdotal success, the clinician has no reliable means to salvage the failing flap. If complications are to be minimized a better understanding of the pathophysiologic mechanisms involved in flap failure is needed. The factors contributing to unsuccessful outcomes can be divided into preoperative, intraoperative and postoperative.¹⁴ Preoperative causes include factors such as improper flap choice, underestimation of the recipient's defect requirements or an inadequate evaluation of any pre morbid conditions of the patient. Intraoperative factors include technical problems, design errors, poor judgement or choice of recipient vessels in cases of free tissue transfers. Postoperative causes of flap failure can be further subdivided into extrinsic and intrinsic. Extrinsic causes include kinking or pressure on the pedicle, infection and vascular thrombosis. The only recognized intrinsic cause for flap failure is insufficient blood flow with inadequate delivery of nutrients and removal of toxic products at the cellular level. Although most perioperative as well as extrinsic causes can either be prevented or treated, the clinician has no way to reverse intrinsic failure and

salvage a failing flap once the process has begun. Despite perfect planning and execution of the surgery, there are still a small number of flap losses in which no obvious cause can be found.

There is a rapid growing body of evidence implicating oxygen derived free radicals to a variety of diseases and pathological processes^{9-10,23-24,39-40}, including the ischemia reperfusion injury. Several mechanisms have been described showing how these highly reactive and toxic radicals can injure tissue by themselves or can participate in a series of chain reactions resulting in metabolic aberrations which ultimately result in tissue death.

Despite general agreement that these radicals can be responsible for an important component of the injury, the exact source of their origin remains poorly defined. Five major biological sources for these radicals have been identified^{10,38-40}: a) the endothelium related xanthine oxidase enzymatic system; b) the activated neutrophil membrane related NADP/NADPH system; c) the disrupted mitochondrial electron transport system; d) the arachidonic acid pathway; and, e) the oxidation of catecholamines. The predominant source and mechanisms of injury vary not only with the biologic model but also with the conditions of the system.

Figure 1: Proposed mechanism of action for the xanthine oxidase enzymatic system in oxygen derived free radical generation during the ischemia reperfusion injury.



Recent laboratory studies, using skin flap ischemia and reperfusion models in the rat, have suggested that the xanthine oxidase enzymatic system may be a major source for generation of these radicals^{3-5,27-30,37} (Figure 1). From the results obtained with the rat, it has been suggested that the same mechanisms might be functioning in other higher species, specifically man. However, it should be recognized that the anatomical and physiologic characteristics of the loose skin rodents are dissimilar to that of humans, and for these reasons fixed skin animals such as the pig are considered superior models. In addition, experimental evidence is available to indicate that the presence of XO in the heart is species specific, and this may also be true in the skin.

In an effort to better understand the role of the xanthine oxidase enzymatic system in an animal model which more closely resembles the clinical setting a series of experiments were designed and divided into three

phases. In the first phase of the project a previously developed experimental pig model that permits simultaneous skin and myocutaneous flap design was selected. In these flaps a dose response effect on standardized ischemic insults was obtained in order to study and compare their survival patterns and overall tolerance to ischemia. In the second phase the ischemia reperfusion injury concept was introduced, and measurements of the enzyme xanthine oxidase in skin were compared between the rat, pig and man at varying intervals of ischemia and reperfusion. In the third and last part of the project the role of the enzyme xanthine oxidase on survival of skin and myocutaneous flaps was further tested, now indirectly, through pharmacologic manipulation with a potent enzyme inhibitor: allopurinol. An attempt was made to correlate measurements of free radical metabolism and blood flow with eventual flap survival. Based on our findings as well as a review of the literature a series of conclusions are drawn and other potential sources of free radicals are briefly discussed.

CHAPTER I

CRITICAL ISCHEMIA TIMES AND SURVIVAL PATTERNS OF EXPERIMENTAL SKIN FLAPS

ABSTRACT

Previous work on critical ischemia time suggested 1) a greater susceptibility of myocutaneous flaps over skin flaps to the ischemia reperfusion injury, and 2) that duration of ischemia may affect the survival area of a flap. Using a pig model the critical ischemia times and survival patterns of the buttock skin (BS, n=85) and latissimus dorsi myocutaneous (LDMC, n=88) island flaps were determined after being submitted to 0, 2, 4, 6, 8, 10, 12, 14, or 16 hours of normothermic ischemia. The average critical ischemia times (CIT₅₀) were determined to be 9 and 10 hours for the BS and LDMC flaps respectively. Percentage of total area surviving (% TAS) in those flaps that did survive was adversely affected by increases in the ischemic interval in both flap models. A statistically significant decrease in % TAS was found after 6 and 8 hours of ischemia for the BS and LDMC flaps respectively.

BACKGROUND AND PURPOSE

If patient morbidity from flap failure is to be minimized an improved understanding of flap hemodynamics and response to ischemia is needed. Previous studies on critical ischemia time (CIT) in myocutaneous flaps⁶⁸ and

skin flaps³¹⁻³² suggested a possible differential response to the ischemic insult as measured by two parameters. First, the primary CIT of latissimus dorsi myocutaneous flaps was 9 hours and that of flank skin flaps was 13 hours. Although not statistically comparable, these data suggested that skin flaps could tolerate a longer ischemic insult than myocutaneous flaps, at least in these initial porcine models. Second, when the survival length of viable flaps was assessed, there appeared to be an inverse relationship between duration of ischemia and length of flap survival. This had not been observed in the flank flap model, where an all or non type of response was seen. A recent case report of prolonged ischemia in a free muscle flap also experienced distal necrosis in a flap design that usually demonstrates full survival.⁶¹

Is there a true difference between skin flaps and myocutaneous flaps in their tolerance to ischemia as measured by critical ischemia time (CIT) and the percent of total area surviving (% TAS)? Using a pig model, buttock skin (BS) and latissimus dorsi myocutaneous (LDMC) flaps were studied to address this question. The buttock flap was chosen in contrast to the flank flap as the latter contains panniculus carnosus, whereas the former does not.

MATERIALS AND METHODS

White Landrace female pigs weighing 20-25 Kg were sedated with intramuscular ketamine (20mg/kg) and xylazine (2mg/kg). Anaesthesia was induced using intravenous sodium thiopental (20-40mg/kg) and maintained by spontaneous inhalation of a mixture of oxygen (4l/min), nitrous oxide (2l/min) and halothane (0.5-1.0%). Normal saline was administered at 80 ml/hr during the operative procedure. Postoperative pig chow and water were offered ad libitum.

Experiment #1 (Critical Ischemia Time)

On each of 27 pigs bilateral LDMC (10 x 20 cm, n= 54) and BS (10 x 18 cm, n=54) island flaps were elevated. Their sizes ensured a distal zone of necrosis.³³ Through separate axillary and groin incisions the neurovascular pedicle of each flap was identified, the nerves were sectioned and the vascular pedicles skeletonized. The latissimus dorsi tendinous insertion was cut and later resutured to prevent pedicle avulsion. Microvascular clamps (Acland V2 or V3) were applied to the arterial and venous pedicles. Ischemia intervals of 0, 2, 4, 6, 8, 10, 12, 14, or 16 hours were randomly assigned to the flaps with 6 LDMC and 6 BS flaps in each

group. The animals were maintained under anaesthesia until the removal of the last clamp. Dermofluorometry indexes (DFI)⁶² were calculated at four time intervals to confirm 1) flap perfusion prior to clamp application, 2) absence of perfusion after clamp application, 3) persistency of ischemia prior to clamp removal at the end of the ischemic interval, and 4) reflow after clamp removal. Postoperatively, the skin of the flaps were observed daily for signs of viability, necrosis and infection. At 7 days, it was noted whether the flap was completely necrotic or had some area of viability. All flaps but one succumbed following 10 hours of ischemia, thus, a second experiment was designed to study the survival patterns in those flaps subjected to 0-10 hours of ischemia.

Experiment #2 (Survival Patterns Following Ischemia)

Enough animals were operated to ensure at least seven surviving flaps of each type at each ischemic interval. Fifty-six LDMC and 56 BS flaps were elevated and made ischemic for 0, 4, 6, 8 or 10 hours. Flap design, operative technique and DFI measurements were carried out as in experiment # 1. After 7 days the animals were sacrificed and if any portion of the skin of the flap had survived the survival necrosis interface was traced on clear plastic. With the aid of a digitizer and computer software (Sigmascan) the % TAS of the skin as well as the surviving length in cm (average of 4 measurements taken at 2.5 cm intervals) was calculated. Two questions were addressed for

each flap. 1) Is the flap completely necrotic or not? These data were used in combination with Experiment 1 to determine the CIT. 2) If viable, what is the survival length in cm and what is % TAS? These data were used to determine the effect of global ischemia on survival patterns.

Statistical Analysis

The method of probit analysis was utilized to study the data on critical ischemia. Probability of total necrosis was calculated from the actual observed total necrosis rates with 95% confidence limits. Data on survival pattern is reported as mean $\pm$ SEM for a) % TAS, and b) survival length in cm, at each ischemic interval. The effect of ischemia time on % TAS was evaluated using regression analysis and one way analysis of variance. Multiple comparisons between survival means for the different ischemia times within one flap model were possible using the Student-Newman-Keuls method⁵⁹. A p value of less than 0.05 was considered significant.

RESULTS

A total of 220 flaps were constructed. Forty-seven flaps were eliminated because of pedicle injury during dissection (n = 11), DFI evidence of incomplete ischemia (n = 8) due to clamp malfunction, absence of a distal

zone of necrosis (n=2), no reflow (n=10) after clamp removal, infection (n=7) or animal death (n=9) before seven days. This left a total of 173 flaps eligible for calculation of CIT.

In all control flaps the distal zone of impending skin necrosis was evident by a purple discoloration soon after flap elevation. At the end of the ischemic period all but ten flaps demonstrated DFI evidence of reflow, although in general the LDMC flaps would take longer to reperfuse as evidenced subjectively by reactive hyperaemia and objectively by positive DFI. Three LDMC flaps took longer than 60 min after clamp removal to reperfuse (two of these flaps eventually survived). Reactive hyperaemia was subjectively observed to be more intense and spread over a smaller area as the ischemic interval increased. Postoperatively, three different patterns were observed. 1) Viable Flaps: proximal zone of skin survival and a distal zone of skin necrosis (n=82). Three of these flaps were associated with large seromas. 2) Early Death: initially proximal zone of reactive hyperaemia followed later by progressive mottled purple discoloration of the whole flap and cessation of bleeding from stab wounds within the first 30 minutes of reperfusion (n=43). 3) Late Death: a similar pattern to early death but occurring between 12 and 36 hours (n=4) and 36 and 60 hours (n=7) after reperfusion. This latter pattern was associated with a flap haematoma in one case and delayed reflow (1 hr) in one case.

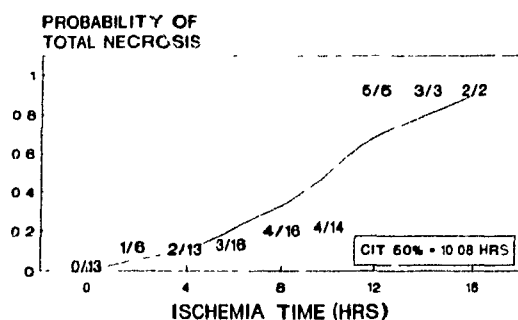
Critical Ischemia Time

From the observed skin necrosis rates and the probit derived curves (Figures 1 & 2) it is predicted that 50% (CIT_{50}) of the LDMC and BS flaps would become totally necrotic at 10 and 9 hours of ischemia respectively. Comparison of the two probit derived curves failed to show a significant difference.

