

**REDUCTION OF POST-SURGICAL PERICARDIAL
ADHESIONS USING A PIG MODEL**

Thesis submitted by

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in

January 2005

in fulfillment of the requirements for the research degree of

Master of Medicine

in the School of Veterinary and Biomedical Sciences

and School of Medicine

James Cook University

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ACKNOWLEDGMENTS

I would like to sincerely thank Professor Phillip Summers for his kind guidance, supervision and provision of facilities and operating funds for this project. I also thank Drs. Ben Bidstrup and Mo Diqer for their contribution and advice throughout the study period. My sincere appreciation goes to Ms. Virginia Boon, School of Pharmacy and Molecular Sciences for her major contribution in establishing the assays and measuring plasma concentrations of indomethacin, rofecoxib, prostaglandin E2 and thromboxane.

I appreciate the kind assistance of Mr. Chris Coleman and Mr. Scott Blyth, Animal Technicians from the School of Veterinary and Biomedical Sciences at James Cook University. Both were responsible for the husbandry of the animals and provided assistance in preparing the animals for surgery and collection of blood. I sincerely thank Dr. Belinda Leslie and Dr. John Potter of the Townsville Veterinary Clinic who provided anaesthesia for the pigs throughout the study.

My appreciation also goes to Dr. David Williams, chief anatomical pathologist at The Townsville Hospital for his histopathological examination of cardiac tissue.

I would also like to thank Ms. Susan Wright and Mr. Paul Jacob, clinical photographers, The Townsville Hospital for their efforts in the preparation and editing of the images throughout the project. My thanks are extended to Dr. Mike Steele of James Cook University-Cairns who gave advice on the statistical analysis of the results.

I also acknowledge the kind assistance of the following companies and institutions for their support during this study:

- The Private Research Trust Fund, The Townsville Hospital for providing me with a two year scholarship and funding for the project.
- The staff at the School of Veterinary and Biomedical Sciences, James Cook University, Townsville.
- The Townsville Vet Clinic.

- Tag Medical Australia, specifically Mrs. Marie Pearce for supplying the Co-Seal throughout the study period.
- Johnson & Johnson, specifically Ms.Cathy Shelly, for their kind donation of all the suture material used.

ABSTRACT

The aim of this study was to reduce pericardial adhesions after open-heart surgery thus enabling re-sternotomies to be much safer and less time consuming for the surgical team. A pig model was developed to test the effects of non-steroidal anti-inflammatory drugs (NSAIDs) and a barrier method in reducing post-surgical pericardial adhesions. Four groups (11 per group) of pigs 8-12 weeks of age were used. Group one was the control group, Group two received indomethacin, Group three received rofecoxib (also a NSAID) and a polyethyleneglycol (Co-Seal) was applied to the pericardium as a barrier in Group four.

After performing a median sternotomy, an adhesion induction model was applied to maximize inflammation in the pericardium. This included abrasion of the heart surface, leaving blood in the pericardium and drying of tissues. The chest was then closed. In Group four, Co-Seal was sprayed on the heart before closure. Post-operatively, Groups two and three received indomethacin and rofecoxib respectively for five days. Plasma markers of inflammation were assessed on days 2, 5 and 10 post-operative. In each group, eight animals were re-opened after 12 weeks and three after 25 weeks to assess adhesions according to adhesion assessment scales. Tissue samples were collected for histopathological examination looking mainly at epicardial and adhesive tissue thickness.

It was observed in this study that adhesions were changed from dense to thin and more easily separable, requiring more blunt rather than sharp dissection. This was seen mainly in Group two, followed by Group four. In Group three, the changes were less in terms of amount of adhesions and tenacity change as compared to the changes in Groups two and four. Comparison was with Group one, which had the densest adhesions.

Adhesive tissue and epicardial thickness was measured. Epicardium was thinnest in Group two. Post-operative inflammatory markers, specifically PGE2 and TXB2 were inhibited mainly in Group two. Less inhibition of these markers was seen in Group three and nearly no inhibition was seen in Groups one and four. The more general

markers used (WCC, ESR and CRP) did not fully show the expected changes in the four groups. The adhesion induction model formulated in this study was successful and may be used in similar future projects.

In conclusion, this model, applied clinically, will reduce adhesions in the pericardium and retrosternal areas after surgery, rendering re-openings safer and less time consuming. Indomethacin has proven to be the best choice to achieve this following a relatively easy and short protocol of administration. The idea of giving patients indomethacin for five days only to achieve significant reduction in adhesion formation after surgery would be attractive to many surgical groups around the world as the short period of administration would minimize any side effects associated with this drug.

Significant reduction in adhesions was also seen following the application of Co-Seal. The attraction here would be the ease of use and the non-pharmacological effects of this barrier method. Rofecoxib was not as effective as indomethacin and Co-seal in adhesion reduction. Future studies in this pig model should examine the extent of adhesion formation following the combined use of indomethacin and Co-Seal.

2.11	The Pig as a laboratory model for human disease	34
2.11.1	Introduction	34
2.11.2	Comparative anatomy and physiology of the pig	34
2.11.2.1	Cardiovascular System	35
2.11.2.2	Digestive System	36
2.11.2.3	Urogenital system	37
2.11.2.4	Integumentary and Lymphatic Systems	38
2.12	Summary of literature review	39
Chapter 3	Material and Methods	41
3.1	Animals	41
3.2	Establishment of drug dosages	41
3.3	Surgical trial	42
3.3.1	Anaesthesia	42
3.3.2	Surgical Procedure	43
3.3.3	Post-operative care	47
3.4	Additional Groups	48
3.5	Inflammatory markers in peripheral blood	48
3.6	Re-openings	49
3.7	Statistical Analysis	51
Chapter 4	Gross and Microscopic Evaluation of Post-Operative Pericardial Adhesions	52
4.1	Introduction	52
4.2	Results	52
4.2.1	Effect of the surgical procedures on the animals	52
4.2.2	Adverse effects of indomethacin treatment	53
4.2.3	Housing period	54
4.2.4	Results of re-sternotomy	55
4.2.5	Gross evaluation of adhesions	55
4.2.6	Effects of omeprazole on adhesion formation	57
4.2.7	Microscopic evaluation of adhesions	59
4.3	Discussion	60
4.3.1	Adhesion Induction Model	60
4.3.2	Gross adhesion evaluation	61
4.3.3	Microscopic evaluation	62
4.3.4	Effect of indomethacin on the gastrointestinal tract	62
4.3.5	Effect of omeprazole on adhesion formation	63
4.3.6	Application of Co-Seal	63
4.4	Summary	64
Chapter 5	Plasma concentrations of anti-inflammatory drugs and inflammatory markers	65
5.1	Introduction	65
5.2	Results	66
5.2.1	Non steroidal anti-inflammatory plasma levels and inflammatory inhibition	66

5.2.1.1	Indomethacin	66
5.2.1.2	Rofecoxib	67
5.2.2	Coagulation screen	67
5.2.2.1	Activated Partial Thromboplastin Time	68
5.2.2.2	Prothrombin Time	69
5.2.2.3	International Normalization Ratio	69
5.2.3	Inflammatory markers results before and after surgery in all groups	70
5.2.3.1	White cell counts	70
5.2.3.2	Erythrocyte sedimentation rate	71
5.2.3.3	C- reactive protein concentrations	73
5.2.3.4	Prostaglandin E2 concentrations	75
5.2.3.5	Thromboxane B2 concentrations	76
5.3	Discussion	77
5.4	Summary	81
Chapter 6	General Discussion	82
6.1	The pig model	83
6.2	Selection of drug doses	84
6.3	Surgical procedures, induction and assessment of cardiac Adhesions	85
6.4	Inflammatory markers in peripheral blood	87
6.5	Coagulation screen assessment	88
6.6	Future research directions	89
6.7	Conclusion	90
References		91

LIST OF TABLES

	Page
Table 2.1 Grading system for pericardial adhesions (Seegar <i>et al.</i> 1997)	13
Table 2.2 Scoring system for adhesive bands.....	13
Table 2.3 Inflammatory actions of eicosanoids.....	26
Table 3.1 Relation between animal weight and endotracheal tube size.....	43
Table 4.1 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for each group re-opened after 12 weeks.....	56
Table 4.2 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for each group re-opened after 25 weeks.....	56
Table 4.3 Probability values of the comparison of the average value for APS and ATTS of each of the treatment groups with the control group in all animals.....	56
Table 4.4 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for the additional groups treated with omeprazole.....	56
Table 4.5 Average thickness in millimeters of epicardium in all groups according to time of re-opening.....	59
Table 4.6 Average thickness in millimeters of retrosternal adhesions in all groups according to time of re-opening.....	59
Table 4.7 Probability values of the comparison of the thickness in millimeters of epicardium of each of the treatment groups with the control group in all animals according to time of re-opening.....	60
Table 4.8 Probability values of the comparison of the thickness in millimeters of retrosternal adhesions of each of the treatment groups with the control group in all animals according to time of re-opening.....	60
Table 5.1 Relationship between indomethacin plasma concentration and plasma concentration of prostaglandin E2 and thromboxane B2 in pig 1.....	67

Table 5.2	Relationship between rofecoxib plasma concentration and plasma concentration of prostaglandin E2 and thromboxane B2 in pig 2.....	68
Table 5.3	Average values for the activated partial thromboplastin time in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).....	68
Table 5.4	Average values for the prothrombin time in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).....	69
Table 5.5	Average values for the international normalization ratio in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).....	69
Table 5.6	Probability values for the activated partial, thromboplastin time change after surgery between all groups.....	69
Table 5.7	Probability values for the prothrombin time change after surgery between all groups.....	70
Table 5.8	Probability values for the international normalization ratio change after surgery between all groups	70
Table 5.9	Mean (\pm SEM) white cell counts $\times 10^9/L$ for the four groups of pigs....	71
Table 5.10	Mean (\pm SEM) erythrocyte sedimentation rate (mm/hr) values for the four groups of pigs.....	73
Table 5.11	Mean (\pm SEM) C-reactive protein (mg/L) concentrations for the four groups of pigs.....	75
Table 5.12	Mean (\pm SEM) prostaglandin E2 (pg/ml) values for the four groups of pigs.....	76
Table 5.13	Mean (\pm SEM) thromboxane B2 (pg/ml) concentrations for the four groups of pigs.....	78

LIST OF FIGURES

	Page
Figure 2.1 Dilated blood vessel during acute inflammation (taken from www.med.und.nodak.edu/depts/path/pathlab/inflammation).....	15
Figure 2.2 Chemical mediators of inflammation (adapted from Cotran <i>et al.</i> 1994).	19
Figure 2.3 Scanning electron microscopy of a moving leukocyte showing the pseudopod (taken from Cotran <i>et al.</i> 1994)	22
Figure 2.4 Generation of arachidonic acid metabolites and their roles in inflammation (taken from Cotran <i>et al.</i> 1994).....	25
Figure 3.1 Heart exposed after opening the pericardium.....	44
Figure 3.2 The placement of aortic and atrial sutures.....	46
Figure 4.1 Group 1 (control) showing extensive adhesions on re-opening the sternum.....	58
Figure 4.2 Group 2 (indomethacin) with thin adhesions and a coronary artery clearly visible	58
Figure 4.3 Group 4 (Co-Seal) with the heart easily dissected off the pericardium...58	58
Figure 4.4 Group 3 (rofecoxib) with more adhesions as the heart was dissected....58	58
Figure 5.1 Mean total white cell count in each group before and after surgery (N = 4 per group).....	72
Figure 5.2 Mean erythrocyte sedimentation rate before and after surgery in each group (N = 4 per group).....	73
Figure 5.3 Mean C-reactive protein concentration before and after surgery in each group (N = 4 per group).....	75
Figure 5.4 Mean prostaglandin E2 concentrations before and after surgery in each group (N = 4 per group).....	76
Figure 5.5 Mean thromboxane B2 concentrations before and after surgery in each group (N = 4 per group)	78

ABBREVIATIONS

AA	Arachidonic acid
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APS	Adhesion percentage scale
APTT	Activated partial thromboplastin time
ASD	Atrial septal defect
ATP	Adenosine triphosphate
ATTS	Adhesive tissue tenacity scale
CAG	Coronary artery grafting
Camp	3', 5'-adenosine monophosphate
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
DAG	Diacylglycerol
ECG	Electrocardiogram
EDTA	Ethylene diamine tetra acetic acid
ELAM-1	Endothelial-leukocyte adhesion molecule-1
ESR	Erythrocyte sedimentation rate
HETEs	Hydroxyeicosatetranoic acids
HPETEs	Hydroperoxyeicosatetranoic acid compounds
HPLC	High pressure liquid chromatography
IC	Inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IL-1	Interleukin 1
IL-8	Interleukin 8
INR	International normalization ratio
IP3	Inositol-1,4,5-triphosphate
LFA	Lymphocyte function-associated antigen

LTA4	Leukotriene A4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MANOVA	Multivariate analysis of variance
MRNA	Messenger ribonucleic acid
N/D	Not detectable
NSAIDs	Non-steroidal anti-inflammatory drugs
NSW	New South Wales
NZ	New Zealand
PEG	Polyethyleneglycol
PGD2	Prostaglandin D2
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PGF1	Prostaglandin F1
PGF2	Prostaglandin F2
PGI2	Prostaglandin I2
PIP2	Phosphatidylinositol-4, 5-biphosphate
PMN	Polymorphonuclear
PT	Prothrombin time
QLD	Queensland
SEM	Standard error of mean
SRS-As	Slow-reacting substances of anaphylaxis
TNF	Tumor necrosis factor
TPA	Tissue plasminogen activator
TxA2	Thromboxane A2
TxB2	Thromboxane B2
VIC	Victoria
VSD	Ventricular septal defect
WA	Western Australia
WBC	White blood cells
WCC	White Cell Count
5-LO	5-lipoxygenase

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Cardiac surgery and pericardial adhesions

The pericardial sac contains the heart and great vessels and forms the middle mediastinal cavity of the chest. Cardiac surgery includes any surgical procedure performed on the heart, pericardium and great vessels within the pericardial sac. This inevitably involves opening the pericardium to access the contents.

Cardiac surgery has increased in popularity over the decades worldwide. In Australia, the number of coronary artery grafting (CAG) procedures has risen dramatically over the past three decades. According to the National Heart Foundation and the Australian Institute of Health and Welfare, in 1970 the number of CAG procedures was only 50 nationwide. In 2000, the number had risen to 17,117 cases raising the rate of operations performed per million populations from 4.0 in 1970 to 890 in the year 2000. The number of heart valve procedures has also risen from 1290 cases in 1976 (91.9 procedures per million population) to 5134 (267.0 procedures per million population). Out of this large number of patients undergoing open-heart surgery every year, 10-20% will require a re-operation at some stage (Hendrix *et al.* 2001). Re-operations are usually due to progression of coronary artery disease in vessels that were not grafted during the first operation, blockage of grafts or failure of implanted heart valves.

During the first operation, tissues are handled, dissected and are exposed to bleeding. This enhances the post-operative inflammatory response leading to adhesion formation in the pericardial sac and in the retro-sternal tissues.

1.2 Complications of post-operative adhesion formation

Post-operative adhesions in general are a cause of increased morbidity and mortality. Abdominal adhesions can be a major cause of post-operative pain, intestinal obstruction, infertility and prolonged hospital stay. Following surgery on the heart, there are usually no clinical complications of adhesion formation, but problems arise

on re-sternotomy in repeat operations. Adhesions form between the epicardium and surrounding structures such as the pericardium, and the sternum. Patients undergoing a second or third time re-sternotomy are at risk of potential injuries to the heart, great vessels, and coronary grafts during re-entry. These injuries mainly occur on re-opening the sternum and dissecting the retrosternal adhesive tissue. The adhesions can thus severely complicate re-operations by impeding orientation and visibility, allowing injury to vital structures, increasing the amount of blood loss and prolonging operative time (Hendrix *et al.* 2001).

With open-heart surgery becoming increasingly prevalent, the incidence of re-operations is also increasing, rendering pericardial adhesions a growing concern. Open-heart surgery in the paediatric population, especially those requiring staged re-operations highlights problems that face surgeons on a daily basis.

1.3 Adhesion formation

Adhesion formation simply means the development of abnormal attachments between tissues and organs as the end result of the inflammatory response to surgical and non-surgical tissue trauma. This is part of the normal healing process after injury and is found in various degrees in daily surgical practice.

Adhesions can start forming within a few hours after surgery. Routine surgical procedures involve various degrees of tissue handling, including abrasion, desiccation, ischaemia, bleeding and exposure to foreign material. Any of these factors can initiate an inflammatory response, which eventually leads to adhesion formation.