Survival Patterns

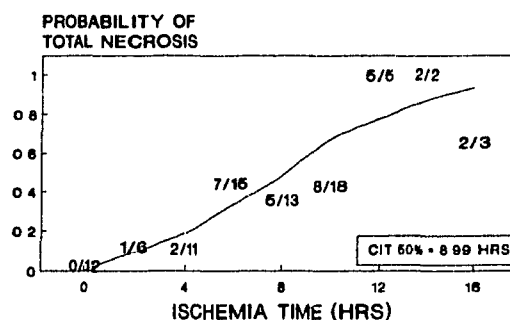
Patterns of survival as measured by length and % TAS (Figures 3a & 3b) yielded similar trends and conclusions. After 60 hours of reflow the survival necrotic interface was stable, well demarcated and correlated well with eventual survival at 7 days. Regression analysis for % TAS and ischemia time showed an inverse relationship with correlation coefficients of $r = -0.70$ and $r = -0.72$ for the LDMC and BS flaps respectively ($p < .001$). When means for the different ischemic intervals were compared by ANOVA they were different ($p < .001$) for both flap models. When the Newman-Keuls method was used for paired comparisons between mean % TAS at different ischemic intervals, statistically significant differences were found for the following time interval pairs: a) in the buttock flap 0/10, 4/10 ($p < .001$); 0/8, 4/8 ($p < .05$) for % TAS and 0/8, 0/10, 4/10 ($p < .001$); 0/6, 4/8 ($p < .05$) for length; b) in the latissimus dorsi 0/8, 0/10, 4/10, 6/10 ($p < .001$) for % TAS and 0/10, 4/10, 6/10 ($p < .001$); 0/8 ($p > .05$) for length. Thus a significant

CRITICAL ISCHEMIA TIME LATISSIMUS DORSI FLAP



1

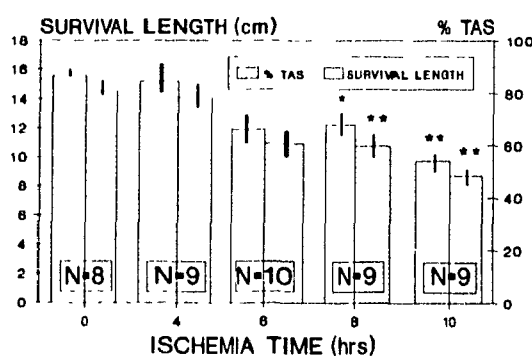
CRITICAL ISCHEMIA TIME BUTTOCK FLAPS



2

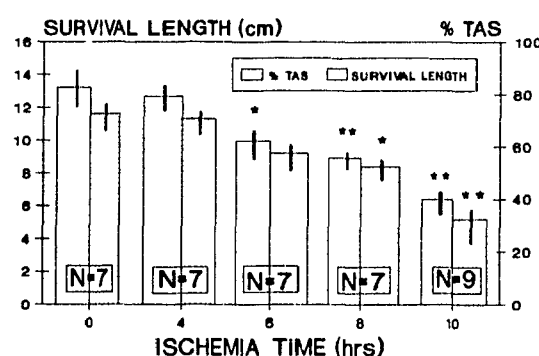
Figures 1 and 2: Observed necrosis rates (fraction) and probit derived curve for probability of total skin necrosis for the Latissimus dorsi flaps ($n=88$, $CIT_{50} = 10.08$ hours, Figure 1) and buttock skin flaps ($n=85$, $CIT_{50} = 8.99$ hours, Figure 2).

LATISSIMUS DORSI FLAP



3a

BUTTOCK SKIN FLAP



3b

Figures 3a and 3b: In the flaps that survived the global ischemic insult both survival length (cm) and percentage of total area surviving (%TAS) skin yielded similar information: decreasing survival with increasing ischemia time in a dose effect type of response, for the a) latissimus dorsi buttock and b) buttock flaps. Results reported as mean $\pm$ SEM. * $p < .05$ and ** $p < .001$ to their control.

decrease in % TAS was not produced until the ischemic insult was extended to 8 hours for both flap models and in length at 6 and 8 hours for the BS and LDMC flap respectively.

DISCUSSION AND CONCLUSIONS

The initial impression that there was a true difference between skin and myocutaneous flaps in their tolerance and response to ischemia was not confirmed by the present data.^{31-32,68} The CIT₅₀ of the BS flap was not statistically different from that of the LDMC flap, but both were similar to the previous LDMC data. These initial studies can be criticized as they were carried out on flank flaps that contained panniculus carnosus and then compared to a different group of pigs with myocutaneous flaps. The current study design with buttock flaps (no panniculus carnosus) and myocutaneous flaps in the same animals eliminates some of these problems. In this series, there were a number of flaps lost for technical reasons. Prolonged application of vascular clamps may be partially responsible as it has previously been demonstrated that there is loss of intima and media necrosis after only 10-15 min of clamp application.¹ The forces of the clamps ranged from 22-37 gm (mean 32 gm) and 25-55 gm (mean 47 gm) for Acland V₂ and V₃ clamp respectively. This corresponds to surprisingly high pressures on

the vessels from 306-674 mmHg. This may explain lack of reflow after prolonged clamping and may shorten the apparent CIT_{50} .

Although there is evidence that muscle has a poorer tolerance to ischemia than skin,^{22,69} it does not seem to endanger the viability of the overlying skin component of the composite skin-muscle flap. Morris et al⁴⁹ using a LDMC flap model in pigs showed that the capillary blood flow remained similar in the viable and non viable muscle component of this flap after 6 hours of ischemia and 48 hours of reperfusion. They also showed that skin overlying this dead muscle may be viable. The present study did not investigate muscle viability but rather viability of the overlying skin.

Pang et al⁶⁰ found good correlation between fluorescein staining 24 hours after flap elevation and eventual survival at 7 days using random skin flaps in the rat. However, in the current study it was not until after 60 hours of reflow, 3 days after flap elevation, that no further total flap deaths occurred. Thus, it appears that flaps subjected to prolonged periods of global ischemia are more unstable in the early postoperative period. The survival necrosis interface after these 60 hours was stable, sharply defined and correlated well with eventual survival at 7 days. In a separate preliminary experiment with 26 flaps (nonreported data) I also found that if the flaps were traced daily for five days we could observe an increase greater than 10% in total area surviving in as many as 11/26 (42%) of the flaps. This was consistent whether the measurements were done with or without fluorescein.

Two factors which could contribute to this are a) shrinkage of the necrotic area of the flap, and or b) expansion of the viable area, secondary to edema of the flap, which occurs in the first days after reperfusion. These fluid content changes in dead or alive flap tissue during this early postoperative period are well recognized.

Survival patterns in those flaps that did survive was adversely affected by increases in the ischemic time. Both skin flaps and myocutaneous flaps demonstrated increasing areas of distal skin necrosis. Why is it that peripheral zones of the vascular territory succumb rather than patchy zones within the whole? Is it simply a matter of decreased perfusion pressure or is there something intrinsically different between the proximal axial zone of the flaps and the more distal random zone? Although this study was not designed to specifically address these questions, one can hypothesize regarding a variety of mechanisms. During the period of global ischemia anaerobic metabolism proceeds with generation of a variety of substances. During the reperfusion period, when many of these substances are further metabolized, toxic free radicals are generated. The tissue in question will require a healthy blood flow and adequate substrate to neutralize these radicals. Flow in the proximal flap is under greater perfusion pressure and the metabolites are perhaps more effectively washed out than in the distal flap. Simultaneously, tissues downstream may be subjected to accumulation of deleterious intravascular substances and thus greater injury.⁴⁷

CHAPTER II

PATHOPHYSIOLOGY OF ISCHEMIC SKIN FLAPS: DIFFERENCES IN XANTHINE OXIDASE LEVELS BETWEEN RAT, PIG AND HUMAN SKIN

ABSTRACT

Oxygen derived free radicals have been implicated in a variety of diseases and pathological processes including the ischemia reperfusion injury (IRI). Based on experimental work with rat skin flap models the enzyme Xanthine Oxidase (XO) has been proposed as a major source of free radicals responsible for tissue injury and flap necrosis. The presence of this enzyme is variable within different tissues of a specific species and between species. XO levels in pig and human skin have not previously been reported. The activity of XO in the skin of rats (N= 16), pigs (N=7) and humans (N=8) was measured after varying intervals of ischemia and reperfusion. Control pig and human skin were found to contain minimal enzyme activity, almost 40 times less than that of the rat. In the rat, XO activity was stable throughout a prolonged period of ischemia and a significant decrease in activity was found after 12 hours of reperfusion ($p < .05$). In man XO activity was unaffected by ischemia time, and in the pig it did not increase until 24 hours of ischemia ($p < .05$). The potential sources of free radicals and mechanisms of action of XO and its inhibitor, allopurinol, in improving flap survival are reviewed. The rat skin is an imprecise model for study of the ischemia reperfusion injury.

BACKGROUND AND PURPOSE

Oxygen derived free radicals have been implicated in a variety of diseases and pathological processes including the ischemia reperfusion injury (IRI).¹⁰ The xanthine oxidase (XO) system has been cited as the major source of free radicals in many organs^{9,25,53} including skin.^{3-5,27-30,37} It has been proposed that the free radicals produced by this enzyme are responsible for the injury associated with ischemia and reperfusion of tissues. By blocking XO activity with allopurinol investigators have been able to improve skin flap survival in rats.^{5,29-30}

It must be recognized that differences exist in the XO activity of different species and within tissues of the same species.^{2,19,34,50,53} The absence of XO in the myocardium and blood of certain species like the rabbit and the pig have lead several researchers to question the importance of XO as a major source of free radicals in the ischemia reperfusion injury of the myocardium of these particular species. To date, all the reports implicating XO as the enzymatic system responsible for the production of free radicals in skin IRI have used the rat as the experimental model. From the results obtained in the rat, it would be easy to assume that the same mechanisms might be functioning in other species, specifically man. Im et al²⁸ studied rat island epigastric skin flaps subjected to global ischemia and found XO

activity in homogenized skin to increase with prolonged intervals of ischemia.

In this study a series of experiments were designed to measure the XO activity in skin specimens from rats, pigs and humans in order to further examine its role in the IRI of skin flaps.

MATERIALS AND METHODS

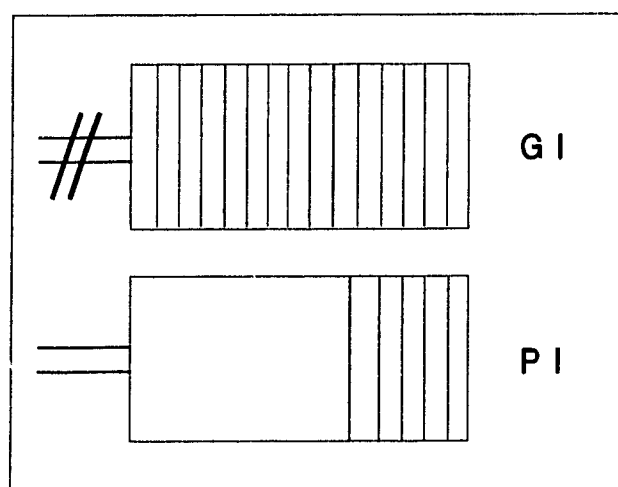
Rats

Following intraperitoneal pentobarbital anaesthesia, epigastric island flaps²¹ (9x9 cm) were elevated on male Sprague Dawley rats (n = 16) weighing 300-325 g. Two groups of eight rats were studied. In group A, global ischemia (GI) was achieved by applying Acland V₂ clamps to the vascular pedicles for 24 hours. In group B, the flaps were made ischemic for eight hours and then allowed to reperfuse for twelve hours. Samples were obtained as three mm punch biopsies at 0, 8 and 24 hours of ischemia in group A (n=8) and at 0 hours and after 12 hours of reperfusion in group B (n=8). All biopsies were obtained 5 cm from the base of the flap; this section of the flap eventually died in all the animals where reperfusion was established but would usually survive in nonischemic flaps.²¹

Pigs

Using intravenous pentobarbital anaesthesia and sterile technique bilateral latissimus dorsi myocutaneous (LDMC, 10x20 cm) and buttock skin (BS, 10x18 cm) island flaps³³ were raised in female white Landrace pigs (n=7) weighing 20-22.5 kg. On one side of the animal, flaps were subjected to 24 hours of global ischemia (GI) by application of Acland V₂ or V₃ clamps to the pedicle, while the contralateral side served as control flaps being subjected to the partial ischemia (PI) conditions associated with flap elevation. See Figure 1 for description of flaps. Skin samples were (n=7) taken as three mm punch biopsies from control nonoperated skin (0 hours) and flap skin at 8 and 24 hours of ischemia. The samples were obtained at 14 cm (LDMC) and 11.5 cm (BS) from the base of the flap, a segment of the flap known to be at high risk of necrosis when reperfusion is established after eight hours of ischemia.⁵⁶

Figure 1: GI & PI Globally and partially ischemic flaps respectively. The crosshatched areas represent the ischemic portion of the flap.



Humans

Skin samples were taken as three mm punch biopsies from fresh surgical specimens. Operative procedures included breast reduction (n=2), abdominoplasty (n=2), rhytidectomy (n=2) and thigh lipectomy (n=2). The skin was wrapped in a moist saline gauze and kept at room temperature (26-27 °C). The samples (n=8) were analyzed following 0, 8 and 24 hours of global ischemia.

Tissue ischemia was confirmed by a negative Dermofluorometry index (DFI)⁶² while successful reperfusion was assessed by reactive hyperaemia and positive DFI. All skin samples were taken as full thickness punch biopsies from which the panniculus carnosus or subcutaneous fat was removed under the microscope. The specimens were then trimmed into 20-30 mg sizes, weighed, immediately frozen in liquid nitrogen and placed in a freezer at -30°C until they were assayed for XO activity.

Xanthine Oxidase Determination

XO activity was assayed by a fluorometric method modified for skin by Im.³⁰ The tissue was cut into 10-14 um thickness slices in a cryostat microtome (Fisher) at -25°C and homogenized in 200 ul of 0.1 M Tris HCl buffer (pH=8.1). The suspension was vortexed and then centrifuged at 15,000 RPM for 30 minutes at 2° C. Twenty microliters of the supernatant was added to 50 ul of reagent mixture. The reagent mixture was prepared

by adding 50 μ l of 0.5 mM of 2-amino 4-hydroxypteridine (AHP) to 1 ml Tris HCl buffer with EDTA; the final concentration of AHP was 20 μ M. Standards were prepared on the day of the experiment by using a 10 μ l aliquot of 0.5 mM solution of isoxanpterine (IXP) into 100 and 200 μ l of Tris HCl buffer (pH= 8.1). Samples were run in triplicate simultaneously with standard tubes. The specimens were placed on a shaking water bath at 37 °C for 60 minutes. One ml of 1 mM sodium acetate buffer (pH=5.3) was added to the samples and vortexed for 1 minute. The fluorescence of the samples was read in a spectrophotometer (Gilford Fluoro IV) at 347 nm excitation and 405 nm emission. Values were calculated from the average of two of the triplicate samples and reported as pmoles/mg of wet tissue/hour. Other investigation has found similar results when XO activity is reported in mg of protein or mg of wet tissue (personal communication with M.J. Im, Johns Hopkins University). All the chemicals used were from Sigma Chemical Co., St. Louis, MO.

Statistical Analysis

Values are reported as mean $\pm$ SEM. A one way analysis of variance and the Newman Keuls method or t test, where appropriate, were used for comparisons between the means of each time interval within one group or species. A value of $p < .05$ was considered to be significant.

RESULTS

In control skin, the rat had 321 ± 18 pmoles/mg/hour of XO activity while pig and human activity was dramatically lower at 8.4 ± 1.6 and 8.6 ± 1.3 pmoles/mg/hour respectively (Table 1).

XANTHINE OXIDASE ACTIVITY CONTROL SKIN

RAT	HUMAN	PIG
320 +/- 18	8.6 +/- 1.3	8.4 +/- 1.6

Table 1: XO activity for control skin. Rat N= 16, Pig N=7, Human N=; Mean +/- SEM pmoles/mg/hour. Activity in rat skin is 38 times higher than pig or human skin

In rats, (Figure 2) XO activity was stable during prolonged ischemia; however, following 8 hours of ischemia and 12 hours of reperfusion, it decreased significantly ($p < .05$). In pig skin, similar data were obtained from the BS and LDMC flaps and for simplicity only the later is presented. There was no difference in XO activity (Figure 3) between PI or GI flaps at 8 or 24 hours of ischemia. XO activity increased in flap skin compared to nonoperated skin after 24 hours of global or partial ischemia but not after 8 hours ($p < .05$). In human skin, (Figure 4) XO activity did not change significantly with prolonged ischemia.

Figure 2: Xanthine oxidase activity in rat skin. Group 1: global ischemia. Group 2: 8 hours of global ischemia and 12 hours of reperfusion. Values reported as mean ($n=8$) $\pm$ SEM (pmoles/mg/hour). * = $p < .05$.

XANTHINE OXIDASE ACTIVITY RAT SKIN

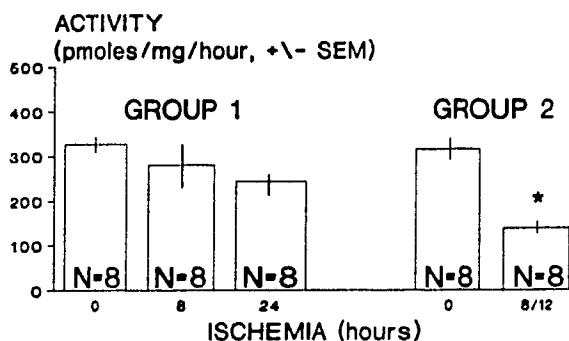


Figure 3: Xanthine oxidase activity in pig skin. Values reported as mean ($n=7$) $\pm$ SEM (pmoles/mg/hour). * $p < .05$, ** $p < .001$.

XANTHINE OXIDASE ACTIVITY PIG SKIN

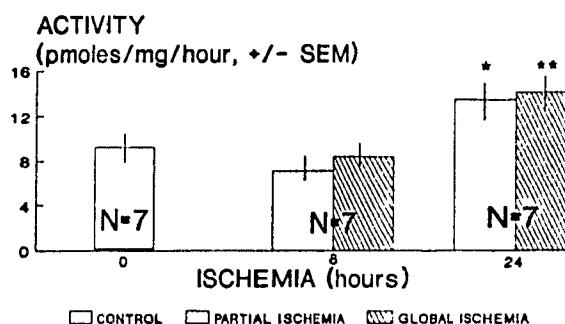
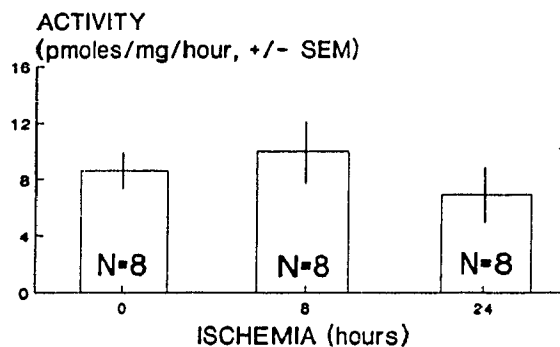


Figure 4: Xanthine oxidase activity in human skin. Values reported as mean ($n=8$) $\pm$ SEM (pmoles/mg/hour).

XANTHINE OXIDASE ACTIVITY HUMAN SKIN



DISCUSSION AND CONCLUSIONS

Free radical injury secondary to periods of ischemia and reperfusion has been implicated in many disease states. Crucial events occur during both the ischemic and the reperfusion period. The recognized biological sources of free radical production are several and include: a) the vascular endothelium related enzyme XO, b) the activated neutrophil membrane related NADP/NADPH oxidase, c) the disrupted mitochondrial electron transport system, d) the arachidonic acid pathway, and e) the oxidation of catecholamines. These sources may be mutually interactive or one may predominate.^{38,40} The present study was aimed specifically at measuring the activity of XO in skin during an ischemic interval and at comparing this activity between different animal species. In the current experiment other possible sources of free radicals have not been investigated nor have free radicals been measured directly.

The proposed mechanism of action of XO involves the irreversible conversion of the enzyme from its physiologic dehydrogenase form into an oxidase form during ischemia or any low energy state (see Figure 1 in the Introduction). XO accumulates in the tissue as the insult continues, while the cell's energy sources are being consumed and degraded to hypoxanthine. When reoxygenation or reperfusion occurs, oxygen can act as an electron acceptor for XO which can then oxidize hypoxanthine and xanthine into uric

acid. This produces the free radicals: superoxide anion and hydrogen peroxide.³⁹ Subsequent reaction of these two compounds in the presence of specific metal ions results in the generation of secondary radicals which can be more toxic than the primary radical species themselves. A subsequent series of chain reactions may result in tissue injury and cell death. There is substantial indirect evidence that free radicals are produced during ischemia and reperfusion injury in skin.^{3-5,27-30,37} Several reports exist of improved flap survival by scavenging radical species^{27,29,37} or chelating iron^{3,48} a cofactor necessary for the peroxidation of tissues by free radical species. My human model could theoretically be criticized since it did not suffer reperfusion injury; however, even though free radicals are generated during reperfusion, XO activity, our subject of interest, rises only during ischemia and is not affected by reperfusion.³⁰

In the present study, XO activity did not rise during the first 8 hours of ischemia. The findings were similar for rats, pigs and human. Unlike rat and humans, following 24 hours of ischemia there was a significant rise in XO activity in the pig. However, this has dubious clinical significance as this amount of XO activity is considered negligible when compared to other organs or species.^{2,53} As well, complete tissue necrosis is the inevitable outcome of reperfusion, even prior to this stage⁵⁶, as is known from the experiments described in Chapter 1. The unpredictability of outcome

following 8 hours of ischemia and subsequent reperfusion, with its potential for pharmacological intervention, is of much greater clinical importance.

XO was assayed using a previously reported fluorometric method modified for skin.³⁰ While an argument could be made that assays using high performance liquid chromatography could be more sensitive these are very difficult to modify for whole tissue homogenates. In any case, I was more concerned in comparing XO activity between different species than on measuring absolute values of one particular species.

XO activity in rat skin was dramatically (40X) higher than pigs and humans. Previous work in skin flap IRI has utilized primarily rat models. Im et al²⁸ found a significant increase in XO levels of rat epigastric free skin flaps submitted to 6 and 24 hours of cold GI. In another study³⁰ he found a significant increase in XO activity after eight hours of venous occlusion and one hour of reperfusion. The increase in XO activity was significantly blocked by treating the animals with allopurinol prior to flap elevation, and this resulted in increased flap survival in both experiments. However, Angel et al⁴ using axial pattern dorsal skin flaps, a model of partial and not global ischemia, were able to improve flap survival while finding no difference in XO activity between the control skin and that of flaps that were up to 48 hours old. Similar to my findings, they found control skin to contain higher levels of XO activity than ischemic skin, although not enough to reach statistical significance. However, these authors were able to demonstrate an increase

in malonyldialdehyde, a product of lipid peroxidation. They concluded from their study that since the enzyme was not elevated it could not be the source of free radical production, although they still believed that free radicals were involved in the overall process.