One of the most potent stimuli for the initiation of an inflammatory response and thus adhesion formation is surgical trauma. This is clearly seen in the peritoneal cavity following abdominal surgery and the pericardial cavity following cardiac surgery. Ischaemia was also shown to be a powerful inflammatory stimulus where ischaemic tissue undergoes neovascularization with the adhesive tissues (Ellis 1962). Other causes of adhesion formation include foreign bodies left in these cavities after surgery, the commonest being suture material. The adhesive bands are usually denser around these areas. Powdered surgical gloves leave magnesium silicate (talc) and

starch, which also enhance the inflammatory response and eventually adhesion formation (Ellis 1971).

1.4 Reduction of adhesion formation

Many studies have been tried to reduce or even totally prevent adhesion formation by either antagonizing the inflammatory response using non-steroidal (NSAIDs) and steroidal anti-inflammatory drugs, or by using various barrier techniques to separate inflamed tissues. Most of these studies have targeted peritoneal adhesions rather than pericardial. Steroids have been abandoned as an unattractive post-operative medication because they have been associated with major wound healing problems such as increase incidence of dehiscence and infection. This was clearly demonstrated in a study by Vander Salm and his team in 1986 where they used methylprednisone to inhibit pericardial adhesions. Non-steroidal anti-inflammatory drugs on the other hand have been investigated in depth to inhibit adhesion formation.

The principle pharmacological effect of NSAIDs is their ability to inhibit prostaglandin synthesis by blocking the activity of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Most NSAIDs currently used are inhibitors of both isoenzymes, though they vary in the degree of inhibition of each (Rang *et al.* 1999). Their therapeutic effectiveness as analgesics, antipyretics, anti-inflammatories and anti-thrombogenics is due to their inhibition of prostanoid synthesis, which also accounts for their side effect profile. Highly selective COX-2 inhibitors are currently available, giving maximum anti-inflammatory response without the negative effects of COX-1 inhibition, namely gastrointestinal and renal side effects.

1.5 The pig model

Pigs have been increasingly utilized as biomedical research models, because they are recognized as a suitable animal model for human disease based on comparative anatomy and physiology (Swindle and Smith 2000). Pigs are used as general surgical models of most organs and systems with particular emphasis for cardiovascular research, and in recent years in transplantation and xenograft research (Swindle and Smith 2000). One of the main concerns in using animals for xenotransplantation,

apart from rejection, is the potential for cross infection. Screening of animals has to include testing for viruses, bacteria, parasites, congenital defects and other inapparent diseases such as neoplasia or metabolic dysfunctions.

Another reason for pigs being explored as models for research is the widespread availability of these animals. They are relatively easy to handle and house, and they also breed well giving large litters usually of 9 to 11 piglets. These features make the pig easy to use in research and have also improved the humane care and use of pigs by research institutions worldwide.

1.6 Hypothesis and aim of the study

The hypothesis being tested in this study was that treatment with an appropriate dose of an anti-inflammatory drug or a barrier method would reduce adhesion formation to an acceptable level. One aim of this study was not to completely prevent nor markedly reduce pericardial adhesions from forming, as this would result in poor healing of tissues. Total adhesion prevention has been attempted and achieved by other surgical teams, mainly using high doses of steroids but with the heavy price of increased wound dehiscence and infection. These methods have therefore never been adopted by any surgical team and never attempted in a human model.

The objective therefore, was to change the tenacity and texture of the adhesive tissue forming after surgery from dense fibrous adhesions that obscure visibility and are extremely difficult to dissect to a thin, transparent and easily dissected tissue, rather than preventing adhesions from forming altogether.

To achieve this, both non-steroidal anti-inflammatory drugs and a barrier method in a pig model were used. Full assessment of adhesive tissue, both grossly and histopathologically was done with measurement of inflammatory markers in the blood to assess the efficacy of the anti-inflammatory regime.

CHAPTER TWO

LITERATURE REVIEW

2.1 Surgical aspects of adhesions

Adhesion formation simply means the development of abnormal attachments between tissues and organs (Ellis 1971; Holmdahl *et al.* 1997). Following any surgical procedure, adhesions form part of the healing process and therefore are found in various degrees in daily surgical practice (Ellis 1971; Monk *et al.* 1994).

Adhesive tissue occurs due to an inflammatory response to surgical trauma, which can start within a few hours of surgery (Ellis 1971). Routine surgical procedures involve tissue handling that entails tissue abrasion, desiccation, ischaemia, bleeding, exposure to foreign material and overheating by lamps (Risberg 1997). All these factors cause an inflammatory response, which eventually leads to adhesion formation.

Adhesions after surgery can cause increased morbidity and re-operation risks (Stone 1993). Following surgery on the heart, adhesions uniformly form between the epicardium and surrounding structures such as the pericardium, mediastinal fat, pleura and sternum (Vander Salm *et al.* 1986). Those patients requiring re-operations are at risk of potential injuries to the heart, great vessels, and cardiac grafts during re-sternotomy. Adhesions can severely complicate re-operations by making re-entry hazardous, impeding orientation and visibility, increasing the amount of blood loss and prolonging operative time (Hendrix *et al.* 2001).

The above highlights the need for adhesion prevention. The goal of any method to reduce or prevent adhesion formation after surgery would be to reduce the incidence, area and severity of adhesive tissue while retaining normal healing and avoiding infection (Risberg 1997). More than sixty years ago, Boys (1942) described five lines of approach to reduce post-operative adhesions. These were:

1. Limitation or prevention of peritoneal injury (during surgery).
2. Prevention of coagulation of serous exudate.
3. Lysis of deposited fibrin (promoting fibrinolysis).
4. Separation of surfaces until the mesothelium regenerates.
5. Inhibition of fibroblastic proliferation (later extended to inhibition of inflammatory response).

2.2 Incidence of adhesions

The majority of the epidemiological work on post-operative adhesions has targeted abdominal adhesions and subsequent clinical symptoms. Cardiac adhesions present an increased risk for sternal re-entry, although assessment of pericardial and retrosternal adhesions prior to re-operations is difficult (Duvernoy *et al.* 1991). Extrapolation based on the anatomical similarity of the mesothelial pericardium and peritoneum suggests the incidence of cardiac adhesions would be similar after cardiac surgery to that following abdominal surgery (Duvernoy *et al.* 1991; Mitchell *et al.* 1994).

Although not all adhesions are the result of surgery, the majority are directly attributable to surgical traumas. Potentially serious clinical complications can occur due to adhesion formation including intestinal obstruction, infertility, pain and difficult re-operative procedures (Canver *et al.* 1993). Most epidemiological work has been on abdominal complications as these patients present with clinical problems of adhesions. Cardiac adhesions on the other hand are silent with no clinical complications, yet they pose a major challenge in re-operations.

Post-surgical adhesions are the chief cause of intestinal obstruction in the Western world, making this a major point for epidemiological research (Holmdahl *et al.* 1997). Menzies (1992) reviewed the incidence of small and large bowel obstruction between 1952 and 1990 in the United States and the United Kingdom. He found that more than 30% were caused by peritoneal adhesions. Abdominal adhesions are the

predominant cause of small bowel obstruction, accounting for 54% to 74% of cases (Menzies 1993).

The majority of such adhesions are attributed to previous surgery (80-90%) with 5-20% arising from inflammatory causes and only 2-5% being congenital (Menzies 1992). Approximately 20% of such adhesive intestinal obstructions occur one-month post-operative and 30% within one year (Menzies 1992). Early small bowel obstruction (within first few weeks) is uncommon, but is a serious complication with a high mortality rate of 17.8% (Stewart *et al.* 1987). In the area of cardiac surgery, it is known that 10-20% of patients undergoing aortic valve replacement and coronary artery bypass grafting will require a re-operation at a later stage in their lives (Hendrix *et al.* 2001).

The prevalence of adhesion-related problems illustrates the need for continued research in this area. Unfortunately, current evidence suggests lack of concern for this ongoing problem. In 1992, Holmdahl and Risberg documented the current concept on adhesion prevention among Swedish surgeons through a postal questionnaire; they found that 78% of respondents had never tried to prevent nor reduce adhesion formation.

2.3 Adhesive problems in cardiac surgery

The consequences of adhesions are directly related to their anatomy. The burden of illness resulting from intra-abdominal adhesions is easily assessable, however the burden of cardiac adhesions is less easily realized (Vander Salm *et al.* 1986). A plethora of studies exist on post-operative peritoneal adhesions, but there is little published work that has assessed the effects of surgical procedures on the formation of cardiac adhesions (Vander Salm *et al.* 1986).

Open-heart surgery is becoming increasingly common and with that the incidence of re-operations, rendering cardiac adhesions a growing concern (Loop 1984; Loging *et al.* 1999; Rao *et al.* 1999). Coronary artery bypass surgery illustrates this risk with one in five patients requiring re-operations (Loging *et al.* 1999). Another cohort of patients affected by adhesions is the pediatric population, where patients are likely to have a scheduled re-operation (Malm *et al.* 1992).

The difficulty in assessing the significance of such scar tissues after cardiac surgery arises from the lack of immediate or obvious morbidity, with the major effects appearing during re-operations (Seeger *et al.* 1997). In repeat coronary bypass surgery, the post-operative complication rate is nearly double the risk of the initial procedure (Loging *et al.* 1999).

The presence of fibrotic adhesions is particularly hazardous between the sternum and the anterior ventricular wall (retrosternal adhesions) (Mitchell *et al.* 1994). This may cause the right ventricle to adhere to the overlying sternal table, making re-sternotomy a challenge (Mitchell *et al.* 1994). The associated difficulty with re-sternotomy and the requirement for surgical adhesiolysis significantly increases operative time (Seeger *et al.* 1997; Loging *et al.* 1999; Hendrix *et al.* 2001). This complicated surgical adhesiolysis can cause injury to the right ventricle, aorta, right atrium, and innominate vein or to an aorto-coronary graft in 2-6% of patients (Loop 1984; Okuyama *et al.* 1998; Okuyama *et al.* 1999). The surgeon's vision can be dramatically obscured by the presence of dense adhesive tissue making identification of vessels difficult and increasing the risk of injury and bleeding (Malm *et al.* 1992). Another consideration with post-operative adhesion formation is the possibility of reducing cardiac output and decreasing coronary graft patency (Bailey *et al.* 1984; Seeger *et al.* 1997), although this is questionable to many in the cardiac surgical community.

Adhesion related problems are difficult to assess but associated morbidity and increased workload incurs considerable healthcare expenditure (Holmdahl *et al.* 1997).

2.4 Economic burden of adhesions

Problems associated with post-operative adhesions pose a significant financial burden as they increase surgical workload and utilize limited health resources (Panay and Lower 1999). Awareness concerning economic consequences resulting from adhesions is rising (Monk *et al.* 1994). A Swedish study estimated the costs incurred for total care, including sick leave expenditure, for adhesive small bowel obstruction was at least US\$20 million a year. Another study in the United States looked at 1988 cases of abdominal adhesion associated complications and found that hospitalization

and treatment costs was up to US\$1.2 billion (Ray *et al.* 1993). These studies did not include outpatient and indirect costs such as loss of function due to long-term disabilities. Although these added costs due to adhesions do not apply to cardiac surgery, they are very evident in other specialties such as general surgery.

2.5 Mesothelial repair

Adhesion formation should be considered a normal pathophysiological response in the mesothelial healing process (Tomizawa 1994). Mesothelial healing occurs in both the pericardium and peritoneum.

Anatomically, the pericardium is a fibroserous membrane of mesothelium and submesothelial connective tissue (Dirckx 1997). It is composed of two layers, the visceral pericardium that immediately coats the heart and great vessels and the parietal pericardium that forms a sac containing the heart and is composed of strong fibrous tissue lined with a serous membrane (Dirckx 1997). A continuous cellular mesothelial layer covers mesenchymal tissue containing blood vessels, collagen and elastin fibers and numerous inflammatory cells such as macrophages.

For many years, it has been recognized that mesothelium possessed excellent regenerative properties. Using a rat model, Ellis *et al.* (1965) established that, unlike dermal defects, both large and small peritoneal defects healed equally fast. Although the process of regeneration of the mesothelial surface of the peritoneum has been investigated extensively, it is still not completely understood for several reasons, including interspecies differences in peritoneal physiology, limitation of animal models and the complexities of interperitoneal circulation and transperitoneal transport.

However, most researchers agree on certain basic facts based on animal research (Raftery 1973; Queralt *et al.* 1987). A layer of macrophages forms over the peritoneal wound approximately 48 hours after injury. Mesothelial cells migrate either from neighboring surfaces or develop from islands of epithelial cells that attach to the wound surface and then proliferate. Large and small defects repair in the same time, and mesothelial healing is complete at approximately seven days.

Therefore, the sequence and timing of mesothelial repair can be summarized as follows:

During the first 12-36 hours following injury, the lesion is infiltrated with phagocytic cells predominantly macrophages. A single macrophage layer forms over the wound surface shortly after injury. These cells are gradually replaced by new mesothelium during the next three to seven days after injury. The wound remains potentially adhesiogenic due to the loss of continuous mesothelial layer for approximately three days after injury.

2.6 Mechanisms of adhesion formation

2.6.1 Causative factors

The most potent stimulus for adhesion formation is peritoneal or pericardial trauma. In animal studies, ischaemia was also shown to be a powerful adhesion stimulus with blood vessels within the adhesions growing into ischaemic tissue (Ellis 1962).

Other causes of adhesion formation include foreign bodies such as suture material, magnesium silicate (talc) and starch from surgical gloves. Lint from surgical gauze also can cause adhesions to form probably because it contains silica, which can cause desquamation of mesothelial cells (Ellis 1971). These foreign bodies tend to accumulate in the traumatized mesothelium and hence, in combination with other factors, possibly prevent the resorption of fibrinous adhesions (Ellis 1971).

2.6.2 Adhesion formation

This is a complex process and involves the absence or modification of fibrinolytic mechanisms with the migration and proliferation of a variety of cell types, including inflammatory cells, mesothelial cells and fibroblasts. In addition, extracellular matrix is synthesized and deposited.

Classically, adhesion formation is triggered by an inflammatory response to tissue injury that releases a fibrin-rich serous exudate consisting of chemical mediators, such as activating factors (prostaglandins, lymphokines, lysozymes), chemotactic agents, permeability factors (bradykinin, serotonin, histamine), and tissue

thromboplastin. This fibrinous coagulum induces the development of fibrinous adhesions between adjacent viscera as early as three hours after the initial insult (Trompke and Signer 1956). Fibrinolysis is required to remove this fibrin matrix. The balance between fibrin deposition and degradation appears to be an important determinant of the events leading to the formation of adhesions (Holmdahl *et al.* 1994).

The fibrinolytic agent of intact peritoneal mesothelial cells has been identified as tissue plasminogen activator (tPA) (Myrhe-Jensen *et al.* 1969), which is diminished following trauma (Porter *et al.* 1969). In addition to decreasing the levels of tPA, tissue injury during surgery also increases the level of plasminogen activator inhibitor, which further inhibits fibrinolysis.

Permanent adhesions form where there is a prolonged suppression in fibrinolytic activity due to peritoneal injury (Pijfman *et al.* 1994). At one to three days, the adhesion is composed of various cellular elements encased in a fibrin matrix. This matrix is gradually replaced by granulation tissue that contains macrophages, fibroblasts, and multinucleated giant cells. Initially there is no evidence of mesothelial cell attachment to the surface of the adhesion. By day four, most of the fibrin is gone, and a larger number of fibroblasts and associated collagen is present in the fibrous attachment.

After day three, macrophages are the predominant leukocyte and are evident in the fibrin mesh, along with a few fibroblasts. At day five, the fibrin network has become generally organized and contains distinct bundles of collagen and fibroblasts. In addition, small vascular channels containing endothelial cells are present. During days five through to ten, fibroblasts become aligned within the adhesion, while collagen deposition and organization advances. At two weeks, the relatively few cells present are predominantly fibroblasts. One to two months after injury, the collagen fibrils become organized into discrete bundles interposed by fibrocytes and a few macrophages. Eventually, the adhesion matures into a fibrous band, often containing small nodules of calcification. Extensive well-defined adhesions often are covered by mesothelium and contain blood vessels and connective tissue fibers, including elastin (Dizerega 1994).

2.7 Assessment of adhesions

When comparing results of different studies on adhesion prevention, there is a lack of uniformity between adhesion assessment criteria used by investigators. Research requires standardization through the use of specific methods for adhesion quantification and definition. Assessment therefore must include the type of adhesions, range, time interval prior to assessment, location and severity (Holmdahl *et al.* 1997). Adhesion examination must be standardized with respect to the animal species used, site of formation and treatment specific parameters (e.g.: dose, administration). Ideally, the assessment should be conducted using both qualitative and quantitative analysis.