Further confusion on the possible role of XO in an ischemia reperfusion models arises from the contradictory reports on the effects of allopurinol on different animals models, being beneficial in some and having no effect on others. Allopurinol, a XO inhibitor, reportedly can improve skin survival in rats ^{5,29-30} presumably by blocking the conversion of the enzyme during ischemia, and thus preventing free radical production upon reperfusion. However, I have been unsuccessful in documenting a significant rise in XO activity or improving flap survival with allopurinol.⁵⁵ Allopurinol has been reported to prevent myocardial ischemic injury in the rabbit⁶, but the rabbit myocardium or blood has no detectable levels of XO.^{34,50,67} Other investigators, while not successful at decreasing infarct size in rabbits with XO inhibitors, have been so with superoxide dismutase¹⁹, a superoxide anion scavenger, or catalase⁵¹, a scavenger of hydrogen peroxide. Further studies using a dog model whose myocardium contains significant XO activity, have found that allopurinol cannot limit infarct size.^{44,58} Pig hearts have low XO activity^{17,50} and XO inhibition cannot limit infarct size nor prevent the free radical induced arrhythmias associated with reperfusion.⁵⁷ Perhaps, the other sources³⁸⁻⁴⁰ of free radicals may be

involved or allopurinol may have an effect through mechanisms other than free radical generation inhibition.^{23,46,52,65}

From the present study I conclude that it is unlikely that XO is the major source of free radical generation in the ischemia reperfusion injury occurring in rat, pig or human skin. The anatomical and biochemical differences between the skin of rats and humans, and the similarities of pigs and humans, are well recognized.^{18,35,45} Those interested in the experimental investigation of the IRI problem and its treatment must be cautious when using the rat as an experimental model.

CHAPTER III

EFFECT OF ALLOPURINOL ON THE SURVIVAL OF PIG AND RAT EXPERIMENTAL FLAPS

ABSTRACT

Allopurinol has been reported to improve cell survival in a variety of conditions including the ischemia reperfusion injury (IRI) occurring in skin flaps. It has been suggested that the beneficial effect of allopurinol on rat skin flaps is through blockage of xanthine oxidase (XO) generated free radicals. In the previous experiments I reported on the lack of XO in the skin of humans and pigs when compared to that of rats. This current study attempts to improve flap survival in pigs using allopurinol at doses of 50 and 300mg/kg, and in rats using allopurinol at a dose of 50mg/kg. At a dose of 50mg/kg (pigs N = 12, rats N = 30) there was no significant difference between the survival of control or treated flaps. In pigs (N = 14) a dose of 300mg/kg resulted in three operative deaths and a decrease in the survival of a myocutaneous flap model. Allopurinol's therapeutic effectiveness and its mechanism of action in an ischemia reperfusion injury model lacking XO activity are discussed.

BACKGROUND AND PURPOSE

While a better knowledge of vascular anatomy has increased the reconstructive surgeon's options for coverage of difficult defects, a reliable

method to treat the failing flap has yet to be developed. Oxygen derived free radicals have been implicated in a variety of pathological processes including the ischemia reperfusion injury (IRI) leading to skin flap necrosis.^{3-4,10,23,28-29,37} Despite a general agreement that free radicals are responsible for an important component of the injury, the exact source of their origin remains poorly defined. Five major biological sources for these radicals have been identified, the predominant mechanisms varying with the biological model.^{3-4,10,28-29,37} In skin flaps, the xanthine oxidase (XO) enzymatic system has been proposed as the major source for these radicals^{3-4,28-30,37} and treatment with allopurinol, a XO inhibitor, has resulted in improved flap survival in two different rat models.^{5,30} The conversion of xanthine dehydrogenase into XO during ischemia is a prerequisite for free radical formation upon reperfusion.³⁸ While Im³⁰ suggested that the beneficial effect of allopurinol in his global ischemia models was secondary to inhibition of free radical production through the XO pathway, Angel⁵ concluded from his partial ischemia model that, although free radicals were involved in the overall process, XO was not the likely source since the enzyme was not elevated in ischemic tissue. In the previous experiments I was unable to document a rise in XO activity in ischemic skin of rats, pigs or humans while finding that control pig and human skin contained negligible enzyme activity when compared to the rat.⁵⁴ Thus, I questioned the efficacy of allopurinol in the treatment of ischemic flaps.

Allopurinol is widely used in thousands of hyperuricemic patients with little if any toxic effects, and has been shown experimentally to benefit the cell in a wide range of ischemia reperfusion conditions²⁶ including animal models which lack XO activity.^{6,51} It has been suggested that allopurinol could benefit the cell by mechanisms not related to inhibition of free radical formation by the XO system.^{17,23,46,52,65} There is a possibility, then, that allopurinol could improve flap survival even in a species whose skin lacks XO activity. Contrary to small rodents like the rat or mice, the pig has proven to be an excellent model for the study of allopurinol pharmacokinetics and purine metabolism^{7,63-64} because of similarities with humans.

The aim of this study was two-fold. First, to test in a swine model, the effect of allopurinol on skin and myocutaneous flap survival under conditions of both partial and global ischemia and correlate this with measurements of free radical metabolism and blood flow and second, test in a rat model, a dose of allopurinol known to produce complete XO inhibition for its effect on the renal function and flap survival in a model of partial ischemia.

EXPERIMENT #1

Materials and Methods

Eighteen white Landrace female pigs weighing 20.5 ± 0.6 kg were randomized into two groups. These groups received either normal saline or

allopurinol (Sigma Chemical Co. St. Louis, MO) 50mg/kg IV, suspended in 500ml of saline 60 minutes prior to flap elevation. Under general intravenous pentobarbital anaesthesia, each animal had bilateral latissimus dorsi myocutaneous (10x20 cm, LDMC) and buttock skin (10x18 cm, BS) island flaps³³ raised and sutured back into their beds. Flap design insured a distal zone of necrosis.⁵⁶ Through separate axillary and groin incisions the vascular pedicles were isolated. One side of each animal served as control and the other was subjected to 8 hours of global ischemia (GI flaps) by clamping the vascular pedicles with Acland V₂ clamps. Flap design was made long enough to include a distal zone of ischemia so that hereinafter the control flaps are referred to as partial ischemic or PI flaps. Following clamp application and removal ischemia and reperfusion were confirmed objectively by Dermofluorometry Index.⁶² The effect of allopurinol was evaluated using three parameters: skin flap survival, malonyldialdehyde determination and blood flow measurements.

Skin Flap Survival

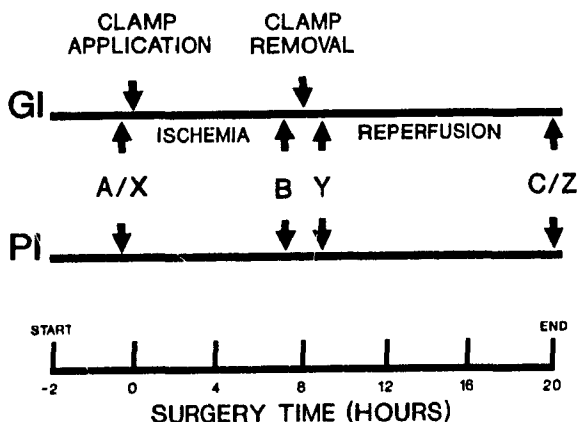
In twelve animals at 5 days postoperatively, flap viability was assessed using two different measurements. First, in those flaps that had undergone 8 hours of global ischemia (GI flaps), flaps were considered necrotic if there was 100% necrosis but viable if even a small percentage of the flap was alive.

This was used to calculate the total necrosis rates (TNR). Second, in all flaps that survived (GI and PI), the survival necrosis interface was traced on clear plastic and, with the aid of a digitizer and computer software (Sigmascan), the percent of total area surviving (% TAS) was calculated. The TNR and % TAS were compared in matching flaps between allopurinol and saline treated animals using a non paired t test. Muscle viability was not assessed.

Malonyldialdehyde (MDA) Determination

MDA is a nonspecific product of lipid peroxidation frequently used as an indicator of free radical mediated tissue necrosis.²⁴ MDA was measured (N = 12, same animals as for survival) using a thiobarbituric acid assay (TBA) modified for skin.^{4,43} Skin biopsies were harvested as 3mm full thickness punch biopsies from (A: non operated flank area between the LDMC and BS flaps) and in each flap at intervals (B: 8 hours of global ischemia or after flap elevation in GI and PI flap respectively) and (C: 12 hours after reflow or 20 hours after elevation in GI and PI flaps respectively. (Figure. 1)

Figure 1: Time intervals for biopsy sites for MDA (A, B, C) and blood flow (X, Y, Z) measurements. Biopsy sites: At 14 and 11.5 cm from the base of the flap for LDMC and BS respectively. A = Control non operated skin for each animal. B = GI flaps after 8 hours of global ischemia but prior to reperfusion; PI flaps, 8 hours after flap elevation. C = GI flaps 12 hrs after reflow; PI flaps 20 hours after flap elevation. X = blood flow after flap elevation just prior to clamp application. Y = blood flow 1 hour after clamp removal and reperfusion of the flap. Z = blood flow 12 hours after reperfusion.



In flaps biopsies harvested at 14 cm (LDMC) and 11.5 cm (BS) from the base, a segment of the flap known to be at high risk of necrosis after 8 hours of ischemia followed by reperfusion.⁵⁶ MDA was a) correlated with ischemia and reperfusion using regression analysis; b) compared between time intervals A, B, and C for flap type and treatment protocol using analysis of variance, non-paired and paired t tests respectively; and c) the change in MDA production between the different time intervals was correlated with eventual flap survival using chi square and Fisher exact probability tests.

MDA Assay: The skin specimens were defatted under the microscope, weighed wet, frozen in liquid nitrogen and stored at -30°C until they were assayed for MDA production. On the day of the assay, each specimen was cut into 10-14µ thickness slices in a cryostat microtome (Fisher) at -25°C and homogenized in 1.2ml of an extraction solution made of butylated hydroxytoluene solution (BHT) which had previously been bubbled in nitrogen gas, and 7% lauryl sulfate. The solution was vortexed and

centrifuged at 5000 RPM at 4°C for 20 minutes. A 1ml aliquot of the extraction solution was added to 500ul of 0.2N HCl and 500ul of 0.50% TBA. The mixture was heated at 100°C for 60 minutes after which 2ml of n-butanol were added. The organic layer is separated by centrifuging at 2000 RPM for 5 minutes and read at 535nm in a spectrophotometer (Bauch & Laumb Spectronic 2000) using n-butanol as blank tubes. The amount of MDA produced was calculated using a coefficient of molar extinction of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and is reported as nmoles of MDA/mg of wet tissue/hour mean $\pm$ SEM. (personal communication with M.J.Im).

Blood Flow

Skin and muscle, in the LDMC, blood flow were measured separately at 2 cm intervals along total length of the flaps on three animals on each group (N=6) using the radioactive labelled microsphere technique. Ce^{141} , Cr^{51} and Ru^{103} labelled 15u microspheres were injected at times (X: 1 hour after flap elevation for all flaps) and again at (times Y and Z corresponding to 1 and 12 hours after reflow in GI flaps or 9 and 20 hours after elevation for PI flaps (Figure 1). Blood flow is expressed as an average of 9 or 10 two cm segments for BS and LDMC flaps respectively, along the flap in ml/g/minute $\pm$ SEM and compared for matching flaps between allopurinol and saline treated animals using non paired t test.

Results

Skin Survival

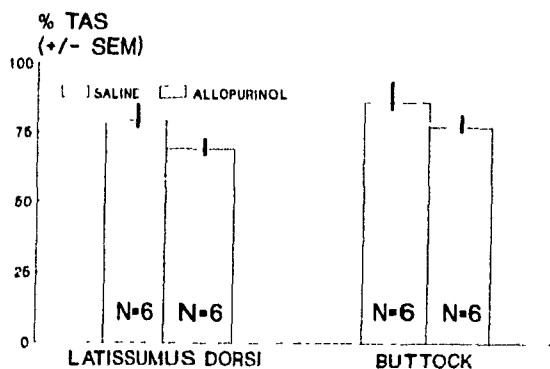
The TNR (Table 1) in GI flaps or % TAS (Figs. 2a & 2b) in surviving GI or PI flaps was not significantly different between allopurinol or saline treated flaps.

PIG FLAPS

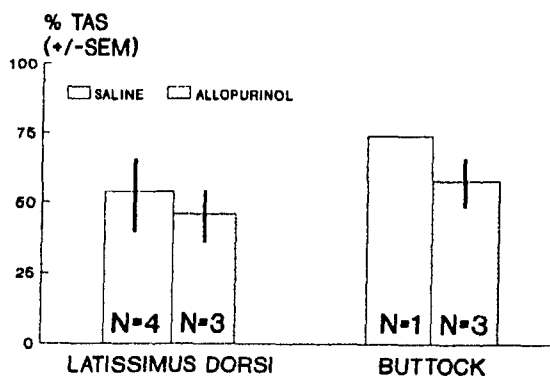
FLAP	GLOBAL ISCHEMIA		PARTIAL ISCHEMIA	
	Allopurinol	Saline	Allopurinol	Saline
Latissimus Dorsi	2/6 (33%)	3/6 (50%)	0/6 (-)	0/6 (-)
Buttock	5/6 (87%)	3/6 (50%)	0/6 (-)	0/6 (-)

Table 1: Total Necrosis Rates. There is no statistical difference between saline and allopurinol treated groups for any of the flap types or ischemia models.

PIG PI FLAPS 50 mg/kg, IV



PIG GI FLAPS 50 mg/kg, IV



Figures 2a and 2b: Skin survival of pig flaps in saline and allopurinol treated (50 mg/kg) animals. (%TAS = percentage total area surviving ± SEM. 2a) PI = Partially Ischemic flaps. 2b) GI = Globally Ischemic flaps, 8 hours.

MDA

There was a wide variability in MDA production between animals but not between different flaps among the same animal. A poor correlation between MDA production and ischemia reperfusion was found ($r=0.1$), some flaps showing a significant amount of MDA upon reperfusion while others not. In general (Tables 2 and 3), all flap skin produced more MDA than its corresponding control skin; however, this increase was statistically significant only in the saline treated buttock flaps after 8 hours of global ischemia ($p<0.04$). This was unexpected since the highest MDA values should correspond to reperfused and not ischemic tissue. Although absolute values of MDA did not correlate with ischemia reperfusion or flap survival, a rise in MDA between time intervals B and C within the same flap was a good predictor of flap death.

LATISSIMUS DORSI MYOCUTANEOUS FLAPS

TIME	<u>ALLOPURINOL</u>		<u>SALINE</u>	
	GI	PI	GI	PI
0 Hours Control Non Operated Skin	1.75 ±.43		1.05 ±.59	
8 Hours Ischemia Flap Skin	2.89 ±.63	2.70 ±.90	1.09* ±.61	1.63 ±.72
12 Hours Reperfusion	2.11 ±.49	1.71 ±.33	1.63 ±.63	1.13 ±.49

Table 2: MDA values for LDMC flaps in nanomoles of MDA/mg wet tissue/hour ± SEM. * $p < .05$

BUTTOCK FLAPS

TIME	<u>ALLOPURINOL</u>		<u>SALINE</u>	
	GI	PI	GI	PI
0 Hours Control Non Operated Skin	1.84 ±.43		1.22 ±.46	
8 Hours Ischemia Flap Skin	2.32 ±.63	2.39 ±.58	1.77* ±.65	1.56 ±.55
12 Hours Reperfusion	1.84 ±.47	1.94 ±.35	1.14 ±.38	1.99 ±.35

Table 3: MDA values for BS flaps in nanomoles of MDA/mg wet tissue/hour. Mean ± SEM. * $p < .05$

MDA AND FLAP OUTCOME

OUTCOME	ALL FLAPS UP DOWN		PI FLAPS UP DOWN		GI FLAPS UP DOWN		ALLOPURINOL FLAPS UP DOWN		SALINE FLAPS UP DOWN	
	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
Survive	12	20	8	14	4	6	7	9	5	11
Die	19	7	7	1	12	6	10	4	9	3
Total	31	27	15	15	16	12	7	13	14	14
χ^2	$p < 0.02$		$p < 0.04$		$p = 0.33$		$p = 0.24$		$p = 0.5$	
Fisher	$p < 0.007$		$p < 0.02$		—		—		$p < 0.03$	

Table 4: Chi square and Fisher Exact Probability Tests. Correlation between a rise in MDA production in a flap from time interval B to C and eventual flap outcome.

Using chi square and Fisher exact probability test (Table 4), a significant correlation between rise in MDA and flap outcome was found for all flaps ($p < 0.007$), all PI flaps ($p < 0.02$), and all saline treated animals ($p < .03$), but not for GI or allopurinol treated flaps ($p > 0.05$). MDA production

was compared between allopurinol and saline treated animals using non-paired t test and no difference was found between any matching pairs.

Blood Flow

The small number of flaps in each subgroup did not permit statistical comparisons. The high variability in flow found in both flap models, specially in the GI flaps, makes interpretation of the results (Tables 5 and 6) difficult. A consistent pattern of progressive decreased blood flow from proximal to distal along the flap until no flow could be detected could be detected. This finding was true for both PI or GI flaps; we could not detect any patchy areas of nonperfused muscle close to the entrance of the pedicle at the base of the flap. Because of a number of technical difficulties, only 88 out a possible 108 (3 measurements x 6 flaps, 4 skin + 2 muscle, x 6 animals) measurements could be obtained. These difficulties included: pedicle injury to one flap (3 measurements), embolic event to one leg (1 measurement), not enough microspheres in 4 flaps (12 measurements), one flap death upon reflow (2 measurements).

BUTTOCK FLAPS

FLOW	PARTIALLY ISCHEMIC FLAPS		GLOBALLY ISCHEMIC FLAPS	
	Allopurinol	Saline	Allopurinol	Saline
X	6.50 $\pm$ 4.14 (N=2)	2.31 $\pm$ 1.59 (N=3)	1.16 $\pm$ 1.02 (N=2)	5.91 $\pm$ 3.14 (N=3)
Y	0.25 $\pm$ 0 (N=1)	0.91 $\pm$ 0.77 (N=3)	0.87 $\pm$ 0 (N=1)	1.74 $\pm$ 0.90 (N=3)
Z	2.27 $\pm$ 0.98 (N=3)	3.54 $\pm$ 1.51 (N=3)	4.08 $\pm$ 2.04 (N=2)	1.06 $\pm$ 0.99 (N=3)

Table 4: Blood Flow in BS flaps in ml/100g/minute $\pm$ SEM. X = flow after flap elevation for both PI and GI flaps, Y = flow 1 hour after reflow in GI flaps which correspond to 9 hours after elevation on PI flaps; Z = 12 hours of reflow for GI flaps which corresponds to 20 hours after flap elevation on PI flaps.

LATISSIMUS DORSI FLAPS

FLOW	PARTIALLY ISCHEMIC FLAPS		GLOBALLY ISCHEMIC FLAPS	
	Allopurinol	Saline	Allopurinol	Saline
X	1.21 $\pm$ 0.75 (N=3)	1.85 $\pm$ 0.83 (N=2)	1.28 $\pm$ 0.64 (N=3)	3.91 $\pm$ 2.32 (N=3)
	4.22 $\pm$ 1.96 (N=3)	2.78 $\pm$ 1.26 (N=2)	1.84 $\pm$ 0.45 (N=3)	2.86 $\pm$ 0.86 (N=3)
Y	1.06 $\pm$ 0.79 (N=2)	1.02 $\pm$ 0.10 (N=2)	2.29 $\pm$ 1.60 (N=2)	2.85 $\pm$ 2.39 (N=3)
	3.38 $\pm$ 1.59 (N=2)	1.33 $\pm$ 0.54 (N=2)	2.18 $\pm$ 0.25 (N=2)	1.19 $\pm$ 0.62 (N=3)
Z	1.90 $\pm$ 0.94 (N=3)	3.81 $\pm$ 2.17 (N=2)	3.55 $\pm$ 2.24 (N=2)	1.73 $\pm$ 1.16 (N=3)
	4.83 $\pm$ 1.64 (N=2)	2.45 $\pm$ 0.40 (N=2)	2.43 $\pm$ 1.30 (N=2)	1.17 $\pm$ 0.70 (N=3)

Table 6: Blood flow measurements in latissimus dorsi flaps: skin (top and muscle (below) for each time interval in cc/100g of tissue/minute $\pm$ SEM. X = flow after flap elevation for both PI and GI flaps; Y = flow 1 hour after reflow in GI flaps which corresponds to 9 hours after flap elevation on PI flaps; Z = 12 hours of reflow in GI and 20 hours after elevation on PI flaps.

Because of the overall high necrosis rate in all GI flaps and the failure of allopurinol to improve survival a second experiment was designed making four changes: first the GI interval was reduced to 6 hours, to decrease the TNR;⁵⁶ second we increased the dose of allopurinol to 300mg/kg, a dose expected to completely inhibit XO activity in white Landrace pigs;⁶³ third muscle survival was also assessed; and fourth the allopurinol powder was injected as a solution instead of a suspension. According to the manufacturers allopurinol is active immediately when injected intravenously even as a saline suspension. Angel⁵ has used it as a suspension in water through a subcutaneous route with apparent success. However, we have found that to keep the suspension homogeneous constant mixing was necessary to avoid the tendency of the allopurinol powder to settle to the bottom. This could have resulted in less than optimal homogeneity of the injected dose in experiment # 1.

EXPERIMENT #2

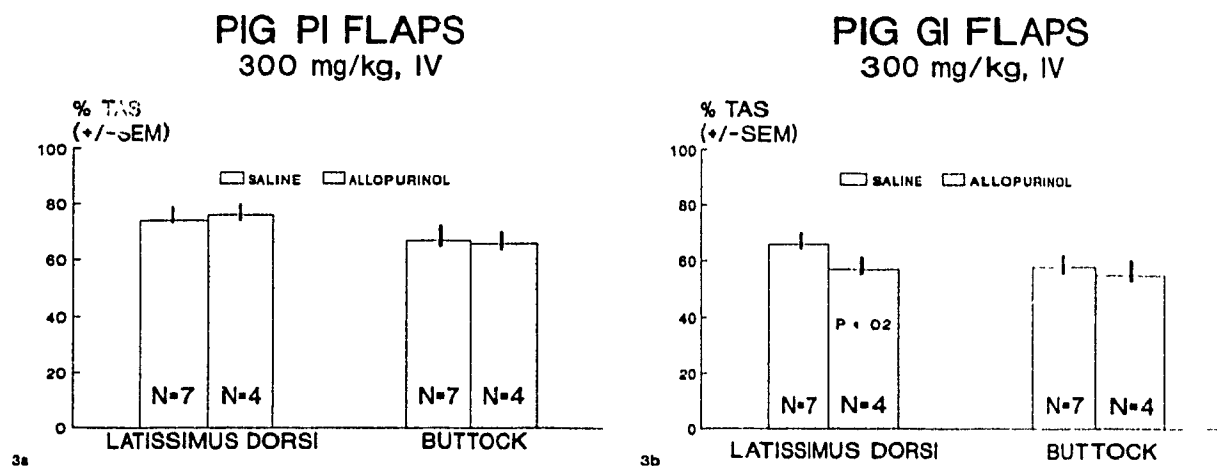
Materials and Methods

Fourteen animals were used in this experiment. Pure allopurinol powder (Wellcome Burroughs, Montreal, Canada) was brought into solution using 1N NaOH (approximately 137mg of allopurinol/ml of NaOH)^{29,30} and diluted into 100 ml of normal saline and given IV 30 minutes prior to flap elevation and again 30 minutes prior to reperfusion. Due to two consecutive deaths in the allopurinol treated animals the second pre reperfusion dose was omitted for the remaining animals. Control animals received a corresponding amount of alkaline vehicle in normal saline. Flap design and operative procedure was carried out as in experiment #1. Skin and muscle survival were assessed 24 hours after reperfusion. In skin, the fluorescent nonfluorescent interface was traced after fluorescein injection with the aid of a Woods lamp, and the % TAS was calculated as in experiment #1. The latissimus dorsi muscle survival was calculated by dividing the whole latissimus dorsi muscle longitudinally into five 2 cm wide segments which were weighed in wet and stained with Nitroblue tetrazolium (NTB) chloride salt.⁸ Using this technique, live muscle stains dark blue while dead muscle remains red. The necrosis survival interface was traced in a standardized fashion and the percent of surviving area was calculated using a digitized image as in experiment #2, Chapter 1. The calculated surviving surface area

was multiplied by the total weight of the muscle to obtain the percentage of total muscle mass surviving (% TMMS).