The presence of adhesions and visual assessment is the main determinant, and is crucial. In a study by Seeger *et al.* (1997), adhesion formation in the pericardium was assessed by three to four observers blinded to the treatment protocol, using a clinical grading scale from zero to four (Table 2.1). In addition, photographic evidence was taken for further comparison and documentation. In 1995, Golan *et al.* tested aspirin in rodents as a preventor for post-operative adhesions. They used a single uniform scoring system based on the parameters of width, strength and thickness (Table 2.2). Here, scores (points) would be added depending on adhesive tissue tenacity and then graded accordingly from 0 to 3. Such a scoring method incorporates both the extent and the tenacity of the adhesions and is markedly different from the many systems used including the system used by Seeger *et al.* (1997).

A study reported by Loging *et al.* (1999) used the same clinical assessment method as Seeger *et al.* (1997), and despite using the same method, the results of the study were much stronger due to the addition of quantification of adhesion formation, using biochemical and histochemical analysis to verify the graded adhesions made by the blinded observers.

Table 2.1 Grading system for pericardial adhesions (Seeger *et al.* 1997).

Pericardial / Epicardial adhesion score	Observation
0	No adhesions
1	Filmy adhesions with easily identifiable plane
2	Mild adhesions with freely identifiable plane
3	Moderate adhesions with difficult plane of dissection
4	Dense adhesions with no plane of dissection

Table 2.2 Scoring system for adhesive bands.

Score (point)	Width (mm)	Thickness (mm)	Strength
1	<2	<1	+
2	2 – 10	1 – 3	++
3	>10	>3	+++

Strength was determined by force needed for tissue separation.

Total scores: Grade 0 = no adhesions (0 points).

Grade 1 = mild adhesions (3 points).

Grade 2 = moderate adhesions (4-6 points).

Grade 3 = severe adhesions (7-9 points).

Quantification of adhesion extensiveness has been conducted using many different methods including:

- Determination of hydroxyprolene content (Loging *et al.* 1999).
- Light microscopy examination (Luciano *et al.* 1983; Vander Salm *et al.* 1986).
- Roentgenograms to determine retrosternal space (Rao *et al.* 1999).
- Fibrinolytic capacity in a biopsy by measurement of various tissue markers (Doody *et al.* 1989; Ivarsson *et al.* 1998).
- Computed Tomography (Duvernoy *et al.* 1991).
- Magnetic resonance imaging (Duvernoy *et al.* 1991).
- Electron microscopy (Treutner *et al.* 1995).
- Measurement of inflammatory marker levels in blood (Malm *et al.* 1992).

While not all the above-mentioned methods of testing are appropriate for every treatment, there is a need for quantification to verify the qualitative findings.

In summary, the scoring system should include several features. A qualitative assessment of the results should be employed through an interval grading system (with a minimum number of intervals) by multiple investigators who are blinded to the treatment group. The grading system should assess the extent of the adhesions (percentage involvement of the affected organ and the anatomy of the surrounding organs) and certain physical characteristics such as strength and tenacity.

Photographic documentation is necessary in case of discrepancy between the assessors. Quantitative assessment of the results should also be employed and these should be specific to the treatment used for adhesion prevention.

Analysis of the various inflammatory processes involved in adhesion formation is not only important for comparison of research findings, but also to fully understand the mechanism of adhesiogenesis and thereby focus future research projects.

2.8 Adhesion re-formation

Most of the research currently being conducted is focused on post-operative adhesion prevention. This is due to the fact that there are no current methods to manage adhesions once they are formed and thus prevention is inherently important.

It is noteworthy that in early re-operations (within around one week of the initial procedure), adhesion formation is still early and dissection is relatively easy (Tomizawa 1994). Once these adhesions have been manually separated, they have a great capacity for reformation. The attachment site of the adhesions is important with regard to their pathogenesis, however, the divided ends of the adhesive tissues represent the site where adhesions typically reform (Group 1991).

A study by Ivarsson *et al.* (1998) investigated the fibrinolytic capacity of adhesive tissue. The authors found that any reduction in this capacity in the peritoneum or in the adhesive tissue itself was associated with increased incidence of formation and reformation of adhesions. This shows that research conducted into adhesion

prevention should also focus on adhesion reformation, since the inflammatory components involved appear to be related, if not the same.

2.9 Role of inflammation in adhesion formation

The inflammatory response can be divided into acute and chronic inflammation.

2.9.1 Acute inflammation

Acute inflammation is defined as the immediate and early response to an injurious agent (Cotran *et al.* 1994). Since the two major defensive components against microbes, antibodies and leukocytes, are normally carried in the blood stream, it is not surprising that vascular phenomena play a major role in acute inflammation.

Therefore, acute inflammation has three major components:

1. **Change in vessel caliber.** The microcirculation consists of the network of small capillaries lying between arteriole, which have a thick muscular wall, and thin-walled venules (Underwood and Stephenson 1996). Changes in the microcirculation occur as a physiologic response (Figure 2.1), for example there is hyperemia in exercising muscle and active endocrine glands. In the skin, this has been shown as the triple response to a blunt injury involving first arteriolar vasoconstriction leading to a momentarily white line followed by a flush, a dull red line due to capillary dilatation. This is followed by the flare stage, a red irregular surrounding zone due to arteriolar dilatation. The end component of this acute inflammatory response is the wheal, a zone of oedema due to fluid exudation into the extravascular space (Underwood and Stephenson 1996).



Figure 2.1 Dilated blood vessel during acute inflammation. (taken from www.med.und.nodak.edu/depts/path/pathlab/inflammation)

2. **Increased vascular permeability.** There is a transient increase in endothelial cell barrier permeability. Electron microscopic examination of venules and small veins showed that gaps of 0.1-0.4 micrometers in diameter had appeared between endothelial cells. These cells are not damaged during this process as they contain contractile proteins such as actin which when stimulated cause contraction of the endothelial cells, pulling open the transient pores (Underwood and Stephenson 1996). This results in leakage of fluid from the intravascular to the extravascular spaces, termed oedema.

The normal Starling forces are not particularly effective in moving this fluid back into the vessels because of a decrease in hydrostatic pressure within the vessel and an increase in colloid osmotic pressure in the extravascular fluid due to the increase in protein content. Most of this leakage is at the level of the post-capillary venules (Sopher 2003). The loss of fluid from the intravascular compartments as blood passes through the capillary venules leads to local stasis and plugging of dilated small vessels with erythrocytes. These changes are reversible following mild injury, and within several minutes to hours the extravascular fluid is cleared through the lymphatics (Rubin *et al.* 1999).

The escaped extravascular fluid is protein-rich (exudate) and this reduces the intravascular osmotic pressure and increases the osmotic pressure of the interstitial fluid (Cotran *et al.* 1994). The protein content of this fluid may be up to 50gm/L and the proteins present include immunoglobulins, which may be important in the destruction of invading micro-organisms, and coagulation factors, including fibrinogen, which result in fibrin deposition on contact with the extravascular tissue (Underwood and Stephenson 1996).

3. **Emigration of leukocytes.** The sequence of events in the journey of leukocytes from the lumen to the interstitial tissue, called extravasation, can be divided into margination, rolling and adhesion, all occurring in the lumen, diapedesis and thirdly migration in interstitial tissues toward a chemotactic stimulus (Cotran *et al.* 1994).

Margination and adhesion involves flowing of leukocytes in the peripheral plasmatic zone of blood vessels and adherence of neutrophils to the vascular endothelium which occurs at sites of acute inflammation is termed pavementing of neutrophils. This appears to be a specific process occurring independently of the eventual slowing of blood flow. The phenomenon is only seen in venules. Increased leukocyte adhesion results from interaction between adhesion molecules on leukocyte and endothelial surfaces.

Leukocyte surface adhesion molecule expression is increased by the complement component C5a, leukotriene B4 and tumor necrosis factor. Endothelial cell expression of endothelial-leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), to which the surface adhesion molecule of leukocytes bind, is increased by interleukin-1, endotoxins and tumor necrosis factor (Underwood and Stephenson 1996).

Neutrophils emigrate first, usually during the first 6-24 hours after the event. Monocytes emigrate later beginning after 24-48 hours (Hand 2003). Red cells may escape from blood vessels. This is a passive process and depends on hydrostatic pressure forcing the red cells out. The process is called diapedesis and the presence of a large number of red cells in the extravascular space implies severe vascular injury, such as a tear in the vessel wall (Underwood and Stephenson 1996).

Migration of leukocytes is by active amoeboid movement through the walls of venules and small veins, but they do not commonly exit from capillaries. Neutrophils, eosinophils and macrophages can insert pseudopodia between endothelial cells. The defect seems to be self sealing, and the endothelial cells are not damaged by this process (Underwood and Stephenson 1996).

2.9.2 Chronic inflammation

Chronic inflammation is inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at healing are proceeding simultaneously (Cotran *et al.* 1994).

2.9.3 Biochemical events

2.9.3.1 Chemical inflammatory mediators

When tissue is traumatized, thromboplastin (tissue factor) is released from vascular endothelium and activates the coagulation cascade (Stone 1993). The conversion of prothrombin to thrombin and the subsequent infiltration of various cells is accompanied by release of chemical inflammatory mediators including cytokines, growth factors, serotonin, histamine, lysosomal enzymes, oxygen free radicals and chemotactic factors (Liebman *et al.* 1993), all playing different roles in inflammation.

These mediators bind to specific receptors on vascular endothelium and smooth muscle cells, causing vasoconstriction or vasodilation. Vasodilation of arterioles increases blood flow and can exacerbate fluid leakage into the tissue.

At the same time, vasoconstriction of postcapillary venules increases the hydrostatic pressure in the capillary bed, potentiating oedema formation. Vasodilation of venules decreases capillary hydrostatic pressures and inhibits the movement of fluid into the extravascular spaces. Therefore, when the role of a particular vasoactive mediator in the development of inflammatory response is being examined, the effects of this mediator on specific tissues and components of the vasculature must be identified (Rubin *et al.* 1999).

The post capillary venule is the primary site at which vasoactive mediators induce endothelial changes. Binding of vasoactive mediators to specific receptors on endothelial cells results in cell activation, causing endothelial cell contraction and gap formation. This break in the endothelial barrier leads to the extravasation (leakage) of intravascular fluids into the extravascular space. Endothelial retraction with gap formation is a reversible process. Local injection of classic vasoactive mediators into the skin results in an acute change in vascular permeability that peaks between 15 and 20 minutes after injection but is corrected within one hour (Rubin *et al.* 1999).

Chemical mediators of inflammation have certain characteristics (Cotran *et al.* 1994). They originate either from plasma or from cells (Figure 2.2). Plasma derived mediators (eg complement) are present in plasma in precursor forms that must be activated, usually by a series of proteolytic cleavages to acquire their biological properties.

Cell derived mediators are normally sequestered in intracellular granules (e.g. histamine in mast cell granules) that need to be secreted or are synthesized de novo (e.g. prostaglandins) in response to a stimulus. The major cellular sources are platelets, neutrophils, monocytes/macrophages, and mast cells.

Most mediators perform their biological activity by initially binding to specific receptors on target cells. Some however, have direct enzymatic activity (eg. lysosomal proteases) or mediate oxidative damage (e.g. oxygen metabolites).

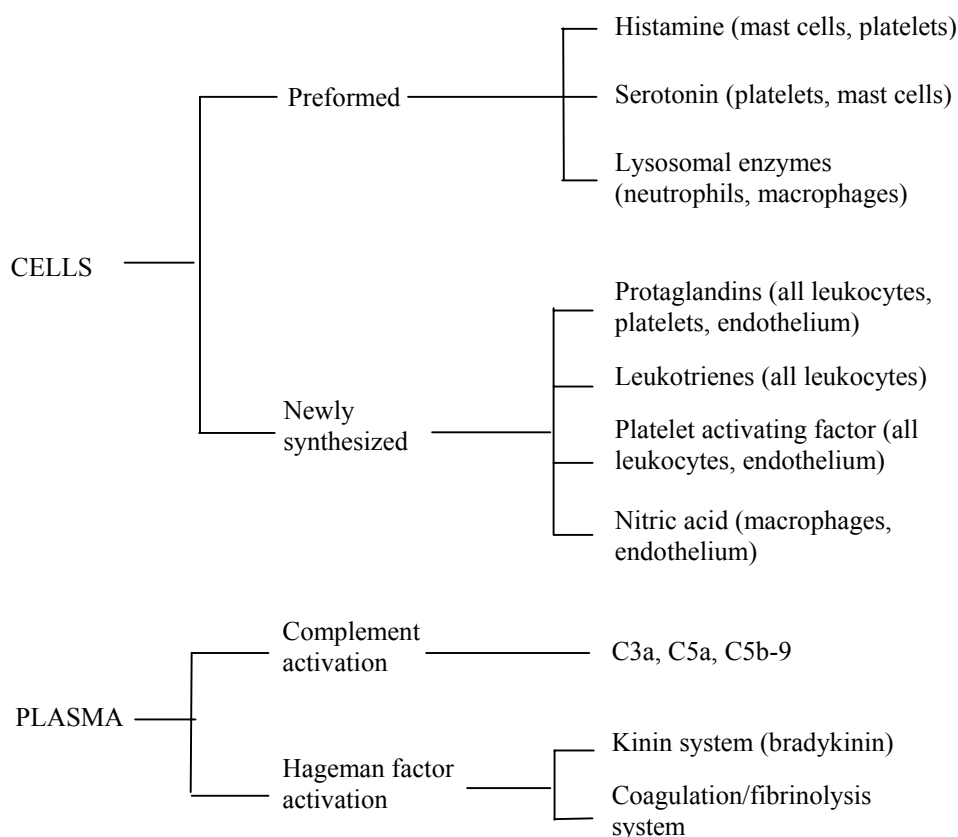


Figure 2.2 Chemical mediators of inflammation (adapted from Cotran *et al.* 1994).

A chemical mediator can stimulate the release of mediators by target cells themselves. These secondary mediators may be identical or similar to the initial mediators but may also have opposing activities. They provide mechanisms for amplifying, or in certain instances counteracting, the initial mediator action. Mediators can act on one or more target cells, have widespread targets, or may even have differing effects, depending on cell and tissue types. Once activated and released from the cell, most of these mediators are short-lived. They quickly decay (eg. arachidonic acid metabolites) or are inactivated by enzymes (eg. kinase inactivates bradykinin) or they are otherwise scavenged (antioxidants scavenge toxic oxygen metabolites) or inhibited (eg complement inhibitors). There is thus a system of checks and balances in the regulation of mediator actions. It is also important to note that most mediators may have harmful effects.

Polymorphonuclear granulocytes (PMN) are potent producers of lipid mediators via the 5-lipoxygenase (5-LO) pathway (leukotrienes) which exert important proinflammatory and immunoregulatory activities, therefore playing a key role in host defense against microbial infections. After severe trauma, PMN show cellular dysfunctions including chemotactic migration, phagocytosis, and bacterial killing (Koller *et al.* 2001).

Kolaczowska *et al.* (2002) looked at early vascular permeability in a murine model for peritonitis and the effect of resident peritoneal macrophages and mast cells. They found that the initial phase of zymosan-induced peritonitis involves an increase in vascular permeability (peak at 30 minutes) that is correlated with high levels of vasoactive eicosanoids, namely, prostaglandins (PGI₂ and PGE₂) of cyclooxygenase-1 origin and cysteinyl-leukotrienes. They concluded that the resident peritoneal macrophages were in fact the main contributors to the vasopermeability at the early stages of peritonitis.

All cells that release chemical mediators are involved in both normal and abnormal mesothelial repair, however whether adhesions form or not depends on the fibrin-plasmin balance (Stone 1993). Fibrinolysis is central to resolving inflammation and thus minimizing adhesion formation (Monk *et al.* 1994).

2.9.3.2 Chemotaxis and leukocyte activation

Following extravasation, leukocytes emigrate in tissues towards the site of injury by a process called chemotaxis, defined most simply as locomotion oriented along a chemical gradient. All granulocytes, macrophages and to lesser extent, lymphocytes respond to chemotactic stimuli with varying rates of speed (Cotran *et al.* 1994).

Both exogenous and endogenous substances can act as chemoattractants. The most common exogenous agents are bacterial and mitochondrial products. Particular peptides are those with a low molecular weight and possessing an N-formyl-methionine terminal amino acid. Others are lipid in nature. Endogenous chemical mediators include components of the complement system (particularly C5a), products of the lipoxygenase pathway (mainly leukotriene B₄) and cytokines, particularly those of the interleukin-8 (IL-8) family (Cotran *et al.* 1994; Rubin *et al.* 1999).

Many of these cytokines are produced at sites of inflammation, and those with chemotactic activity are referred to as chemokines. Two families of chemokines (alpha and beta) are defined by the molecular relationship of specific cysteine residues within the molecule. Alpha-chemokines include IL-8 and are potent chemoattractants for neutrophils. The beta-chemokines are preferentially chemotactic for monocytes (Rubin *et al.* 1999).

But how does the leukocyte 'see' or 'smell' the chemotactic agent and how do these substances induce direct cell movement? Although not all the answers are known, several important steps and second messengers are recognized. Binding of chemotactic agents to specific receptors on the cell membranes of leukocytes results in activation of phospholipase C (mediated by unique G- proteins), leading to the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP₂) to inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), and the release of calcium, first from intracellular stores and subsequently from the influx of extracellular calcium.

It is the increased cytosolic calcium that triggers the assembly of contractile elements responsible for cell movement (Cotran *et al.* 1994). The leukocyte moves by extending a pseudopod (lamellipod) that pulls the remainder of the cell towards the

direction of extension, just as an automobile with front wheel drive is pulled by the wheels in front (Figure 2.3.)



Figure 2.3 Scanning electron microscopy of a moving leukocyte showing the pseudopod (taken from Cotran *et al.* 1994).

The interior of the pseudopod consists of a branching network of filaments composed of actin as well as the contractile protein myosin. Locomotion involves rapid assembly of actin monomers into linear polymers at the pseudopod's leading edge, cross-linking of filaments followed by disassembly of such filaments away from the leading edge.

These complex events are controlled by the effects of calcium ions and phosphoinositols on a number of actin-regulating proteins such as actin binding protein (filamin), gelsolin, profilin, and calmodulin. In addition to stimulating locomotion, many chemotactic factors, particularly in high concentrations, induce other responses in leukocytes, referred to under the rubric of leukocyte activation.

Such responses which can also be induced by phagocytosis and antigen-antibody complexes, include the following:

1. Production of arachidonic acid metabolites from phospholipids, due to activation of phospholipase A2 by DAG and increased intracellular calcium.

2. Degranulation and secretion of lysosomal enzymes, and activation of the oxidative burst. These two processes are induced by DAG-mediated activation of protein kinase. Activation of intracellular phospholipase D by the increased calcium influx contributes to the sustained DAG accumulation.
3. Modulation of leukocyte adhesion molecules. Certain chemoattractants cause increased surface expression and increased adhesive avidity of the LFA-1 integrin, allowing firm adhesion of activated neutrophils to ICAM-1 on endothelium. In contrast, neutrophils shed L-selectin from their surface, making them less adhesive to the L-selectin ligand on endothelium (Cotran *et al.* 1994).

A newly appreciated phenomenon in leukocyte activation is priming, denoting an increased rate and extent of leukocyte activation by exposure to a mediator that itself causes little activation. The cytokine, tumour necrosis factor, in particular, markedly increases leukocyte activation by other chemotactic agents, accounting for its powerful *in vivo* effects (Cotran *et al.* 1994).

2.9.3.3 Arachidonic acid (AA) metabolites - Prostaglandins and Leukotrienes (Eicosanoids)

An important aspect of the inflammatory response is the arachidonic acid (AA) pathway and metabolites. Products derived from the metabolism of AA (called eicosanoids) affect a variety of biological processes, including inflammation and hemostasis. They are best thought of as autocoids, or local short-range hormones, which are formed rapidly, exert their effects locally, and then either decay spontaneously or are destroyed enzymatically (Cotran *et al.* 1994). Arachidonic acid is a 20-carbon polyunsaturated fatty acid (5,8,11,14-eicosatetraenoic acid). Depending on the specific inflammatory cell and the nature of the stimulus, activated cells generate AA metabolites by one of two pathways. One pathway involves stimulus-induced activation of the phospholipase A₂, an enzyme that cleaves AA from the glycerol backbone of membrane phospholipids. Phosphatidylcholine, an important substrate of phospholipase A₂, is the major source of AA in inflammatory cells. The other mechanism for the generation of AA metabolites is the metabolism

of phosphatidylinositol phosphates to diacylglycerol and inositol phosphates by phospholipase C. Diacylglycerol lipase then cleaves AA from diacylglycerol (Cotran *et al.* 1994; Rubin *et al.* 1999).

Arachidonic acid metabolism proceeds along one of two major pathways named after the enzyme that initiates the reactions (Figure 2.4):

1. The *Cyclooxygenase Pathway* leads to the generation of prostaglandins. These include PGE₂, PGD₂, PGF₂-alpha, PGI₂ (prostacyclin), and thromboxane A₂ (TxA₂), each of which is derived by the action of a specific enzyme. Some of these enzymes have restricted tissue distribution. For example, platelets contain the enzyme thromboxane synthetase, and hence TxA₂ is the major product in these cells (Cotran *et al.* 1994).

Thromboxane A₂, a potent platelet aggregating agent and vasoconstrictor, is itself unstable and is rapidly converted to its inactive form, thromboxane B₂ (TxB₂). Vascular endothelium, on the other hand, lacks thromboxane synthetase but processes prostacyclin synthetase which leads to the formation of prostacyclin (PGI₂) and its stable end product PGF₁-alpha.

Prostacyclin is a vasodilator and a potent inhibitor of platelet aggregation. Prostaglandin D₂ is the major metabolite of the cyclooxygenase pathway in mast cells; along with PGE₂ and PGF₂ (which are more widely distributed) it potentiates vasodilation and oedema formation (Cotran *et al.* 1994). Prostacyclin and PGE₂, owing to their vasodilating effects, have been used clinically to enhance tissue perfusion and in the lung to improve tissue oxygenation (Rubin *et al.* 1999). Aspirin and nonsteroidal anti-inflammatory agents, such as indomethacin, inhibit cyclooxygenase and thus inhibit prostaglandin synthesis. Lipoxygenase, however, is not affected by these agents (Cotran *et al.* 1994).

2. In the *Lipoxygenase pathway*, 5-lipoxygenase is the predominant enzyme in neutrophils, and the metabolites derived by its actions are the best characterized. Lipoxygenation in inflammatory cells leads to the production of hydroperoxyeicosatetraenoic acid compounds (HPETEs)

(Rubin *et al.* 1999). The hydroperoxy compounds may be metabolized to hydroxyeicosatetraenoic acids (HETEs) and the main product 5-HETE is chemotactic for neutrophils. This is converted into a family of compounds collectively called leukotrienes.

Leukotriene B₄ (metabolized from LTA₄) is a potent chemotactic agent and causes aggregation of neutrophils and macrophages. Leukotriene C₄, LTD₄ and LTE₄ cause vasoconstriction, bronchospasm and increased vascular permeability and are collectively known as slow-reacting substances of anaphylaxis (SRS-As).

Leukocytes also produce trihydroxymetabolites of AA called lipoxins. Lipoxins have both anti and pro-inflammatory effects, and their role *in vivo* is currently being pursued (Cotran *et al.* 1994; Rubin *et al.* 1999).



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Figure 2.4 Generation of arachidonic acid metabolites and their roles in inflammation (taken from Cotran *et al.* 1994).

Eicosanoids can mediate virtually every step of acute inflammation (Cotran *et al.* 1994; Rubin *et al.* 1999) and the main effects are summarized in Table 2.3.

Prostaglandin E₂ and prostacyclin are important mediators of inflammatory vasodilation. They also markedly potentiate the permeability-increasing and

chemotactic effect of other mediators and have been used clinically to improve tissue perfusion (Cotran *et al.* 1994; Rubin *et al.* 1999). The cysteinyl-containing leukotrienes, C4, D4 and E4 (SRS-As) cause intense vasoconstriction and increase vascular permeability. The vascular leakage, as with histamine, is restricted to venules (Cotran *et al.* 1994). They are also potent broncho-constrictors and are responsible for many of the symptoms of allergic-type reactions (Rubin *et al.* 1999).

Leukotriene B4 causes aggregation and adhesion of leukocytes to venular endothelium and is a powerful chemotactic agent. Some of the other products of lipoxygenase metabolism such as HETE are also chemotactic. The prostaglandins are also involved in development of fever and pain in inflammation. Prostaglandin E2 causes a marked increase in pain caused by intradermal injection of suboptimal concentrations of histamine and bradykinin and interacts with cytokines in causing fever during infections. Eicosanoids can be found in inflammatory exudates, and agents that suppress cyclooxygenase (aspirin, indomethacin) also suppress inflammation *in vivo*. Glucocorticoids, which are powerful anti-inflammatory agents, may act at least in part by inducing the synthesis of a protein that inhibits phospholipase A2.

Finally, variations in AA metabolism may account for some of the beneficial effects of fish oil. Diets rich in fish oil contain essential fatty acids of the omega 3 variety (eg, linoleic acid) rather than omega 2 linoleic acid found in most animal and vegetable fat. The omega 3 fatty acids serve as poor substrates for conversion to active metabolites of the cyclooxygenase and particularly the lipoxygenase series. Such diets inhibit platelet aggregation and thrombosis and prevent certain inflammatory processes (Cotran *et al.* 1994).

Table 2.3 Inflammatory actions of eicosanoids.

Inflammatory action	Metabolite
Vasoconstriction	TxA2, Leukotrienes C4, D4, E4
Vasodilation	PGI2, PGE1, PGE2, PGD2
Increased vascular permeability	Leukotrienes C4, D4, E4
Chemotaxis	Leukotrienes B4, HETE

2.10 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

2.10.1 History

The use of medicinal substances to relieve pain and fever dates back to ancient Egypt (Cabral and Hoffman 1999), where a decoction of dried leaves of myrtle applied to the back and abdomen of patients was used to relieve pains from the uterus. Later, in Greece the bitter extracts from the bark of the poplar tree were used in patients with eye disease.

Willow bark was chewed to relieve childbirth pains and the benefits remained largely unknown until the first published report on the medicinal effect of willow bark by Reverend Edward Stone in 1763. Later the active component of willow bark was identified as Salicin, which is metabolized to salicylate (Cabral and Hoffman 1999). In 1899, Heinrich Dresser named the compound “Aspirin”, the “a” referring to the acetyl grouping and the “spirin” recalling the botanical genus *spiraea*, from which salicylates could be extracted. During the beginning of the 20th century, aspirin was recognized as an anti-pyretic, anti-inflammatory, and an analgesic drug.

Later on, the development of drugs with similar clinical effects like phenylbutazone in 1949 and indomethacin in 1963 was an inspiration for a common mode of action. Little was known except that they produced an anti-inflammatory effect different in quantity and quality to the more potent glucocorticoids (Cabral and Hoffman 1999).

During the 1960's, Harry Collier, a British pharmacist, suggested that aspirin and related drugs inhibited some underlying cellular mechanism that takes part, to different extents in different responses, and was mediated by different endogenous substances (Collier 1969).

A purified and enzymatically active cyclooxygenase (COX) was isolated in 1976 (Vane 1998), and it wasn't till 1990 that the possible existence of two different COX enzymes was suggested. In 1992, this new enzyme was isolated from humans (Hla and Nelson 1992) and later from animal sources (Herschman 1996) and was called cyclooxygenase-2 (COX-2).

2.10.2 Mechanism of action of NSAIDs

The principle pharmacological effect of aspirin and related NSAIDs is due to their ability to inhibit prostaglandin synthesis by blocking the activity of both COX-1 and COX-2 (Cabral and Hoffman 1999). Most NSAIDs currently used are inhibitors of both isoenzymes, though they vary in the degree of inhibition of each. Clearly, the anti-inflammatory action of the NSAIDs is mainly related to their inhibition of COX-2 and it is probable that, when used as anti-inflammatory agents, their unwanted effects are due largely to their inhibition of COX-1 (Rang *et al.* 1999). Their therapeutic effectiveness as analgesics, antipyretics, anti-inflammatories and anti-thrombogenics is due to their inhibition of prostanoid synthesis, which also accounts for their side effect profile.

Traditional NSAIDs can be grouped into three classes based on their modes of inhibition of COX (Cabral and Hoffman 1999):

CLASS 1: Simple, competitive, reversible inhibitors that compete with arachidonic acid for binding to the COX active site. Included in this class are ibuprofen, piroxicam, sulindac sulfide, flufenamate, mefenamic acid and naproxen.

CLASS 2: Competitive, time dependent, reversible inhibitors that bind to the COX active site in a first phase to form reversible enzyme inhibitor complexes that if retained for a sufficient time, cause a non covalent conformational change in the protein. Included in this class are indomethacin, flurbiprofen, meclofenamic acid and diclofenac.

CLASS 3: Competitive, time dependent, irreversible inhibitors that form an enzyme inhibitor complex after a covalent conformational change in the protein. Included in this class is aspirin.

Non-steroidal anti-inflammatory drugs have three major types of effects:

1. *Anti-inflammatory effects:* NSAIDs reduce mainly those components of the inflammatory and immune response in which the products of COX-2 action play a significant part, namely:
 - a. Vasodilation
 - b. Oedema (by an indirect action, the vasodilation facilitates and potentiates the action of mediators such as histamine which increase the permeability of post-capillary venules).
 - c. Pain.

2. *Antipyretic effects:* Normal body temperature is regulated by the anterior portion of the hypothalamus, especially the pre-optic area which is sensitive to the temperature of blood flowing through it. This ensures a balance between heat loss and heat production (Guyton and Hall 2000). Fever occurs when there is a disturbance of this hypothalamic ‘thermostat’ that leads to the set point of body temperature being raised. Non-steroidal anti-inflammatory drugs reset the thermostat. Once there has been a return to a normal set- point, the temperature-regulating mechanisms (dilatation of superficial blood vessels, sweating, etc) then operate to reduce temperature. Normal temperature is not affected by NSAIDs (Rang *et al.* 1999). The mechanism of the antipyretic action of the NSAIDs is thought to be largely due to inhibition of prostaglandin production in the hypothalamus. During an inflammatory reaction, bacterial endotoxins cause the release from macrophages of a pyrogen-interleukin 1 (IL-1), which stimulates the generation in the hypothalamus of E-type prostaglandins and these, in turn, cause the elevation of the set-point for temperature. There is some evidence that prostaglandins are not the only mediators of fever; hence NSAIDs may have an additional antipyretic effect by mechanisms as yet unknown (Rang *et al.* 1999).

3. *Analgesic effect:* NSAIDs are effective mainly against pain associated with inflammation or tissue damage because they decrease the production of prostaglandins that sensitize receptors to inflammatory mediators such as bradykinin. Therefore they are effective in arthritis, bursitis, pain of muscular and vascular origin, toothache, dysmenorrhoea,

the pain of post - partum states, and the pain of cancer metastasis in bone – all conditions that are associated with increased prostaglandin synthesis (Rang *et al.* 1999).

In combination with opioids, they decrease post-operative pain and in some cases can reduce the requirement for opioids by as much as one third. Their ability to relieve headache may be related to the abrogation of the vasodilator effect of the prostaglandins on cerebral vessels. There is evidence that they have a central effect by an action mainly in the spinal cord.

2.10.3. Cyclooxygenase-1 and Cyclooxygenase-2

Cyclooxygenase-1 was presumed to be the major target of NSAIDs acting in their analgesic and anti- inflammatory capacities. The enzyme was originally purified from ovine and bovine vesicular glands in 1976 (Cabral and Hoffman 1999).

In the early nineties, the existence of two different cyclooxygenases was discovered and COX-2 isolated (Hla and Nelson 1992) based on the evidence that steroids inhibit the increase in COX activity induced by bacterial lipopolysaccharides in macrophages, without any effects on the basal production of prostaglandins or leukotrienes. This led to the isolation of the COX-2 isoenzyme from both human and animal sources. It was originally discovered as a transcript that was decreased by steroids and upregulated by cellular transformation and inflammation.

Tissue localization studies under physiologic conditions found a constitutive expression of COX-1 in virtually all tissues, whereas COX-2 appeared to be constitutively restricted to the brain, kidney, bones, testicles, ovaries, uterus, tracheal epithelial cells and small intestine in very low levels (Smith and DeWitt 1995; Komhoff 1997).

Cyclooxygenase-1 is responsible for the ‘housekeeping’ prostaglandins critical to the maintenance of normal function, gastric mucosal integrity, vascular hemostasis and the autocrine response to circulating hormones.

Cyclooxygenase-2 on the other hand is an inducible enzyme, upregulated 20-fold in macrophages, monocytes, synoviocytes, chondrocytes, fibroblasts, osteoblasts and endothelial cells by various inflammatory stimuli such as IL-1, tumor necrosis factor, lipopolysaccharides, mitogens (phorbol esters), growth factors, platelet derived growth factor, transforming growth factor beta, epidermal growth factor, fibroblast growth factor and reactive oxygen intermediates (Smith and DeWitt 1995; Spangler and Ronald 1996).

An upregulation in the expression of COX-2 has been noted in colorectal adenomas and carcinomas with an increase in COX-2 mRNA in 86% of carcinomas compared with normal mucosa. Transcription is likely to be increased also in breast, head and neck cancers (Crofford 1997). After these discoveries, it was suggested that the anti-inflammatory actions of NSAIDs were due to COX-2 inhibition and the unwanted side effects due to the inhibition of COX-1 (Smith and DeWitt 1995; Lipsky *et al.* 1997; DeBrum-Fernandez 1997; Vane *et al.* 1998; Brooks 1998; Silas *et al.* 1999).