Results

The first two consecutive allopurinol treated animals, receiving a preop and another pre reperfusion dosages, died shortly after reperfusion while the animals were still under mechanical ventilatory support. The usual sequence of events was initiated by a progressive sinus bradycardia shortly followed by ventricular tachycardia, fibrillation and cardiac arrest unresponsive to therapeutic measures. Autopsies performed in these animals failed to reveal any macroscopic abnormalities except for nitro tetrazolium blue staining evidence of a full thickness myocardial infarct of the left ventricle in one of the animals. In the subsequent five allopurinol treated animals (only one dose prior to flap elevation there was one more operative death occurring in a similar pattern as the other two animals. Because of the three operative deaths, only four animals in the allopurinol group remained for analysis. Despite the small number in the treatment group, the low variability within each group and the dramatic differences between treated and control group, statistical comparison is still possible. Percent TAS was not statistically different in allopurinol treated versus controls BS flaps (PI and GI), or the skin and muscle components of LDMC flaps (PI) (Figures 3a, 3b & 4). However there was a



Figures 3a and 3b: Skin survival of pig flaps in saline and allopurinol treated (300 mg/kg) animals. (%) TAS = percentage total area surviving $\pm$ SEM. 3a) PI = Partially Ischemic flaps. 3b) GI = Globally Ischemic flaps, 6 hours.

a significant decrease in the survival of the skin (Figure 3b, $p < .02$) or the muscle component Figure 4, $p < .005$) of the Latissimus dorsi myocutaneous flap.

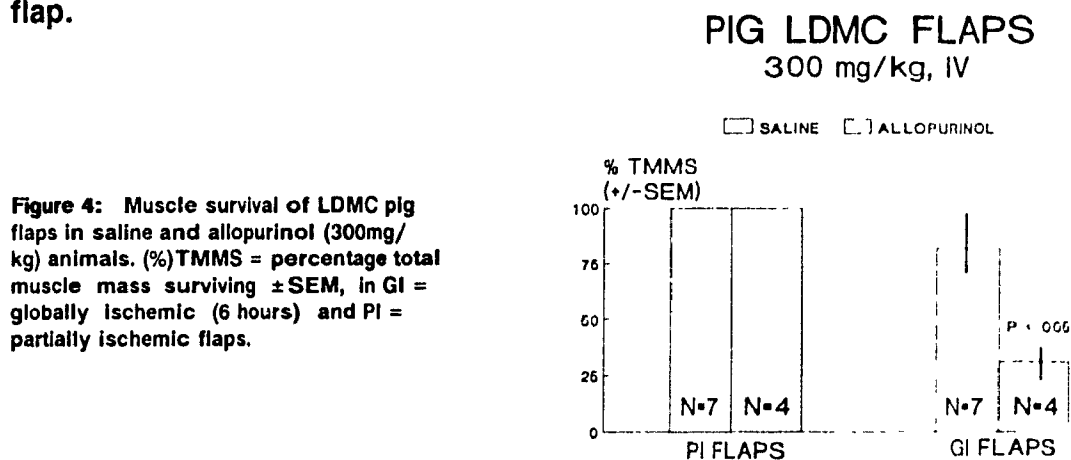


Figure 4: Muscle survival of LDMC pig flaps in saline and allopurinol (300mg/kg) animals. (%)TMMS = percentage total muscle mass surviving $\pm$ SEM, in GI = globally Ischemic (6 hours) and PI = partially ischemic flaps.

Not being able to improve flap survival in the first two pig experiments and knowing that a reported successful dose regimen in a rat distal ischemia model⁵ had included a dose double of that needed to inhibit XO activity I decided to test the effect of allopurinol in such a model with a smaller dose regimen expected to just inhibit XO activity but hopefully have no other effects on the flap.

EXPERIMENT #3

Materials and Methods

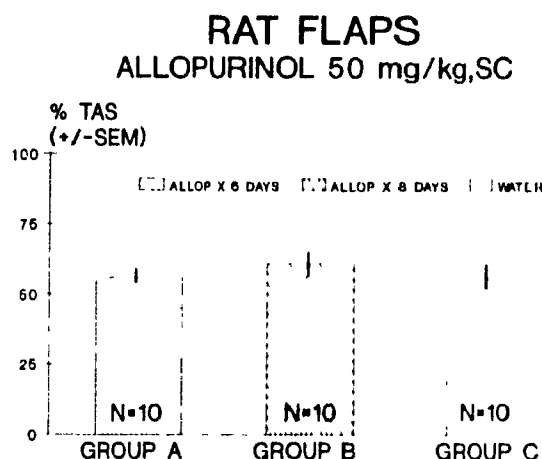
In thirty male Sprague Dawley rats weighing 339 ± 3 g, 10x3 cm caudally based dorsal skin flaps were elevated. Flap design, drug preparation and route of administration were the same as those used by Angel.⁵ The animals had previously been randomized into three groups to receive a daily subcutaneous dose of either sterile water or 50mg/kg allopurinol (Sigma Chemical Co.) in sterile water suspension. Group A received sterile water for 2 days preoperatively followed by allopurinol 1 dose 1 hour prior to flap elevation and continued for 5 days postoperatively (6 days total). Group B received allopurinol for 3 days preoperatively (one dose being 1 hour prior to flap elevation) plus 5 days postoperatively (8 days total). Group C received sterile water pre and postoperatively. All animals

received the same volume and number of injections and the surgeon was blinded in regards to the treatment received by each animal. On the sixth postoperative day a blood sample was drawn for biochemical measurements to assess the effect of allopurinol on the renal function. The animals were then sacrificed and skin survival (% TAS) was calculated from the necrosis survival interface as in Chapter #1. The % TAS as well as the serum biochemical results were compared using analysis of variance and t tests.

Results

Allopurinol treated animals did not show increased survival over saline treated animals as manifested by % TAS (Figure. 5). The Group B dose regimen resulted in a significant amount of renal impairment (Table 5) as manifested by elevated uric acid, creatinine, BUN, potassium, Calcium and PO_4 and decreased HCO_3 , all of which were significantly different to their controls ($p < .05$).

Figure 5: Skin survival in rat flaps. (% TAS = percentage total area surviving $\pm$ SEM in rat flaps. Group A = sterile water for 2 days preop and Allopurinol 1 dose 1 hour pre flap elevation followed by 5 days post-operatively. Group B = allopurinol 3 days preop plus 5 days post operatively. Group C = (control) sterile water for 3 days preop plus 5 days post operatively.



BIOCHEMICAL MEASUREMENTS IN RAT SERUM

	<u>GROUP A</u>	<u>GROUP B</u>	<u>GROUP C</u>
GLUCOSE (MMOL/L)	7.9 ± 1	8.5 ± 2	6.0 ± 0.3
UREA (MMOL/L)	6.5 ± 0.2 *	6.5 ± 0.2*	5.7 ± 0.3
CREATININE (UMOL/L)	71 ± 7 *	50 ± 4	46 ± 8
URIC ACID (UMOL/L)	364 ± 86 *	141 ± 28	154 ± 20
CALCIUM (MMOL/L)	2.3 ± 1**	2.6 ± 0.1	2.5 ± 0
PO ₄ (MMOL/L)	6.6 ± 0.9 **	4.4 ± 0.3 *	3.7 ± 0.1
SODIUM (MMOL/L)	144 ± 0.6	144 ± 0.8	143 ± 0.8
POTASSIUM (MMOL/L)	17.6 ± 0.6 **	10 ± 0.8	9.3 ± 0.4
CHLORIDE (MMOL/L)	100 ± 0.9	102 ± 0.9	101 ± 0.6
HCO ₃ (MMOL/L)	27 ± 0.2 **	34 ± 0.8	34 ± 1
CA/C (MMOL/L)	3.5 ± 0.1 *	3.1 ± 0.1	3.0 ± 0
PROTEINS (G/L)	50 ± 2	47 ± 1	46 ± 1
ALBUMIN (G/L)	31 ± 1	30 ± 1	28 ± 0.6
BILIRUBIN (UMOL/L)	0.34 ± 0.2	0.5 ± 0.3	1.2 ± 1
CHOLESTEROL (MMOL/L)	1.6 ± 0.9	1.4 ± 0.1	.5 ± 0.6
ALK. PHOSP. (U/L)	242 ± 24	237 ± 20	215 ± 19
AST (U/L)	239 ± 34	266 ± 34	257 ± 42
ALT (U/L)	117 ± 30 *	48 ± 4	42 ± 3
LDH (U/L)	2350 ± 250	2542 ± 232	2634 ± 221
AGAP (MMOL/L)	16.3 ± 3 *	8.8 ± 1	7.3 ± 0.8

Table 5: Groups A and B received Allopurinol 50 mg/kg/day s.c., while Group C received an equal volume and number of injections as the treatment groups. See text for dose regimens. * p <.05, ** p<.01.

DISCUSSION AND CONCLUSIONS

This study was designed to test allopurinol's ability to improve flap survival and subsequently to indirectly investigate the role of xanthine oxidase in an ischemia reperfusion flap model. According to the mechanism of action of XO proposed by McCord³⁸⁻⁴⁰, the xanthine dehydrogenase is converted during ischemia. During reperfusion, XO is capable of metabolizing hypoxanthine and xanthine into uric acid with generation of free radicals as byproducts. This mechanism was first described using the intestinal mucosa of the cat which is rich in XO but has been questioned in models which lack a significant amount of XO. As well the models tested are usually of global ischemia. Could the XO system still play an important role as a source of free radicals in organs which lack XO activity? What about its role in partial ischemia models? Could allopurinol be beneficial to the flap by mechanisms other than through XO inhibition?

Im et al²⁸⁻³⁰ using models of global ischemia proposed XO as the major source of free radicals in the IRI of skin flaps when, they were able to both block XO activity rise and improve survival with a single dose (50 mg/kg) prior to flap elevation. They concluded that the beneficial effect of allopurinol in their model was a result of a selective blockage of the production of free radicals during reperfusion. Perhaps a single dose given before flap elevation may be all that is needed to protect the flap at the time

of reperfusion and peak free radical production in such a model. I have not tested a GI model in the rat.

Angel et al using a model of partial ischemia while not being able to document a rise in XO activity did show a rise in MDA in those skin flaps with an underlying haematoma and destined to die.⁴ They concluded that although free radicals were probably involved XO was not their source since the enzyme was not elevated. In a different study,⁵ also using a model of partial ischemia, they were able to improve survival without producing renal damage with allopurinol (100 mg/kg) for 3 days prior to flap elevation and continued treatment for 5 days postoperatively. Using a dose twice the amount required for complete XO inhibition,⁵² added to the short half life in the rodent and the long dosing interval (24 hours), leads one to suspect that the beneficial effects of allopurinol were not related to inhibition of XO generated free radicals. My data in rats support this idea since I could not improve flap survival using a dose of 50 mg/kg expected to completely inhibit XO activity in this animal.