Cyclooxygenase-2 Selectivity:

The inhibition of COX-1 and COX-2 has been estimated through the development of *in-vitro* assay systems (Vane *et al.* 1998). The best models measured COX-1 activity by assessing platelet aggregation and thromboxane production. Cyclooxygenase-2 was assessed by measuring PGE₂ production by monocytes stimulated by lipopolysaccharides. The *in-vitro* studies take whole blood from healthy patients and expose it to the drug. An *ex-vivo* study used whole blood from healthy patients exposed to the drugs (Fenner 1997).

Cryer and Feldman (1998) published an *in-vitro* whole blood study evaluating currently available NSAIDs in six concentrations and also incubated gastric mucosal biopsies with the same drugs. The drug concentration causing a 50% inhibition of COX activity was reported as the IC (inhibitory concentration) 50; for each drug a COX-2 / COX-1 IC 50 ratio was reported. A low ratio implied relative selectivity for COX-2. There was however, a broad degree of variability in the ratios, and the author's conclusion was that these were not clinically useful because the known effective serum concentration of each drug were well above the COX-1 IC 50.

The definition of COX-2 specificity was given to drugs that inhibited COX-2 but not COX-1 across the therapeutic dose range, using the *ex-vivo* whole blood assay. But this does not imply that COX-2 specific agents have a better safety profile.

Until recently, all available NSAIDs inhibited both COX-1 and COX-2 in their therapeutic dose range. Two agents (nimesulide and meloxicam) with some preferential COX-2 inhibition were introduced several years ago, suggesting that with their greater selectivity for COX-2, they would have superior gastrointestinal safety profiles (Vane *et al.* 1998). These drugs were also inhibitors of COX-1 at therapeutic dosages.

The currently approved COX-2 inhibitors for human use, celecoxib (Celebrex) and rofecoxib (Vioxx) are highly selective for COX-2 in comparison with traditional NSAIDs (375-fold and 1000-fold respectively) (Cabral and Hoffman 1999). Celecoxib has been approved for the treatment of osteoarthritis and rheumatoid arthritis, but has been criticized for its side effects, mainly arterial thrombosis and renal hemorrhage. Celecoxib has been found to inhibit not only COX-2, but also inhibit adenylyl cyclase, an important enzyme forming the intracellular second messenger 3', 5'- adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) (Saini *et al.* 2003). Up to now, rofecoxib has been approved for the treatment of pain, osteoarthritis and dysmenorrhea.

2.10.4 Side effects of NSAIDs

Non-steroidal anti-inflammatory drugs have a range of side effects which has made them unsuitable for some patients. These include:

1. Haematologic: These include thrombocytopenia, haemolytic anaemia, aplastic anaemia and agranulocytosis. Decreased platelet aggregation and increased bleeding time can be seen with NSAID treatment.
2. Cardiovascular: Patients with hypertension and congestive heart failure can have their condition exacerbated when starting NSAIDs.

Angina and other ischaemic heart events may be seen and NSAIDs may interfere with some medications such as beta-blockers and angiotensin converting enzyme inhibitors (Cabral and Hoffman 1999).

3. Central Nervous System: These include headaches, confusion and drowsiness. Some patients may experience psychological disturbances such as depression, hallucinations and behavioral symptoms. Other side effects may include aseptic meningitis, neuropathy and tremors. Vertigo and tinnitus have been reported.
4. Gastrointestinal: Gastrointestinal problems are the commonest and most well known side effects of NSAIDs, including nausea, vomiting, dyspepsia, diarrhoea and constipation. More sinister effects can be in the form of oesophagitis and oesophageal strictures, gastritis, gastric ulcers and mucosal irritation (Cabral and Hoffman 1999). Gastrointestinal hemorrhage occurs more in patients with underlying gastric and duodenal disease. Small and large bowel erosion and strictures may also occur. Non-steroidal anti-inflammatory drugs may also cause hepatotoxicity, hepatitis and fulminant hepatic failure. Treatment with COX-2 inhibitors was associated with an improvement of mucosal damage in studies in rabbits on the treatment of oesophagitis induced by acidified pepsin (Lanas *et al.* 2003).
5. Renal: Non-steroidal anti-inflammatory drugs should be used with caution in patients with renal impairment as they can cause renal failure although this is usually reversible (Cabral and Hoffman 1999). Other renal problems associated with NSAID use are the nephrotic syndrome, interstitial nephritis and uric acid uroliths.
6. Hypersensitivity: Exacerbation of asthma, rashes, urticaria and photosensitivity (Cabral and Hoffman 1999).

2.11 The Pig as a laboratory model for human disease

2.11.1 Introduction

Pigs have been increasingly utilized as biomedical research models in the last twenty five years (Swindle and Smith 2000). This increased use as an animal model is because pigs are recognized as a suitable animal model for human disease based on their comparative anatomy and physiology. Pigs are used as general surgical models of most organs and systems with particular emphasis for cardiovascular research including atherosclerosis, for digestive system models, and in recent years in transplantation and xenograft research (Swindle and Smith 2000). Pigs are expected to be used as xenograft donors for both whole-organ and cellular transplantation. Screening of animals will have to include testing for viruses, bacteria, parasites, congenital defects and other inapparent diseases such as neoplasia or metabolic dysfunctions (Swindle 1998b).

A few examples of research projects where pigs have been used include testing fibrin haemostatic bandages after bleeding the femoral artery (Larson *et al.* 1995), testing the effect of haemofilter pore size on the efficacy of continuous arteriovenous haemofiltration (Lee *et al.* 1998) and the use of pigs for testing chronic intravascular catheters, cannulas and fistulas (Swindle *et al.* 1998).

They are being explored as models for many other body systems because of the widespread availability of both domestic and miniature breeds. Hand in hand with this increase in the use of pigs in biomedical research have come technical developments in surgery, anesthesia, husbandry and handling techniques. These technical advancements have made it easier to use this species in research and have also improved the humane care and use of pigs by research institutions worldwide.

2.11.2 Comparative anatomy and physiology of the pig

All pigs commonly used in research and testing are *Sus scrofa domesticus*, whether they are farm or miniature breeds. The main difference between breeds is size at sexual maturity. Domestic breeds typically reach 100kg by four months of age and miniature breeds typically range from 25-50kg at the same age. The predominant

breeds of miniature swine used in research are *Yucatan, Hanford, Gottingen and Sinclair Hormel*, although dozens of other breeds have been reported in the scientific literature as being used in biomedical research (Swindle *et al.* 1994).

2.11.2.1 Cardiovascular System

The heart of the pig is anatomically similar to humans with a notable exception being the presence of the left azygous (hemiazzygous) vein, which drains the intercostal system into the coronary sinus (Swindle *et al.* 1986). The coronary system is similar to 90% of the human population in anatomy and function. There are no preexisting collateral vessels in the myocardium (Bloor *et al.* 1992). The heart of a 40-50kg miniature pig is approximately the same size as an adult human heart.

The aorta in the pig contains vasavasorum like humans. It also has a comparable microscopic anatomy, however, blood vessels and the atria in pigs tend to be more friable than other species, especially in neonates (Swindle 1983; Swindle *et al.* 1986; Swindle *et al.* 1988; Swindle *et al.* 1998).

Vascular access may be readily obtained with standard size needles from the cephalic, internal and external jugular, anterior vena cava, auricular, anterior abdominal, saphenous and femoral veins with practice. All these vessels as well as internal abdominal and thoracic vessels may be chronically catheterized using surgical technique (Swindle 1983; Swindle *et al.* 1986; Swindle *et al.* 1988; Swindle *et al.* 1998).

Haemodynamically, pigs have been demonstrated to be similar in cardiac function to humans although there are variations between breed and age that need to be taken into consideration. When repeating studies and comparing results between laboratories, caution should be taken in comparing haemodynamics between different breeds. Animals should be age and weight matched (Smith *et al.* 1990; Swindle 1998a).

The development of atherosclerosis occurs both spontaneously and by experimental induction in pigs fed an atherogenic diet. The metabolism of lipoproteins is similar to humans. The distribution of atherosclerotic plaques is similar to humans if allowed

to develop spontaneously over time and the histology and pathogenesis of the plaques appears to be similar to humans also (Gal and Isner 1992; White *et al.* 1992).

Pigs do have congenital cardiovascular anomalies such as atrial and ventricular septal defects (ASD and VSD). The model of VSD has been developed as a genetically reproducible model, which has been shown to be analogous to human infants with VSD and failure to thrive syndrome (Swindle *et al.* 1998).

The characteristics that have led to the use of pigs over other species for these models are related to the anatomical and physiological characteristics described above. The porcine model develops an infarction pattern like the human and develops arrhythmogenic activity with reperfusion. The pattern of infarction and healing of the myocardium is almost identical to humans (Stanton and Mersmann 1986; Bloor *et al.* 1992; Gal and Isner 1992; White *et al.* 1992; Swindle 1998a).

Likewise, the wound healing characteristics in the cardiovascular system mimic those in humans following implantation of some devices, such as intra-coronary stents (Swindle 1998 a). The myocardial wound healing characteristics of pigs are more analogous to humans than sheep models since healing in ruminants is characterized by the formation of collagenous scars (Von Recum 1986; Mehran *et al.* 1991).

2.11.2.2 Digestive System

The digestive system of pigs has anatomic differences from humans, however the physiology of digestion remains similar to humans. Pigs are true omnivores. In spite of anatomic differences, the pig has been used extensively as a gastrointestinal model for humans. Most of the classic models involving the digestive system have been related to nutritional studies to study digestion of the pig and for studying human digestive phenomena. Pigs will readily ingest such test substances as alcohol in its various forms. The metabolic functions, intestinal transport times, and characteristics of absorption of nutrients have made them useful in basic nutritional research.

Other specific functional characteristics of swine that relate directly to humans include iron transport and motility, neonatal development of the gastrointestinal tract and splanchnic blood flow characteristics.

Development of host defenses and endotoxic shock studies has made pigs useful as biomedical models in these areas. Like the human, these physiological characteristics of the gastrointestinal tract are probably due to the omnivorous diet that they consume, unlike that of carnivores, ruminants, rabbits and rodents (Brown and Terris 1996; Reeds and Odle 1996; Tumbleson and Schook 1996).

More recently, endoscopic and laparoscopic surgical models have been developed and used extensively in the pig. The size and function of structures such as the biliary system and pancreatic duct make them amenable for studying human size equipment and biomaterial implants (Swindle 1998 a).

Pigs have a similar cytochrome P450 system to humans except for the absence of CYP2C19 and CYP2D6 (Skaanild and Friis 1997). However, metabolically the liver functions in a similar way to humans and has been used for xenoperfusion protocols for humans in hepatic comas (Collins *et al.* 1994; Swindle 1998b).

2.11.2.3 Urogenital system

The female reproductive system has a bicornuate uterus with torturous Fallopian tubes. The Fallopian tubes of an adult female are the same diameter as those of humans, however they are much longer (Swindle 1998a).

The sow has been used in studies of foetal surgery to create models that mimic the human situation. Even though the placentation is unlike that in humans, the physiologic characteristics of transplacental transfer of antiarrhythmics has been shown to be more similar to the human situation than the traditional ewe model. This transplacental transfer of therapeutic agents may make the sow a predictable model of teratogenicity and efficacy of pharmaceutical agents (Weist *et al.* 1996).

The male reproductive system has the same structures as humans, however, the accessory sex glands which predominate are different. The penis has a corkscrew shaped tip and this makes it impossible to catheterize a male pig through the penile

opening. Catheterization has to be performed in the perineal urethra percutaneously (Swindle 1983; Swindle 1998a).

The kidneys of the pig are more like humans in anatomy and function than most other species of animals. The adrenal glands are located near the cranial poles of both kidneys and the right gland is intimately associated with the wall of the posterior vena cava. The kidneys are multirenculate and multipapillate like humans (Terris 1986; Pennington 1992; Sachs 1992; Brown and Terris 1996).

The anatomical and physiological characteristics of the porcine kidney may make it useful for the study of pharmacological agents since the anatomy of the kidney is more similar to humans than even non-human primates. Pigs can be utilized in studies of renal hypertension and can be developed as a model of intact renal salt induced hypertension or as surgical ablation models of rennin induced hypertension (Terris 1986; Swindle *et al.* 1988; Swindle 1998a).

2.11.2.4 Integumentary and Lymphatic Systems

Pigs are relatively hairless animals with a fixed skin tightly attached to the subcutaneous tissues like humans. Overall, the skin is thicker and less vascular than humans, however the cutaneous blood supply characteristics are similar. Apocrine sweat glands are absent. The skin tends to be thicker on the neck and dorsum of the animal (Kerrigan *et al.* 1986; Bolton *et al.* 1988; Chvapil and Chvapil 1992; Monteiro-Riviere and Riviere 1996).

The anatomy and physiology of the cutaneous blood supply and the wound healing characteristics have made the pig a standard model for plastic surgical and wound healing studies (Kerrigan *et al.* 1986).

Besides the anatomical similarities, pigs are equivalent to primates for percutaneous absorption studies and have similar lipid biophysical properties, epidermal turnover kinetics and carbohydrate metabolism in the skin (Monteiro-Riviere and Riviere 1996).

In summary, the pig is physiologically similar to humans in many aspects, especially the gastrointestinal and cardiovascular systems and has been used in many research projects around the world especially in recent xenotransplant projects.

One of the major institutes looking into xenotransplantation is the Islet Foundation that deals with management of diabetes. In 1998, an international workshop on xenotransplantation was held in New York. Gordon gave a full report on the workshop, which highlighted the use of pigs for pancreatic islet xenotransplantation for diabetic patients. This was found to be a safe mode of treatment when compared to organ xenotransplantation. No immunosuppression was required and there was no hyperacute rejection and no acute vascular rejection. There was no report of cross infection. The consequences of graft failure would be minimal, as the patient would revert to the normal insulin therapy before the transplant (Gordon 1998).

2.12 Summary of literature review

Adhesion formation simply means the development of abnormal attachments between tissues and organs. Adhesions in the pericardium after cardiac surgery can cause increase morbidity and re-operation risks by potential injuries to the heart, great vessels, and cardiac grafts during the re-sternotomy. Other complications include impeded orientation and visibility, increasing the amount of blood loss and prolonging operative time.

The most potent stimulus for adhesion formation is peritoneal or pericardial trauma where adhesive tissue forms as an end result of the acute inflammatory response to surgery. The use of anti-inflammatory agents would therefore inhibit the formation of adhesive tissue formation.

Non-steroidal anti-inflammatory drugs are commonly used in daily medical practice. Until recently, all available NSAIDs inhibited both COX-1 and COX-2 in their therapeutic dose range. The currently approved COX-2 inhibitors for human use, celecoxib (Celebrex) and rofecoxib (Vioxx) are highly selective for COX-2 in comparison with traditional NSAIDs.

Pigs have been increasingly used in research because they are recognized as a suitable animal model for human disease based on their comparative anatomy and physiology. They have been widely used for many years in cardiovascular research. Currently there is considerable research activity in the field of using pigs as xenograft donors for both whole-organ and cellular transplantation although comprehensive screening of animals will be necessary to exclude microbes, congenital defects and other inapparent diseases such as neoplasia or metabolic dysfunctions

CHAPTER THREE

MATERIAL AND METHODS

3.1 Animals

Weaner piglets of the Large White breed type were purchased at six to seven weeks of age from a local piggery or were obtained from litters bred in the large animal facilities of the Biomedical and Tropical Veterinary Science precinct, Douglas Campus, James Cook University, Townsville. Purchased animals were allowed to adapt to the husbandry conditions for about two to four weeks before they were used in the experiments. The pigs were housed indoors in concrete pigpens adjacent to the large animal surgery in the building referred to as “The Piggery”. Pens were cleaned twice a day and the animals fed twice a day with purchased pig feed (Barastoc pig grower pellets; Ridley Agriproducts, Toowoomba, QLD) using protocols developed by staff of the Australian Institute of Tropical Veterinary and Animal Science, James Cook University. Water was available ad libitum via automatic nipple waterers. The animals were identified with numbered plastic ear tags (Allflex Tag System; Delta Plastics, Palmerston North, NZ). The project received ethics approval from the James Cook University Ethics Review Committee (approval no. A943-02).

3.2 Establishment of drug dosages

An initial trial was conducted to confirm that the calculated dosages of a non-steroidal anti-inflammatory drug, indomethacin (Indocid; Merck Sharp & Dohme Pty Ltd, South Granville, NSW) and a cyclooxygenase-2 (COX-2) inhibitor, rofecoxib (Vioxx; Merck Sharp & Dohme Pty Ltd, South Granville, NSW) would produce the desired plasma concentrations of the drugs and to correlate this with the pharmacodynamic response, in order to assess the effectiveness of the administered dose.

Two eight week-old pigs were used. One pig was dosed orally with 3 mg/kg of indomethacin in pure powder form in a gelatin capsule and the other pig was dosed orally with 0.55mg/kg of rofecoxib in tablet form similar to the human tablets. These doses were based on human doses and were calculated with the assistance of a

pharmacological software program (PharmaCalc). Blood samples (2ml-5mls) were collected from the anterior vena cava using a 10ml syringe and a size 21G 1.5 TW needle at 0.5, 2, 3, 4, 6, 8 and 24 hrs after dosing with rofecoxib.

For the pig dosed with indomethacin, blood samples (2ml) were collected using the same size syringes and needles at 2, 3, 3.5, 4, 8 and 24 hrs after drug administration. All blood was collected in anti-coagulant tubes with K3 ethylene diamine tetra acetic acid (EDTA) additive. Plasma was separated by centrifugation and samples were analyzed for drug levels using high-pressure liquid chromatography (HPLC) at the School of Pharmacy and Molecular Sciences, James Cook University, Townsville. All plasma samples were stored at -20 degrees centigrade until analysed.

3.3 Surgical trial

Forty-four mostly nine to ten week-old piglets were randomly allocated to four treatment groups (eleven per group) with a near equal mix of males and females.

Group 1: Control group

Group 2: Non-Steroidal Anti-inflammatory group, using indomethacin (intramuscular administration, as oral route was less tolerated by the animals).

Group 3: Cox-2 inhibitor group using rofecoxib (oral administration).

Group 4: Co-Seal group. Local administration of Co-Seal (Baxter Healthcare, Old Toongabbie, NSW), a synthetic non-toxic polyethylene glycol sealant as a barrier to adhesions.

An additional group of four control and two Co-Seal animals were given omeprazole 1mg/kg (Losec; AstraZeneca Pty Ltd, North Ryde, NSW) once daily for seven days to assess any effect on adhesion formation. These results were compared with the major control group.

3.3.1 Anaesthesia

All animals undergoing surgery were deprived of food and water approximately 12 hours before the operation and were weighed on the morning of surgery. General

anaesthesia and tracheal intubation was performed and maintained by veterinary anaesthetists throughout the surgical procedure. Endotracheal tubes were sized according to animal weight as shown in Table 3.1.

Table 3.1 Relation between animal weight and endotracheal tube size.

Weight	Size of tube
10 – 15 kg	5 – 7 mm
15 – 20 kg	8 mm
20 – 30 kg	8 – 10 mm

Induction of anaesthesia was by intramuscular administration of ketamine 5mg/kg (Ketamil; Troy Laboratories Pty Ltd, Smithfield, NSW), azaperone 2mg/kg (Stresnil; Boeringer Ingelheim Pty Ltd, North Ryde, NSW) and inhaled isoflurothane 2.5%. (Isoflurane; Rhodia Australia Pty Ltd, Notting Hill, VIC). Maintenance of anaesthesia was achieved with isoflurane 2% in oxygen. All animals were extubated shortly after the surgery was completed without the use of anaesthetic reversing agents.

3.3.2 Surgical Procedure

The ventral chest wall of the animal was shaved with hair clippers from the lower aspect of the neck to the anterior two mammary nipples. The skin was thoroughly cleaned using chlorhexidine in alcohol 70% (Pharmacia Pty Ltd, Bentley, WA) surface disinfectant solution. This was followed by sterile draping of the chest. All animals were monitored using an electrocardiogram (ECG) device throughout anaesthesia and surgery. Four ECG leads were used, two placed on the ventral aspect of each foreleg and two on the ventral aspect of each thigh. The areas were shaved and lubricated with conduction gel beforehand to increase electrical conductivity. This gave a reading of ECG leads I, II and III. After incision of the skin, a sternotomy was performed on each animal using an oscillating electric saw.

The chest cavity was held open using a self-retaining retractor and the pericardial sac was opened similar to the human technique and the heart exposed (Figure 3.1). The right atrium, aorta and pulmonary artery were identified.

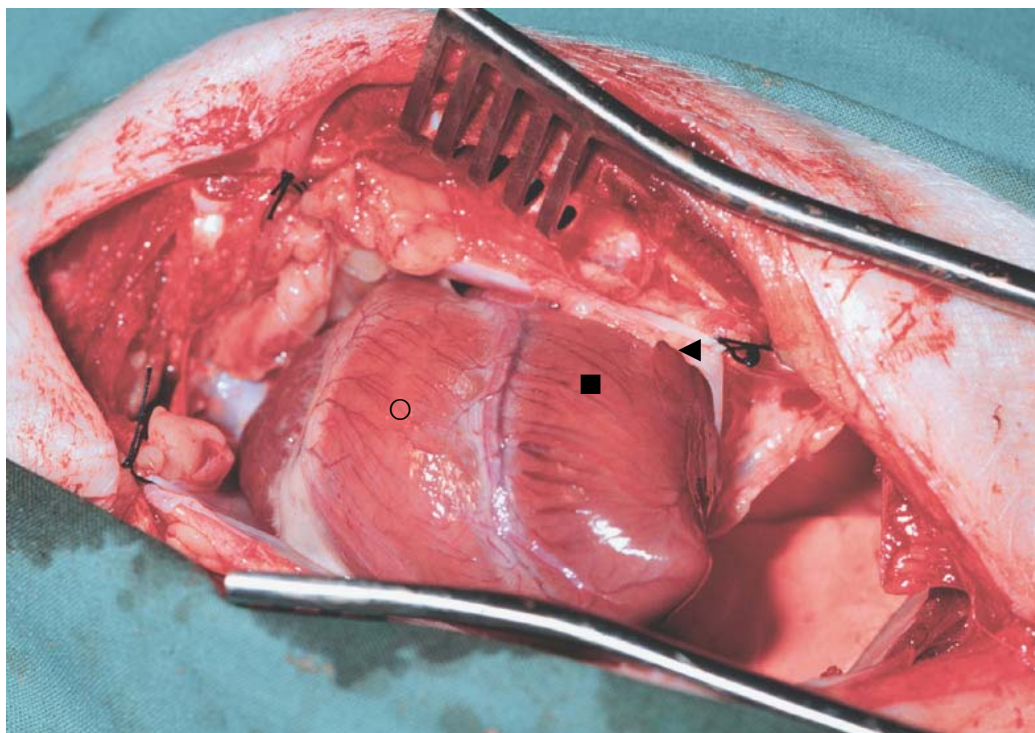


Figure 3.1 Heart exposed after opening the pericardium.

○ Right Ventricle ■ Left Ventricle ◀ Pericardium

This was followed by application of an adhesion induction model that was formulated to induce a maximal inflammatory response and therefore maximum adhesion formation in the pericardial sac. This model consisted of the following steps:

1. A 2-0 Ethibond Excel polyester suture on a 26 mm needle (Ethicon. Inc., Johnson & Johnson, Somerville, New Jersey, USA) was used to apply a purse-string suture on the aorta (Figure 3.2).
2. A second purse-string suture using 3-0 monofilament polypropylene on a 26 mm needle (Prolene; Ethicon. Inc., Johnson & Johnson, Somerville, New Jersey, USA) was applied to the right atrial appendage (Figure 3.2).

These two sutures are identical to the ones used in humans for cannulation before heart bypass procedures. The idea behind using these sutures in this model was to simulate human cardiac surgical procedures and to increase the inflammatory response and therefore adhesion formation in the pericardial sac.

3. Rubbing the pericardium, retrosternal surfaces, the aortic area and the epicardium with gauze (approximately 15-20 rubs to each surface). The reason for this procedure was to irritate these surfaces thereby increasing the inflammatory response.
4. Aspiration of five ml of blood directly from the right atrium using a size 21G 1.5 TW needle on a 5ml syringe. This blood was left in the pericardial cavity. This blood will form multiple clots, which will adhere to the surface of the heart and the pericardium leading to adherence of these surfaces with each other and increasing the inflammatory reaction.
5. Drying the heart surface by leaving it exposed for 10 - 15 minutes. This will cause more irritation to tissues and increased inflammatory response.

The chest was closed using titanium sternal wires size 6, identical to the wires used in human patients. The subcutaneous tissue was closed with a continuous running 2-0 polyglactin 910 suture (Coated Vicryl; Ethicon. Inc., Johnson & Johnson, Somerville, New Jersey, USA) on a 36mm needle, and the skin closed with a subcuticular 3-0 monofilament (poliglecaprone 25) suture (Monocryl; Ethicon. Inc., Johnson & Johnson, Somerville, New Jersey, USA) on a 24mm needle.

In the Co-Seal group, eight ml of Co-Seal were applied locally using a spraying device under air pressure of 25psi just before chest closure. The retrosternal surfaces, the pericardium and the epicardium were sprayed achieving at least a 0.5 - 1mm thick layer. After a 120 second wait, the Co-Seal solidified into a transparent non-adhesive gel layer. The chest was then closed as previously described.

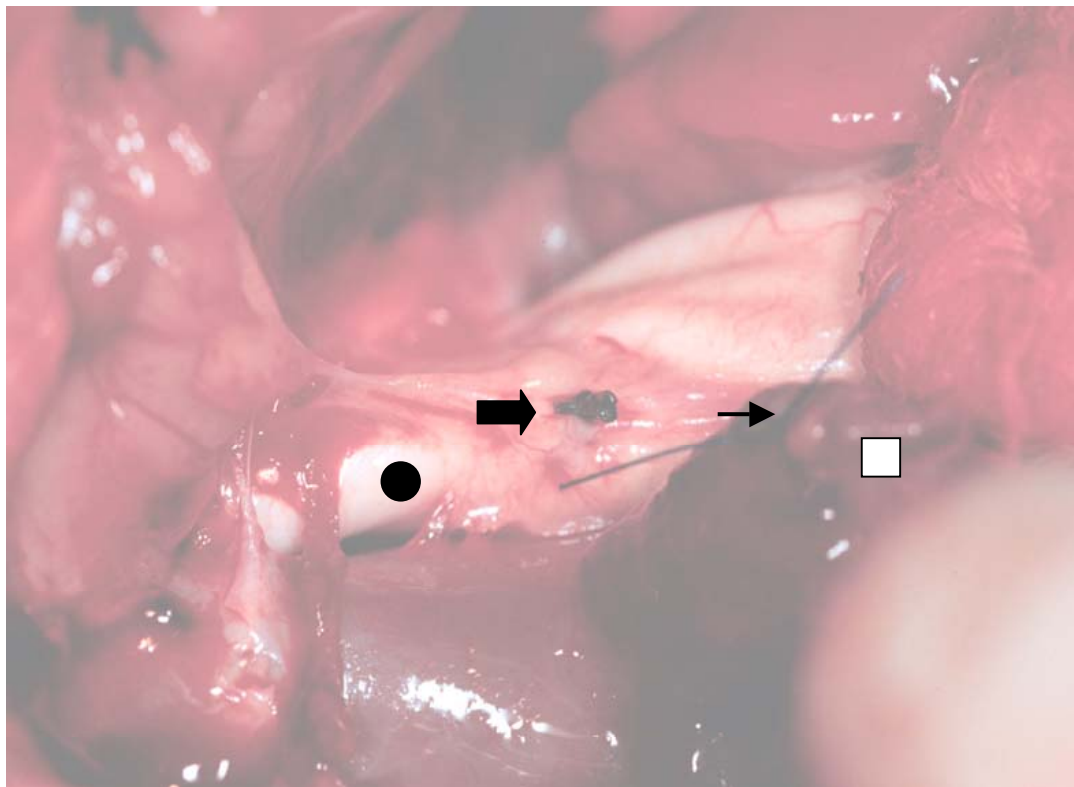


Figure 3.2 The placement of aortic and atrial sutures.

● Aorta → Aortic suture □ Right Atrium → Atrial suture

An average of two animals were operated on in any given operating day. The average surgical time was 40 - 50 minutes and the pericardial sac was opened for an average of 30 minutes. The average time for a whole single operation including anaesthetic and turn over time was around two hours. The initial recovery time for the animals was approximately three hours after surgery, after which they were given access to food and water.

All animals received antibiotic cover with combined lincomycin + spectinomycin 1ml/10kg (Linco-spectin; Pharmacia & Upjohn Pty Ltd, Rydalmere, NSW) intra-operatively by intramuscular injection. Intramuscular analgesia, buprenorphine hydrochloride 0.004-0.008mg/kg (Temgesic; Reckitt & Coleman Pharmaceuticals

Pty Ltd, West Ryde, NSW) was used on all animals for 24 hours after the operation to minimize any discomfort.

After surgery, the pigs were allowed to recover from anaesthesia in a small individual pen or cage. The animals were monitored for bleeding, any acute wound problems or any other complication for one to two days after surgery. They were then returned to the pens containing their littermates.

3.3.3 Post-operative care

Post-operative care was similar for the four animal groups in terms of housing in pens and twice daily feeding and cleaning. All animals received Linco-spectin 1mg/10kg each day for three days given intramuscularly in the muscles of the hind leg on alternate sides. No other site of injection was used. Group one (control) and Group four (Co-Seal) received no further treatment till slaughter. Group two was treated with intramuscular indomethacin 1mg/kg/dose suspended in sterile normal saline into the muscles of the hind legs as follows:

1. An initial dose of indomethacin was administered two to three hours before surgery to achieve a therapeutic level during the operation.
2. Eight hourly dosing with indomethacin was continued post-operatively for five days, giving a total of 15 post-operative injections. The injection sites were rotated on the hind leg muscles and sides alternated each day to reduce discomfort. No other site of injection was used for this group. A 21G 1.5 TW needle on a 3ml syringe was used.

Group three was treated with rofecoxib (Vioxx) 0.5mg/kg orally in tablet form as follows:

1. An initial dose of rofecoxib was administered two to three hours before surgery to achieve a therapeutic level during the operation.
2. Once daily dosing with rofecoxib was continued post-operatively for five days.

All animals in Groups two and three received omeprazole 1mg/kg once daily for seven days orally in tablet form similar to human tablets as a precaution against any gastrointestinal irritation that may have been caused by the anti-inflammatory treatment. No further treatment was administered in these two groups till slaughter.

The animals in Groups two and three were also weighed daily for seven days to confirm that they were gaining weight similar to Groups one and four. They were closely monitored for any early signs of any anti-inflammatory side effects especially gastrointestinal disturbances. Their feeding habits were also monitored for any signs of decreased appetite.

3.4 Additional Groups

Two additional groups were used to assess any effect of omeprazole on adhesion formation in groups one and four as omeprazole was not used in these two groups initially. Four control and two Co-Seal animals underwent the same initial surgery as the original groups. They all received omeprazole in similar fashion as groups two and three for seven days. They were re-opened after 25 weeks. Gross and histopathological evaluation of adhesions was done.

3.5 Inflammatory markers in peripheral blood

Inflammatory markers in peripheral blood were measured in four animals only in each group pre- and post-operatively. This was due to cost containment. The inflammatory markers measured were total white blood cell count and differential count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), prostaglandin E2 (PGE2) and thromboxane B2 (TXB2).

In addition, the erythrocyte count, haemoglobin concentration, and measurements of coagulation including International Normalization Ratio (INR), Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) were determined.

Approximately five ml of blood were aspirated from the anterior vena cava as described in section 3.2. One ml samples were placed in small blood collection tubes (MiniCollect, microtainer, Becton Dickinson, Franklin Lakes, USA) as follows: for the full blood count (includes WBC and differential count), ESR, CRP and PGE2,

tubes containing K3 EDTA anti-coagulant additive were used. For the coagulation studies, the tubes used contained citrate 3 Na (3.8%). Blood samples for the measurement of TXB2 were placed in a tube containing Z serum/gel additive.

The first sample was taken approximately 30 minutes pre-operatively on the same day of the operation as a baseline for inflammatory markers. Three more samples were taken in the same fashion on days two, five and ten after the surgery. Blood samples for CRP, PGE2 and TXB2 were immediately centrifuged in the animal operating theatre, stored at 4 degrees centigrade and then sent within two hours in a cool box to the Queensland Pathology laboratory at The Townsville Hospital for analysis.

3.6 Re-openings

Eight animals in each group were re-opened (re-sternotomy) at approximately 12 weeks and the remaining three in each group were re-opened at approximately 25 weeks after the initial operation.

All re-openings were performed at the Charters Towers abattoir, approximately 130 kilometers from James Cook University. The animals were transported to the abattoir during the morning that they were slaughtered. Each animal was first slaughtered in routine fashion and then transferred to a specially prepared table at the abattoir. A re-sternotomy was then performed and the sternal wires from the first operation were removed. The sternal tables were then separated using two self-retaining retractors and the adhesions retrosternally assessed grossly. The heart and pericardium were dissected and separated from each other using sharp, blunt or both sharp and blunt dissection as needed. The type of dissection needed formed an important part of the gross evaluation of adhesive tissue formation.

Five tissue samples were taken from each animal and fixed in 4% formaldehyde solution. The samples collected were:

1. Retrosternal adhesive tissue.
2. Adhesive tissues over the right ventricle.

3. Adhesive tissues over the left ventricle.
4. The pericardium.
5. The myocardium.

All these samples were submitted to the Queensland Pathology laboratory at The Townsville Hospital to be assessed by an independent pathologist. Tissue sections were stained with haematoxylin and eosin and Masson trichrome stains.