Allopurinol has been shown to benefit the cell in a wide range of ischemia reperfusion conditions where tissue injury is thought to be free radical mediated;²⁶ however, the exact mechanisms of the beneficial effect remains unclear, especially when it is noted that allopurinol can benefit a system lacking in XO activity.^{6,34,41,50-51,67}

There are two recognized ways to prevent free radical injury from occurring: first, through a selective blockage of the pathway of generation, or second, through a non-selective scavenging effect after the radicals have been generated.

The metabolism of allopurinol is very complex and varies among different organs and species according to their particular enzymatic and renal system.^{7,20,63-64} A complete review of the pharmacokinetics of allopurinol and purine metabolism is beyond the scope of my project; however, some important points should be mentioned. Allopurinol and its active metabolite, oxypurinol, can act as inhibitors as well as substrate for the enzyme XO. It will reduce the urinary production of allantoin (pig and rats) or uric acid (humans) and increase the production of hypoxanthine and xanthine. When the enzyme is totally saturated, allantoin or uric acid excretion cannot be further reduced and, concomitantly, one will obtain unchanged levels of urine oxypurinol with progressively increasing amounts of unchanged allopurinol. In rodents, allantoin and not uric acid is the end product of purine metabolism; consequently, assessment of allopurinol's effect by measuring the decrease in blood uric acid as reported by others⁵ could be inaccurate and misleading.²⁰ Small laboratory rodents have many disadvantages for the study of purine metabolism in relation to human diseases. They normally pass their purine metabolites in much smaller volumes of urine, both in relation to their body weight and filtration rate.⁷

Because of the former, the large doses of allopurinol that are usually required to inhibit 100% of XO activity will frequently result in precipitation with crystal formation and renal damage.⁵² Although Angel⁵ did not find this my data suggest that this is true since a dose regimen of 50mg/kg, for 8 days total) produced significant renal impairment. We do not know if the renal damage was reversible since the animals were sacrificed acutely. In addition, the distribution of enzymes of purine metabolism in these rodents differ from man.^{7,34,54} Based on studies of radioactive label allopurinol, 90% of an IV or PO dose is excreted in the urine after 24 hours as unchanged allopurinol or one of its active metabolites: allopurinol riboside or oxypurinol. The remaining will be excreted in feces slowly over the next few days, and less than 1% will be incorporated into tissues. Allopurinol is rapidly excreted through the kidney, therefore having a very short half life. In humans as well as in white landrace pigs, oxypurinol is reabsorbed at the level of the renal tubular system, a phenomenon which does not occur in small rodents like the rat. This reabsorption results in a half life of more than 18 hours in the pig and man vs less than 6 hours in rats.⁶⁴ In rats, improvement in skin flap survival following a single daily dose⁵ of allopurinol is hard to interpret. The inhibition of XO should be so short that any beneficial effects are likely through other mechanisms. The complicated pharmacokinetics of allopurinol and the differences in purine metabolism between humans and rodents question the rat as a reliable experimental model in this setting. The pig, in

contrast, has skin and renal systems which structurally and functionally more closely resemble those of man.

In the current experiments, no improvement in flap survival could be detected in pigs in either the global or partially ischemic flap model at doses of allopurinol (50 mg/kg) expected to produce incomplete saturation of the XO enzyme. When the dose was increased to produce complete XO saturation (300 mg/kg), this proved to be detrimental both to the survival of the animals and the myocutaneous flaps. At this time we do not know if the deaths were due to an isolated effect of allopurinol on the pigs or a potentiation of the depressant effect of barbiturics on the myocardium.

Several alternative mechanisms of action for allopurinol have been postulated.^{17,65} Nelson⁵² has documented conversion of allopurinol into a ribonucleotide which inhibits 5-nucleotidase resulting in sparing of purine nucleotides by preventing the dephosphorylation of inosine and adenosine monophosphates. This allows more efficient ATP resynthesis making the cell more resistant to ischemia. It has also been reported that allopurinol and its active metabolite, oxypurinol, may act as a hydroxyl radical scavenger⁴⁶ or as an inhibitor of other oxidase enzymes which generate free radicals.²³ My current results do not support any of these proposed mechanisms as allopurinol did not improve flap survival, at least not at the doses tested.

Direct free radical measurements have not been performed in a skin flap model; however, a popular method of assessing free radical mediated

tissue necrosis is to quantify the amount of lipid peroxidation by measuring malonyldialdehyde production by the Thiobarbituric Acid method.^{10,24} The popularity of this method lies in its simplicity and it is because of its simplicity that several assumptions must be made when interpreting TBA-MDA results. First, most of the MDA production occurs not in vivo but during the acid heating stage of the assay. Second, several other aldehydes other than MDA could be misread as MDA. Despite taking the necessary technical precautions to decrease the error margin, we found the TBA assay to correlate poorly with tissue injury. We would recommend to those interested in measuring MDA to use the high performance lipid chromatography method which is much more accurate.³⁶ Despite the inaccuracy of our assay our results suggest further indirect evidence of free radical mediated tissue injury in these pig models.

In conclusion, in these experiments, allopurinol does not improve skin or myocutaneous flap survival in the pig or skin flap survival in the rat at the doses and models tested. Based on these and previous results showing low levels of XO in pigs and humans, it is unlikely that the xanthine oxidase enzymatic system is of major importance in free radical mediated skin flap necrosis.

CONCLUSIONS

A better understanding of the pathophysiologic mechanisms leading to tissue injury and flap necrosis is needed before the clinician has reliable means of not only preventing flap failure but of salvaging the failing flap. A rapidly growing body of scientific evidence implicates oxygen derived free radicals in the overall process; however, the mechanism by which they produce tissue injury and their precise origin still elucidates us. Five major biological sources of free radicals have been identified: a) the vascular endothelium related xanthine oxidase system, b) the activated neutrophil membrane related NADP/NADPH oxidase system, c) the disrupted mitochondrial electron transport system, d) the arachidonic acid pathway, and e) the oxidation of catecholamines. In the ischemia reperfusion injury that occurs in many organ systems, xanthine oxidase and the neutrophil appear to be the most important sources for these free radicals. Very little work has been reported regarding the role of these two potential sources in skin or myocutaneous flap models. Most of the current literature reports on the role of xanthine oxidase using rodent models which may not be the most clinically appropriate ones.

In the first phase of this project I investigated the pig buttock skin and the latissimus dorsi myocutaneous flap models for their survival patterns and tolerance to ischemia under conditions of both partial and global ischemia. A similar dose response to an ischemic insult was observed in the two flap models. The average critical ischemia times were 9 and 10 hours

respectively for the BS and LDMC flaps. Percentage of total area surviving (%TAS) in those flaps that did survive was adversely affected by increases in the ischemic interval in both flap models. Having established a reproducible model in an animal whose skin characteristics are very similar to that of humans, I was ready to begin testing the effect of ischemia on the xanthine oxidase system.

In the second group of experiments there was no significant increase in xanthine oxidase activity in ischemic skin for any of the three species tested. This is in direct contrast to previous data on the literature. In addition, and perhaps of greater clinical relevance both pig and human skin had a negligible amount of xanthine oxidase under normal as well as ischemic conditions.

In the third group of experiments I further tested the effect of allopurinol, a xanthine oxidase inhibitor, on flap survival in pigs and rats. Despite the use of several dosing regimens no benefit could be demonstrated in either animal. There was however some indirect evidence to suggest that free radicals were involved in the overall process of flap necrosis as determined by our measurements of malonyldialdehyde on pig skin.

In conclusion, in our laboratory allopurinol does not improve skin or myocutaneous flap survival in the pig or skin survival in the rat at the doses tested. Based on these results and the low levels of xanthine oxidase found

in the pig and human skin, it is unlikely that the xanthine oxidase enzymatic system is of major importance in free radical mediated skin flap necrosis. I cannot support the theory that the xanthine oxidase system is a key player in the ischemia reperfusion injury leading to flap failure in our patients. I would also like to caution those interested in flap research to carefully select an experimental model taking into consideration the possible limitations when extrapolating its behaviour to that of man.

While the present thesis was unable to define any role played by the xanthine oxidase system in the ischemia reperfusion injury in pig flaps free radical induced injury remains a significant clinical problem following prolonged periods of ischemia of flaps. It is likely that investigation of alternate sources of free radical production, such as the neutrophil and others will significantly enhance our understanding of flap pathophysiology.

REFERENCES

1. **Acland, R.D., and Trachtenberg, L. "The Histopathology of Small Arteries Following Experimental Microvascular Anastomosis." Plast. Reconstr. Surg., 59:868, 1977.**
2. **Al-Khalidi, U.A., and Chaglassian, T.H. "The Species Distribution of Xanthine Oxidase." Biochem. J. 97:318, 1965.**
3. **Angel, M.F.; Narayanan, K.; Swartz, W.M.; et al. "Deferoxamine Increases Skin Flap Survival: Additional Evidence of Free Radical Involvement in Ischemic Flap Surgery." Br. J. Plast. Surg. 39:469, 1986.**
4. **Angel, M.F.; Narayanan, K.; Swartz, W.M.; et al. "The Etiologic Role of Free Radicals in Haematoma Induced Flap Necrosis." Plast. Reconstr. Surg. 77:795, 1986.**
5. **Angel, M.F.; Ramasastry, S.S.; Swartz, W.M.; et al. "Augmentation of Skin Flap Survival With Allopurinol." Ann. Plast. Surg. 18:494, 1987.**

6. Bhimji, S.; Godin, D.V.; McNeill, J.H.; et al. "Effect of Allopurinol on Myocardial Ischemia Damage." Fed. Proc. 44:1480, 1985. (abstract).
7. Cameron, J.S.; Simmonds, H.A.; Hatfield, P.J.; et al. "The Pig as an Animal Model for Purine Metabolic Studies." In: Purine Metabolism in Man. (Ed.) P. Sperling, A. de Vries, J.B. Wyngaarden, New York, Plenum Press, 1974, p. 691.
8. 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, August, 1985.
9. Chambers, D.E.; Parks, D.A.; Patterson, G.; et al. "Xanthine oxidase as a Source of Free Radical Damage in Myocardial Ischemia." J. Mol. Cell. Cardiol. 17:145, 1985.
10. Cross, C.E. "Oxygen Radicals and Human Disease." Ann. Int. Med. 107:4:526, 1987.
11. Daniel, R.K. "Direct Transfer of Skin Flaps by Microvascular Anastomosis." MSc. Thesis, McGill University, 1973.

12. Daniel, R.K. "The Anatomical and Hemodynamical Characteristics of the Cutaneous Circulation and their Influence of Skin Flap Design." In Grabb, W.C., and Myers, M.B., (Eds.): Skin Flaps, Boston, Little, Brown & Company, pp. 111-134, 1975.
13. Daniel, R.K. "Toward an Anatomical and Hemodynamic Classification of Skin Flaps." Plast. Reconst. Surg., 56:330, 1975.
14. Daniel, R.K., and Kerrigan, C.L. "Principles and Physiology of Skin Flap Surgery." In McCarthy, J.G., (Ed.), Plastic Surgery, Montreal, W.B. Saunders. pp. 275-328, 1990.
15. Daniel, R.K., and Taylor, G.I. "Distant Transfer of an Island Flap by Microvascular Anastomoses: A Clinical Technique." Plast. Reconst. Surg., 52:111, 1973.
16. Daniel, R.K., and Williams, H.B. "The Free Transfer of Skin Flaps by Microvascular Anastomosis: An Experimental Study and a Reappraisal." Plast. Reconst. Surg., 52:16, 1973.