Gross evaluation was performed by three assessors, all unaware to the group of animals being re-opened as was the pathologist performing the histopathological analysis of the tissue samples. Adhesions were evaluated according to two adhesion grading schemes. The first was a gross evaluation scheme and used the following criteria:

1. Adhesion formation: Present or absent
2. Adhesion percentage scale (APS):
 - 0 = No adhesions.
 - 1 = Adhesions covering 1 – 25% of area.
 - 2 = Adhesions covering 26 – 50% of area.
 - 3 = Adhesions covering 51 – 75% of area.
 - 4 = Adhesions covering 76 – 100% of area.
3. Adhesive tissue tenacity scale (ATTS):
 - 0 = No adhesions.
 - 1 = Filmy adhesions, easily separable.
 - 2 = Moderate adhesions requiring blunt and sharp dissection (mainly blunt).
 - 3 = Dense but patchy adhesions requiring blunt and sharp dissection.
 - 4 = Dense diffused adhesions requiring mainly sharp dissection.

The second evaluation scheme was based on histopathological analysis, and included assessment of the following features in the tissue samples:

1. Fibrosis.

2. Necrosis.
3. Inflammation grading: None, mild, moderate and marked.
4. Absence or presence of inflammatory cells and type of cells if present.
5. Distribution of inflammatory cells in relation to tissue site.
6. Evaluation of myocardial tissue for all above in addition to collagen deposition, epicardial thickening and mesothelial layer re-establishment.

3.7 Statistical Analysis

An Analysis of Variance (ANOVA) or a Multivariate Analysis of Variance (MANOVA) was not possible as two of the major assumptions (homogeneity of variances and normality) were not satisfied with the dependent variables (APS and ATTS) being defined on a scale from 0 to 4.

Because of this it was decided to perform three separate Mann-Whitney tests to determine if there was, overall, significantly less APS and ATTS in each of the three treatment groups compared to the control group (Group 2 v Group 1, Group 3 v Group 1 and Group 4 v Group 1). The Mann-Whitney test is a non-parametric statistical test which does not rely on the assumptions of the ANOVA as identified above. As there were three comparisons it was decided to use a more conservative (i.e. harder to reject the null hypothesis) Bonferroni correction. This adjustment maintained the overall significance level of the test at 0.05 by dividing 0.05 by the number of individual tests. This makes the adjusted significance level $0.05/3 = 0.0166$ for each of the three Mann-Whitney tests.

CHAPTER FOUR

GROSS AND MICROSCOPIC EVALUATION OF POST- OPERATIVE PERICARDIAL ADHESIONS

4.1 Introduction

The reduction and prevention of adhesions from forming whether pericardial or peritoneal adhesions has been the objective and aim of many research groups around the world over the decades (Boys 1942). The process of adhesion formation is part of the normal healing process after surgery. Any major reduction in adhesion formation would therefore result in delayed wound healing. Teams that have achieved prevention or marked reduction of post-operative adhesions, mainly using high doses of steroids, have all had increased wound dehiscence and infection rates (Vander Salm *et al.* 1986). The aim of this study, therefore, was neither the prevention nor the marked reduction of adhesion formation as this would result in poor healing of tissues. The aim was to moderately reduce and most importantly change the tenacity and texture of the adhesive tissue forming after surgery from dense fibrous adhesions to more thin, and easy to dissect tissues. This would reduce operative and post-operative morbidity and mortality.

In this study, adhesions were assessed by both a gross and a histopathological evaluation scheme on re-opening the sternum after slaughter of the animals. Three assessors unaware of the animal groups being re-opened performed the gross adhesion evaluation according to the gross evaluation scores (see Chapter three, section 3.6). Multiple tissue samples were taken and sent for histopathological analysis by an independent pathologist.

4.2 Results

4.2.1 Effect of the surgical procedures on the animals

All animals of the four groups underwent a sternotomy and a pericardiectomy after which adhesions were induced using the adhesion induction model. Surgery was

performed under general anaesthesia with endotracheal intubation. None of the animals had complications with induction and maintenance of anaesthesia.

During the surgery there were minimal complications. Two animals, one from Group one and the other from Group three had a short episode of atrial fibrillation lasting for around 1-2 minutes when the heart and pericardium were rubbed with gauze as part of the adhesion induction model. These episodes were reversed in both animals by tapping the right atrium lightly with forceps. There were no residual effects. There was only one intra-operative death during the study, where an animal from Group four died due to an episode of ventricular fibrillation during rubbing of the heart. One animal from Group one had moderate bleeding from the anterior vena cava due to injury on entering the chest and this was successfully controlled by suturing the damaged area with a 4-0 Prolene suture. There was no further bleeding and an extra 250 ml of intravenous Hartmann's solution were given to replace the lost volume in addition to the baseline fluid rate of 10ml/kg/hr. The animal was not compromised by the blood loss. One animal from Group three had a delayed recovery from anaesthesia by over half an hour but eventually recovered and was returned to the recovery cage. All the other animals of the four Groups underwent uneventful initial surgery with a good anaesthetic recovery period.

One animal from Group two died three to four hours post-operatively due to an unknown cause. Another from Group three died 24 hrs after surgery due to no apparent cause. Post-mortem examination did not reveal any abnormality in both animals. Apart from these two animals, all the other animals in the study were stable for the first 24 hours in the recovery cage and were returned to their littermates in a healthy condition.

4.2.2 Adverse effects of indomethacin treatment

At the beginning of the experimental work, pigs in Group two were treated orally with indomethacin at a dose rate of 3mg/kg/dose eight hourly but this was not tolerated well. Five out of six animals started on this regime died within 2-21 days after surgery.

Three animals died within 48-72 hours after surgery and in all three, post-mortem examination revealed extensive acute peritonitis. The peritonitis was due to a 1.5-2 cm rupture of the greater curvature of the stomach in the pyloric gland region. A fourth animal in this group exhibited weight loss and poor appetite around one week after surgery. These clinical signs continued for a further two weeks, after which the animal died. On autopsy, the stomach mucosa was normal but extensive necrotising colitis involving a 20 cm length of the mid to lower spiral colon was found. At this stage, the mode of administration of indomethacin was changed from oral to intramuscular using the same dose of 3mg/kg. This was trialed on a fifth animal in this initial experimental group, and again symptoms of poor appetite, weight loss and this time vomiting were seen around one week after surgery. The animal died 10 days post-operative. Autopsy revealed widespread peritonitis subsequent to a ruptured stomach. The peritonitis in this animal had been present for a few days with relative containment of the stomach contents around the site of rupture.

Histopathological examination of the stomach at the site of rupture did not reveal any other contributing factor such as fungal infection in any of the examined specimens. A range of other tissues in all the deceased animals was also examined histopathologically but no abnormalities that could be attributed to indomethacin toxicity were identified. All the deceased animals were replaced by new ones.

Only one animal given this initial dosing regime intramuscularly survived but it suffered a sternal wound infection and dehiscence around 3-4 weeks after the initial operation. This required debridement and re-wiring of the sternum in addition to five days of antibiotic treatment. The animal recovered well but was also replaced on the trial.

Because of the adverse effects of indomethacin, the dose was changed to 1mg/kg/dose 8 hourly administered intramuscularly. There were no ill effects in any of the animals given this dose and all continued to gain weight normally till the time of re-opening.

4.2.3 Housing period

All animals were kept alive, some for three months and some for six months. During

this period, they all maintained a good appetite with adequate weight gain. None of the animals suffered from post-operative bleeding or wound infection. The animals from each group that underwent blood sampling during this period for inflammatory markers tolerated this procedure well with no residual neck haematomas.

4.2.4 Results of re-sternotomy

All animals underwent a re-sternotomy after being slaughtered at the Charters Towers abattoir. Two to three animals were slaughtered and then re-opened in one day. Trips to the abattoir for re-openings were performed an average of once a week. All animals tolerated the transport to the abattoir well and there were no complications.

Re-sternotomy was performed successfully in all animals. In two animals from Group one, a small laceration on the right ventricle was noted on re-sternotomy caused by the oscillating saw. This had no effect on gross adhesive tissue evaluation or on tissue sampling. All other animals underwent a re-sternotomy with no injury to underlying structures. Exposure and lighting was adequate for gross adhesion evaluation to be performed accurately. All tissue samples were taken successfully from each animal with no tissue distortion or damage.

4.2.5 Gross evaluation of adhesions

The average value for the Adhesion Percentage Scale (APS) and the Adhesive Tissue Tenacity Scale (ATTS) for each group with re-opening after 12 weeks are shown in Table 4.1. The average value for the Adhesion Percentage Scale (APS) and the Adhesive Tissue Tenacity Scale (ATTS) for each group with re-opening after 25 weeks are shown in Table 4.2. The probability values for the statistical comparison between the treatment groups and the control group in all the animals are shown in Table 4.3.

Table 4.1 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for each group re-opened after 12 weeks.

	N	Average APS	Average ATTS
Group 1	8	3.9 (3-4)	3.6 (2-4)
Group 2	8	2.7 (2-4)	2.1 (1-4)
Group 3	8	3.6 (2-4)	2.7 (2-4)
Group 4	8	3.3 (2-4)	2.3 (2-4)

N = number of animals in each group.
Range in values is given in parenthesis.

Table 4.2 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for each group re-opened after 25 weeks.

	N	Average APS	Average ATTS
Group 1	3	4.0 (4-4)	3.6 (3-4)
Group 2	3	2.6 (2-4)	2.0 (1-3)
Group 3	3	3.3 (2-4)	2.9 (1-3)
Group 4	3	3.1 (1-4)	2.5 (1-3)

N = number of animals in each group.
Range in values is given in parenthesis.

Table 4.3 Probability values of the comparison of the average value for APS and ATTS of each of the treatment groups with the control group in all animals.

Comparison	P-value (APS)	P-value (ATTS)
Group 2 v Group 1	0.003	0.001
Group 3 v Group 1	0.241	0.005
Group 4 v Group 1	0.025	0.002

The maximum values for the average APS and ATTS were found in the control group. The least was in Group two, followed by Group four then Group three. Examples of the adhesions present in the four groups of animals are illustrated in Figures 4.1 - 4.4. The results for APS and ATTS were statistically analyzed between the three treatment groups and the control group. This was done for the 11 animals in

each group as the differences in average values of the APS and ATTS between the animals re-opened after 12 weeks and those re-opened after 25 weeks were insignificant. There was no interest at this stage providing differences between Groups two, three and four.

Both the APS and ATTS values were significantly less in Group two than the control group (P -values 0.003 and 0.001 respectively). When Group three were compared Group one, the APS value was not significantly less than the control (P -value 0.241), whereas the ATTS values of Group three were significantly less than the ATTS values in the control group (P -value 0.005).

Although generally less than the control group, the APS values for Group four were not significantly different (P -value $0.025 > 0.05/3$ under the Bonferroni adjustment). It should be noted that the Bonferroni correction does make it harder to reject the null hypothesis and as the p -value of 0.025 is still reasonably low, further investigations of Group four versus Group one might need to be considered with a larger sample size. The ATTS values for Group four were shown to be significantly lower than the control group (P -value 0.002).

4.2.6 Effects of omeprazole on adhesion formation

Omeprazole was used in two additional groups, an additional control group (four animals) and an additional Co-Seal group (two animals) to evaluate any effect of this drug on adhesive tissue formation as it was not used in the original control or Co-Seal groups. Both the APS and ATTS were evaluated on these additional animals. The results are given in Table 4.4.

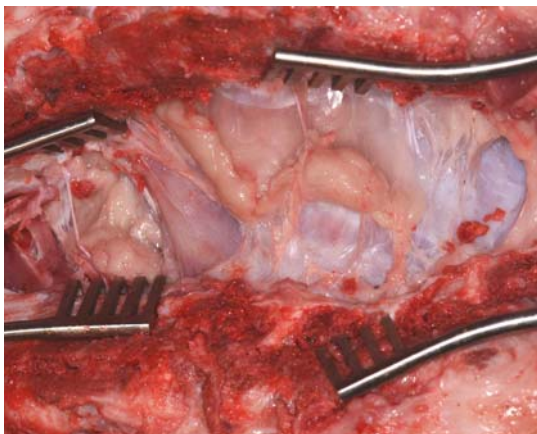


Figure 4.1. Group 1(control) showing extensive adhesions on re-opening the sternum.

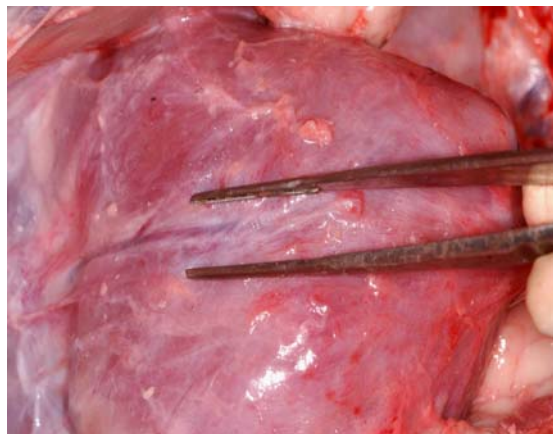


Figure 4.2. Group 2 (indomethacin) with thin adhesions and a coronary artery clearly visible.

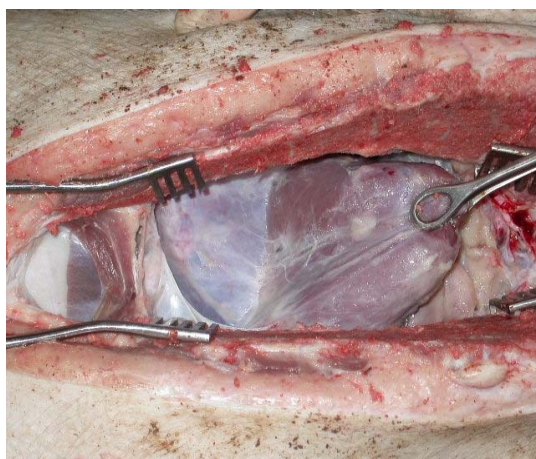


Figure 4.3. Group 4 (Co-Seal) with the heart easily dissected off the pericardium.



Figure 4.4. Group 3 (rofecoxib) with more adhesions as the heart was dissected.

Table 4.4 Averages for the Adhesion Percentage Scale (APS) and Adhesive Tissue Tenacity Scale (ATTS) for the additional groups treated with omeprazole.

	N	Average APS	Average ATTS
Additional control group	4	3.75 (3-4)	3.75 (3-4)
Additional Co-Seal group	2	2.9 (2-3)	2.0 (2)

Range in values is given in parenthesis.
N = number of animals in each group.

4.2.7 Microscopic evaluation of adhesions

As mentioned in Chapter three (section 3.6), multiple biopsies were taken from the heart and surrounding tissues to assess the thickness of the adhesive bands that formed post-operatively. The most important samples that were examined were the myocardial biopsies to assess mainly any epicardial thickening. The types of inflammatory cells were observed in all samples taken and the degree of inflammation was assessed.

Inflammation was minimal in all tissue samples examined throughout the study. Lymphocytes were the predominant inflammatory cells when present and necrosis was absent in all samples. Adhesive tissue thickness was seen least in Group two (indomethacin Group); this was observed mainly in the epicardial thickening (Table 4.5) and retrosternal samples (Table 4.6).

Table 4.5 Average thickness in millimeters of epicardium in all groups according to time of re-opening.

Group	Re-opening time		
	3 months (n=8)	6 months (n=3)	Total average (n=11)
Group 1	1.51 (0.6-3.2)	1.45 (0.2-2.7)	1.50 (0.2-3.2)
Group 2	1.00 (0.1-2.4)	0.56 (0.2-0.9)	0.88 (0.1-2.4)
Group 3	1.41 (0.1-3.2)	1.93 (0.3-4.3)	1.55 (0.1-4.3)
Group 4	2.07 (0.5-3.6)	2.45 (1.1-3.7)	2.15 (0.5-3.7)

Range in values is given in parenthesis.

Table 4.6 Average thickness in millimeters of retrosternal adhesions in all groups according to time of re-opening.

Group	Re-opening time		
	3 months (n=8)	6 months (n=3)	Total average (n=11)
Group1	2.85 (0.5-9.5)	2.57 (1.9-3.2)	2.8 (0.5-9.5)
Group 2	2.50 (0.6-6.7)	0.68 (0.3-1.1)	1.96 (0.3-6.7)
Group 3	2.47 (0.1-4.5)	2.58 (1.2-4.9)	2.50 (0.1-4.9)
Group 4	2.54 (1.2-6.5)	0.70 (0.2-1.2)	2.17 (0.2-6.5)

Range in values is given in parenthesis

Table 4.7 Probability values of the comparison of the thickness in millimeters of epicardium of each of the treatment groups with the control group in all animals according to time of re-opening.