17. Das, D.K.; Engelman, R.M.; and Clemente, R. "Role of Xanthine Oxidase Inhibitors as Free Radical Scavengers. A Mechanism of Action of Allopurinol and Oxypurinol in Myocardial Salvage." Biochem. Biophys. Res. Commun. 148:314, 1987.
18. Douglas, W.R. "Of Pigs and Men and Research: A Review of Applications and Analogies of the Pig Suscrofan in Human Medical Research." Space Life Sci. 3:226, 1976.
19. Downey, J.M. "Species Specificity of Free Radical Source and the Pharmacological Consequences." FASEB J. 2:A1477, 1988. (abstract)
20. Ellison, G.B., and Nelson, D.J. In Ribonucleotides of Allopurinol and Oxipurinol in Rat Tissue and Their Significance in Purine Metabolism in Man. (Ed) P. Sperling, A. de Vries, and J.B. Wyngaarden, New York, Plenum Press, 1974, p.639.
21. Finseth, F., and Cutting, C. "An Experimental Neurovascular Island Skin Flap for Study of the Delay Phenomenon." Plast. Reconst. Surg. 61:412, 1978.

22. Gordon, L.; Buncke, H.J.; and Townsend, J.J. "Histological Changes In Skeletal Muscle After Temporary Independent Occlusion of Arterial and Venous Supply." Plast. Reconstr. Surg., 61:576, 1978.
23. Grisham, M.B.; Hernandez, L.A.; and Granger, D.N. "Xanthine Oxidase and Neutrophil Infiltration in Intestinal Ischemia." Am. J. Physiol. (Gastrointestinal Liver Physiology 14): G567, 1986.
24. Halliwell, B., and Guthridge, J. "The Importance of Free Radicals and Catalytic Metal Ions in Human Disease." Ch. 3, In Peroxidation, its Measurements and Significance. Molecular Aspects of Medicine. 8:2:119, 1985.
25. Hearse, D.J.; Manning, A.S.; and Downey, J.M. "Xanthine Oxidase a Critical Mediator of Myocardial Injury During Ischemia and Reperfusion." Acta Physiol. Scand. Suppl. 48:67, 1986.
26. Hernandez, L.A., and Granger, N. "Role of Antioxidants in Organ Preservation and Transplantation." Critical Care Medicine 16:5:543, 1988.

27. Huang, L.; Privalle, C.T.; Serafin, D.; et al. "Increased Survival of Skin Flaps by Scavengers of Superoxide Radical." FASEB J. 1:129, 1987.
28. Im, M.J.; Hoopes, J.E.; Yoshimura, Y.; et al. "Xanthine Acceptor Oxidoreductase Activities in Ischemic Rat Skin Flap." J. Surg. Res. 46:230, 1989.
29. Im, M.J.; Manson, P.N.; Bulkley, G.B.; et al. "Effects of Superoxide Dismutase and Allopurinol on Survival of Acute Island Skin Flaps." Ann. Surg. 201:4:357, 1985.
30. Im, M.J.; Shen, W.H.; and Pak, C.J. "Effect of Allopurinol on Survival of Hyperaemic Island Skin Flaps." Plast. Reconstr. Surg. 73:276, 1984.
31. Kerrigan, C.L., and Daniel, R.K. "Critical Ischemia Time and the Failing Skin Flap." Plast. Reconstr. Surg., 69:986, 1982.
32. Kerrigan, C.L.; Zelt, R.G.; and Daniel, R.K. "Secondary Critical Ischemia Time in Experimental Skin Flaps." Plast. Reconstr. Surg., 74:522, 1984.

33. Kerrigan, C.L.; Zelt, R.G.; Thomson, J.G.; et al. "The Pig as an Experimental Animal In Plastic Surgery Research for Study of Skin Flaps, Myocutaneous Flaps and Fasciocutaneous Flaps." Lab. Animal Sci., 36:408, 1986.
34. Ketel, L.; Grum, C.; and Myers, C. "Purine Efflux from Ischemic Rabbit Heart, Lack of Xanthine Oxidase Activity, Relevance to Allopurinol Cardioprotection." Circulation 72 (Supp. III):357, 1985.
35. Klain, G.J.; Boonner, S.J.; and Bell, W.G. "The Distribution of Selected Metabolic Process in the Pig and Human Skin." Swine In Biomedical Research. Vol.1.(Ed.) M.E. Tumbleson, Plenum Press, New York, 1986, p 667.
36. Lang, J.; Heckenast, P.; Esterbauer, H.; et al. "Detection of Malonyldialdehyde by HPLC." In Oxygen Radicals Chemistry and Biology. Walter de Gruyter & Co., Berlin, 1984, p. 351.
37. Manson, P.N.; Anthenelli, R.M.; Im, M.J., et al. "The Role of Oxygen Free Radicals in Ischemic Tissue Injury of Island Skin Flaps." Ann. Surg. 198:1:87, 1983.

38. McCord, J.M. "Oxygen Derived Free Radicals in Postischemic Tissue Injury." N. Eng. J. Med. 312;159, 1985.
39. McCord, J.M. "Oxygen Derived Free Radicals: A Link Between Reperfusion Injury and Inflammation." Fed. Proc. 46:2402, 1987.
40. McCord, J.M. "Free Radicals and Myocardial Ischemia Overview and Outlook." Free Radic. Biol. 4:9, 1988.
41. Mes, L. G. "Improving Flap Survival by Sustaining Cell Metabolism Within Ischemic Cells: A Study Using Rabbits." Plast. Reconst. Surg. 65:56, 1980.
42. Milton, S.H. "Pedicled Skin Flaps: The Fallacy of the Length to Width Ratio." Br. J. Surg., 52:502, 1970.
43. Mitsuru, U., and Mihara, M. "Determination of Malonyldialdehyde Precursor in Tissues by Thiobarbituric Acid Test." Analytical Biochemistry. 86:27, 1978.

44. Miura, T.; Yellon, D.M.; and Kingma, J. "Protection Afforded by Allopurinol in the First 24 Hours of Coronary Occlusion Is Diminished After 48 Hours." Free Radic.Biol. 4:25, 1988.
45. Monteiro-Riviera, N.A. "Ultrastructural Evaluation of Porcine Integument." In: Swine In Biochemical Research. Vol. 1. (Ed.) M.E. Tumbleson, Plenum Press, New York, 1986, p 641.
46. Moorehouse, P.C.; Grootveld, M.; Halliwell, B.; et al. "Allopurinol and Oxypurinol are Hydroxyl Radical Scavengers." FEBS Lett. 213:23, 1987.
47. Morris, S.F. "Pathogenesis and Treatment of Ischemia Induced Tissue Injury in the Latissimus Dorsi Myocutaneous Flap in Pigs." MSc. Thesis, University of Toronto, 1988.
48. Morris, S.F.; Pang, C.Y.; and Lindsay, W.K. "Deferoxamine Prevents Ischemia Induced Reperfusion Injury in Porcine Island Latissimus Dorsi Myocutaneous Flaps Subjected to Global Ischemia." 33rd Annual Meeting Plastic Surgery Research Council Meeting, San Francisco, May, 1988.

49. Morris, S.; Pang, C.Y.; Lindsay, W.K., et al. "Pathogenesis of Ischemia Induced Tissue Damage in Myocutaneous Flaps in Pigs." Presented at the Plastic Surgery Research Council Meeting, Boston, 1987.
50. Muxfeldt, M., and Schaper, W. "The Activity of Xanthine Oxidase in Hearts of Pigs, Guinea Pigs, Rats, Rabbits and Humans." Basic Res. Cardiol. 82:486, 1987.
51. Myers, C.L.; Weiss, S.J.; Kirsh, M.M.; et al. "Effects of Supplementing Hypothermic Crystalloid Cardioplejic Solution with Catalase, Superoxide Dismutase, Allopurinol or Deferoxamine on Functional Recovery of Globally Ischemic and Reperfused Isolated Hearts. J. Thorac. Cardiovasc. Surg. 91:281, 1986.
52. Nelson, D.; Bugge, C.; Crasny, H.; et al. "Formation of Nucleotides of 6-¹⁴C Allopurinol and 6-¹⁴C Oxypurinol on Rat Tissues and Effects on Uridine Nucleotide Pools." Biochem. Pharmacol. 22:2003, 1973.
53. Parks, D.A., and Granger, D.N. "Xanthine Oxidase: Biochemistry, Distribution and Physiology." Acta Physiol. Scand. Suppl. 548:87, 1986.

54. Picard-Ami, L.A.; MacKay, A.; and Kerrigan, C.L. "Pathophysiology of Ischemic Skin Flaps: Differences in Xanthine Oxidase Levels Between Rat, Pig and Man." Accepted for publication Plast. Reconst. Surg.
55. Picard-Ami, L.A.; MacKay, A.; and Kerrigan, C.L. "Effect of Allopurinol on the Survival of Pig and Rat Experimental Flaps." Plast. Reconst. Surg. (Submitted for publication).
56. Picard-Ami, L.A.; Thomson, J.G.; and Kerrigan, C.L. "Critical Ischemia Times and Survival Patterns of Experimental Pig Flaps." Plast. Reconst. Surg. 86:739, 1990.
57. Podzuweit, T.; Baun, W.; Muller, A.; et al. "Arrhythmias and Infarction in Ischemic Pig Heart Are Not Mediated by Xanthine Oxidase Derived Free Radicals." Basic Res. Cardiol. 82:493, 1987.
58. Richard, V.J.; Murray, C.E.; and Jennings, R.B. "The Xanthine Oxidase Inhibitor Oxypurinol Does Not Limit Myocardial Infarct Size After 30 Minutes of Ischemia Followed by 4 Days of Reperfusion in Dogs." FASEB. J. 2:A487, 1988. (abstract)

59. Rosner, B., Payne, M. Analysis of Variance, Fundamentals of Biostatistics. Boston, Duxbury Press, 1986.
60. Sasaki, G.H., and Pang, C.Y. "Hemodynamics and Viability of Acute Neurovascular Island Flaps in Rats." Plast. Reconstr. Surg., 65:152, 1980.
61. Schubert, W.; Hunter, D.W.; Guzman-Stein, G., et al. "Use of Streptokinase for the Salvage of a Free Flap: Case Report and Review of the Use of Thrombolytic Therapy." Microsurgery, 8:117, 1987.
62. Silverman, D.G.; LaRossa, D.D.; Barlow, T.G.; et al. "Quantification of Tissue Fluorescein Delivery and Prediction of Flap Viability with the Fiberoptic Dermofluorometer." Plast. Reconstr. Surg., 66:545, 1980.
63. Simmonds, H.A.; Hatfield, P.J.; Cameron J.S.; et al. "Metabolic Studies of Purine Metabolism in the Pig During the Oral Administration of Guanine and Allopurinol." Biochem. Pharmacol. 22:2537, 1973.
64. Simmonds, H.A.; Rising, T.J.; Cadenhead, A.; et al. "Radioisotope Studies of Purine Metabolism During Administration of Guanine and Allopurinol in the Pig." Biochem. Pharmacol. 22:2553, 1973.

65. Simpson, P.J.; Mickelson, J.K.; and Lucchesi, B.R. "Free Radical Scavengers in Myocardial Ischemia." Fed. Proc. 46:2413, 1987.
66. Strock, P.E., and Majno, G. "Microvascular Changes in Acutely Ischemic Rat Muscle." Surg. Gynec. & Obst., 129:1213, 1969.
67. Yellon, D.M.; Richard, V.; Hearse, D.J.; et al. "The Effect of Allopurinol on Myocardial Infarct Size in Rats vs Rabbits. The Contribution of Xanthine Oxidase." Fed. Proc. 44:1480, 1985. (abstract)
68. Zelt, R.G.; Olding, M.; Kerrigan, C.L.; and Daniel, R.K. "Primary and Secondary Critical Ischemia Time of Myocutaneous Flaps." Plast. Reconstr. Surg., 78:498, 1986.