Comparison	<i>P</i>-value (3 months)	<i>P</i>-value (6 months)
Group 2 v Group 1	0.366	0.351
Group 3 v Group 1	0.854	0.675
Group 4 v Group 1	0.319	0.308

Table 4.8 Probability values of the comparison of the thickness in millimeters of Retrosternal adhesions of each of the treatment groups with the control group in all animals according to time of re-opening.

Comparison	<i>P</i>-value (3 months)	<i>P</i>-value (6 months)
Group 2 v Group 1	0.657	0.020
Group 3 v Group 1	0.757	0.922
Group 4 v Group 1	0.919	0.020

4.3 Discussion

4.3.1 Adhesion Induction Model

The adhesion induction model used in this study is clearly explained in Chapter 3 (section 3.3.2). The model was very successful in terms of inducing a maximum inflammatory response and dense adhesion induction as seen by the gross evaluation results of the control group. The main methods that were used to induce inflammation and adhesion formation included:

1. Rubbing tissue surfaces with gauze. The reason for this procedure was to irritate these surfaces thereby increasing the inflammatory response. This simulates the frequent use of gauze material during human surgery.
2. Aspiration of five ml of blood directly from the right atrium and leaving this blood in the pericardial cavity. This blood formed multiple clots, which

adhere to the surface of the heart and the pericardium leading to adherence of these surfaces with each other and increasing the formation of adhesions. This simulates the blood usually left in the pericardial sac in humans as a result of the operation.

3. Drying the heart surface by leaving it exposed for 10 - 15 minutes. This causes more irritation to tissues and increases the inflammatory response. This simulates the drying of tissues due to exposure to air during human surgery and also the use of air blower devices during open-heart procedures. Air blowers are used to facilitate coronary grafting by making the field more accessible after blowing the blood away from the coronary arteries, but will lead to drying of the heart surfaces.
4. The use of aortic and atrial purse-string sutures. The idea behind using these sutures in this model was to simulate human cardiac surgical procedures where these sutures are applied for cannulation of the aorta and the atrium and to increase the inflammatory response and therefore adhesion formation in the pericardial sac.

4.3.2 Gross adhesion evaluation

The reduction of adhesion percentage has been successful in comparison of Group two v Group one as seen by the *P*-value of the APS ($P = 0.003$). There has also been a very effective change in tenacity of adhesive tissue in Group two as compared to Group one as seen by the *P*-value of the ATTS between these two groups ($P = 0.001$). This coincides with the clinical findings and ease of dissection on re-opening the Group two animals as compared to Group one. This suggests that indomethacin is a potent anti-inflammatory agent with major effects on adhesive tissue formation, in terms of amount and texture.

Although there was no statistical difference in the values of the APS between Groups three and one, there was a statistical difference in the ATTS between these two groups. This indicates that rofecoxib is a weaker agent for reducing adhesion formation and changing tenacity of adhesive tissue compared to indomethacin but still enough to cause desired changes in adhesive tissue tenacity.

Comparison of the results of Group four with Group one shows that Co-Seal as a barrier to adhesion formation is very effective in terms of changing adhesive tissue tenacity but not as effective in actually reducing the amount of adhesive tissue formed. It was shown to be superior to rofecoxib in both aspects but less effective than indomethacin.

It was concluded that, based on the use of the APS and ATTS for gross adhesion evaluation, indomethacin is foremost in reduction of adhesion formation and in changing tenacity of adhesive tissue from dense fibrous adhesions to thinner and more transparent adhesions, which are easier to dissect on re-openings.

Co-Seal followed indomethacin in effects on adhesion formation although a larger group of animals will be required to clearly demonstrate statistical significance. Rofecoxib follows these two agents in its effect on adhesion formation with its main effect being on adhesive tissue tenacity rather than the amount of adhesion.

4.3.3 Microscopic evaluation

All tissue samples taken from the animals were assessed microscopically for adhesive tissue thickness. Special interest and focus was on retrosternal adhesions as these represent the first layer of adhesions encountered during re-sternotomy procedures. Special interest was also given to epicardial thickening seen on myocardial biopsies as the amount of thickening on the surface of the heart affects the visibility of the heart surface and coronary vessels after re-sternotomy. Retrosternal adhesive tissue thickness was least in the indomethacin group (Group two); this was followed by Group four, Group three and finally, the thickest tissues were found in Group one (control group).

Epicardial thickening as examined on the myocardial biopsies was least in Group two; this was followed by Group one, Group three and finally the thickest epicardium was found in Group four (Co-Seal).

4.3.4 Effect of indomethacin on the gastrointestinal tract

During the study, initial problems with gastrointestinal complications, mainly a ruptured stomach, were encountered due to the high dose of indomethacin used.

Changing the mode of administration from oral to intramuscular did not solve the problem.

It is well known that indomethacin is a potent anti-inflammatory agent that inhibits both COX-1 and COX-2 isoenzymes although it is relatively selective for COX-1 (Rang *et al* 1999). Cyclooxygenase-1 is responsible for the cytoprotective prostaglandins critical to the maintenance of gastric mucosal integrity.

In a more general sense, regulation of gastric epithelial permeability is important for the protection of gastric mucosa from secreted acid. However, the exact mechanism(s) for this regulation in gastric mucus cells remains unknown although prostaglandins are known to be important in the regulation of gastric epithelial permeability (Takezono *et al* 2004). When gastric surface mucus cells are exposed to acid, epithelial permeability is rapidly decreased to inhibit back diffusion of acid. Prostaglandins dependent upon COX-1 (particularly PGE₂) play an important role in this protective response to acid exposure.

Cyclooxygenase inhibitors such as indomethacin may inhibit the regulation of epithelial permeability by reducing PGE₂, thereby making acid injury more likely (Takezono *et al* 2004). This leads to gastric mucosal damage that can lead to inflammation and subsequent ulceration (Cabral and Hoffman 1999). This is a systemic rather than a local effect and for this reason, parenteral indomethacin gives a similar inhibition of PGE₂ as seen with the oral route.

It has been suggested that the anti-inflammatory actions of NSAIDs were due to COX-2 inhibition while the unwanted effects were due to inhibition of COX-1 (Silas and Clegg 1999). For this reason, treatment with COX-2 inhibitors is associated with lessening of gastric and oesophageal mucosal damage as seen experimentally in rabbits in which oesophagitis was induced by acidified pepsin (Lanas *et al.* 2003).

By reducing the dose from 3mg/kg/dose to 1mg/kg/dose eight hourly, the pigs in the study responded well clinically. There was no further decrease in appetite and weight gain was within normal limits until the time of slaughter. There was no wound infection in any of the animals treated with the lower dose of indomethacin throughout the post-operative period.

4.3.5 Effect of omeprazole on adhesion formation

The gross evaluation of adhesions for both the Group one and Group four additional groups mentioned in Chapter three (section 3.4) showed average APS and ATTS values nearly identical to their original groups. This indicates that the use of omeprazole for seven days in these two groups had no effect on adhesion formation.

4.3.6 Application of Co-Seal

Co-Seal was applied locally on the heart, the pericardium and retrosternal tissues via a spraying technique using pressurized air and special applicators. The layer applied was a thin coat, approximately 1-2 mm in thickness. This was not accurately measured, but applying one to two coatings of Co-Seal for each surface would suffice as advised by the manufacturing company. This would then solidify within 60-120 seconds into a thin transparent gel. Eight mls of Co-Seal per animal were enough to provide an adequate coating of all tissues.

4.4 Summary

Post-operative adhesion reduction has been a problem studied by many research groups over the years. Adhesions form as a result of post-operative inflammation. The use of anti-inflammatory drugs and barriers in this study was aimed at reducing this response and therefore adhesion formation.

Adhesion formation in this study was assessed by both gross and microscopic evaluation according to two main scales, the APS and ATTS for gross evaluation and microscopic evaluation on tissue samples taken after re-opening of the pigs.

It was concluded that indomethacin was superior to Co-Seal and rofecoxib in reducing and changing the tenacity of adhesions from dense to thin tissues requiring more blunt rather than sharp dissection. This made re-opening of animals that had been treated with indomethacin a lot easier with much less dissection needed to isolate the heart from the pericardium.

CHAPTER FIVE

PLASMA CONCENTRATIONS OF ANTI-INFLAMMATORY DRUGS AND INFLAMMATORY MARKERS

5.1 Introduction

The principle pharmacological effect of non-steroidal anti-inflammatory drugs (NSAIDs) is due to their ability to inhibit prostaglandin synthesis by blocking the activity of both COX-1 and COX-2 (Cabral and Hoffman 1999). Most NSAIDs currently in use are inhibitors of both isoenzymes. The anti-inflammatory action of the NSAIDs is mainly related to their inhibition of COX-2 and it is probable that, when used as anti-inflammatory agents, their unwanted effects are due largely to their inhibition of COX-1 (Rang *et al.* 1999). This has triggered the introduction of highly selective COX-2 inhibitors as potent anti-inflammatory drugs and at the same time have minimal side effects due to lack of inhibition of COX-1 isoenzyme. An example in this group is rofecoxib (Vioxx) which was used in Group three in this study. The idea was to deliberately use both non-selective and highly selective COX-2 NSAIDs to compare the effects on the inflammatory process with the control group.

Two animals were used initially to determine if the doses of indomethacin and rofecoxib given in this study would reach an adequate plasma concentration and give an effective inhibition of inflammation. One animal was dosed with indomethacin (pig 1) at 1mg/kg/dose eight hourly orally and the other with rofecoxib (pig 2) at 0.5mg/kg once daily given orally. Dosing was for five days in both animals. Plasma drug levels were determined using HPLC while inflammatory inhibition was assessed by measuring peripheral blood concentrations of PGE₂ and TXB₂ in both animals.

With the anti-inflammatory effect of these two drugs used, it was expected that an inhibition in the systemic inflammatory response after surgery would be reflected in the measured inflammatory markers in the blood. For this reason, several inflammatory markers were tested post-operatively in four animals in each group to

assess their inhibition by these drugs. The markers measured (see Chapter 3, section 3.5) were total and differential white cell count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) as general markers, and prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) as specific markers. A coagulation screen was also performed on the same four animals in each group to assess any pro- or anti-thrombotic effect of NSAIDs. To assess this, Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and International Normalization Ratio (INR) were measured in the same blood samples taken for inflammatory markers.

5.2 Results

5.2.1 Non-steroidal anti-inflammatory plasma levels and inflammatory inhibition.

5.2.1.1 Indomethacin

No relationship was found between plasma concentrations of indomethacin and inhibition of the COX pathway as determined by plasma concentrations of PGE2 and TXB2 (Table 5.1). Even when indomethacin concentrations were below the detection level of the assay, inhibition of both PGE2 and TXB2 continued in most samples as compared to the baseline values

Table 5.1 Relationship between indomethacin plasma concentration and plasma concentration of prostaglandin E2 and thromboxane B2 in pig 1.

Sampling Day	Plasma concentration		
	Indomethacin (ng/ml)	PGE2 (pg/ml)	TXB2 (pg/ml)
Baseline	-	250	85
Day 1	N/D	110	65
Day 2	N/D	200	50
Day 3	N/D	200	130
Day 4	187.8	40	50
Day 5	82.3	90	30
Day 6	N/D	150	50

N/D Not detectable

5.2.1.2 Rofecoxib

There was a relationship between plasma concentrations of rofecoxib and plasma concentrations of PGE2 and TXB2 (Table 5.2). The highest concentration of rofecoxib was on day 2 and this was associated with the lowest concentration on PGE2 and TXB2 relative to the baseline values.

Table 5.2 Relationship between rofecoxib plasma concentration and plasma concentration of prostaglandin E2 and thromboxane B2 in pig 2.

Sampling Day	Plasma concentration		
	rofecoxib (ng/ml)	PGE2 (pg/ml)	TXB2 (pg/ml)
Baseline	-	200	350
Day 1	N/D	150	200
Day 2	322	60	166
Day 3	217	110	235
Day 4	184	200	200
Day 5	65.9	200	350
Day 6	31.6	200	350

N/D Not detectable

5.2.2 Coagulation screen

Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and International Normalization Ratio (INR) in blood plasma were assessed for significant rate of change after surgery. Average values of APTT, PT and INR for each group are shown in Tables 5.3, 5.4 and 5.5.

Table 5.3 Average values for the activated partial thromboplastin time in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).

Group	Baseline	Day 2	Day 5	Day 10
One	43.5 (31-56)	38.5 (20-57)	21.0 (20-22)	17.5 (15-20)
Two	37.5 (25-67)	107.3 (20-240)	24.7 (20-39)	27.0 (20-41)
Three	102.2 (46-240)	86.5 (26-240)	24.2 (20-31)	30.0 (22-48)
Four	41.0 (24-63)	28.7 (20-40)	25.0 (20-30)	38.2 (27-60)

Range shown in parenthesis
N = 4 for each group.

Table 5.4 Average values for the prothrombin time in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).

Group	Baseline	Day 2	Day 5	Day 10
One	9.5 (9-10)	10.5 (9-12)	10 (9-11)	9.5 (9-10)
Two	8.0 (7-9)	9.6 (9-10)	9.5 (9-10)	9.3 (9-10)
Three	8.5 (8-10)	8.5 (7-10)	10 (9-11)	8.5 (8-9)
Four	8.7 (8-9)	9.2 (9-10)	9.0 (8-10)	9.0 (8-10)

Range shown in parenthesis
N = 4 for each group.

Table 5.5 Average values for the international normalization ratio in all animal groups before surgery (baseline) and after surgery (days 2, 5, and 10).

Group	Baseline	Day 2	Day 5	Day 10
One	0.90 (0.8-1.0)	0.90 (0.9-1.1)	0.90 (0.8-1.0)	0.90 (0.9-1.0)
Two	0.80 (0.7-0.9)	0.90 (0.8-1.0)	0.95 (0.9-1.0)	0.86 (0.8-0.9)
Three	0.82 (0.8-0.9)	0.82 (0.7-0.9)	0.90 (0.7-1.0)	0.80 (0.7-0.9)
Four	0.85 (0.8-0.9)	0.87 (0.8-1.0)	0.85 (0.8-0.9)	0.85 (0.8-0.9)

Range shown in parenthesis
N= 4 in each group

5.2.2.1 Activated Partial Thromboplastin Time

No significant difference was found in the rate of change of activated partial thromboplastin time (APTT) between the four animal groups on samples taken on days 2, 5 and 10 post-operative from the baseline sample (Table 5.6).

Table 5.6 Probability values for the activated partial thromboplastin time change after surgery between all groups.

	Control	Indomethacin	Rofecoxib
Control	-	-	-
Indomethacin	0.508	-	-
Rofecoxib	0.499	0.116	-
Co-Seal	0.924	0.340	0.412

5.2.2.2 Prothrombin Time

No significant difference was found in the prothrombin time between the four animal groups in samples taken on days 2, 5 and 10 post-operative from the baseline sample (Table 5.7).

Table 5.7 Probability values for the prothrombin time change after surgery between all groups.

	Control	Indomethacin	Rofecoxib
Control	-	-	-
Indomethacin	1.0	-	-
Rofecoxib	0.827	0.80	-
Co-Seal	1.0	1.0	0.412

5.2.2.3 International Normalization Ratio

No significant difference was found in the international normalization ratio between the four animal groups on samples taken on days 2, 5 and 10 post-operative from the baseline sample (Table 5.8).

Table 5.8 Probability values for the international normalization ratio change after surgery between all groups.

	Control	Indomethacin	Rofecoxib
Control	-	-	-
Indomethacin	0.315	-	-
Rofecoxib	0.888	0.258	-
Co-Seal	0.891	0.155	0.696

From the above findings, the P values for the coagulation cascade were $P>0.05$ and therefore not statistically significant.

5.2.3 Inflammatory markers results before and after surgery in all groups.

5.2.3.1 White cell counts.

Figure 5.1 and Table 5.9 show the results for the total white cell count (WCC) measured as a baseline before surgery and on days 2, 5 and 10 post-operative for all the groups. There was a marked increase at days 2 and 5 days after surgery in the Control group followed by a decline to baseline values at day 10. In the indomethacin group, the WCC was mildly elevated at day 2 post-operative followed by a decrease towards day 5 then a gradual increase at day 10 after the drug had been stopped. In Group three (rofecoxib), the WCC increased by a small amount by day 2 and then there was a rapid decline at days 5 and 10. The Co-Seal group had a marked increase in WCC by the second day followed by a plateau towards day 5 then a decline by day 10. When the increment in WCC in both the control and Co-Seal groups was compared to the two drug groups, it was found that both drugs were effective in inhibiting a significant increase in WCC post-operative.

Table 5.9 Mean (\pm SEM) white cell counts $\times 10^9/L$ for the four groups of pigs.

Day of test	Group			
	Control	Indomethacin	Rofecoxib	Co-Seal
Baseline	20.4 (± 2.9)	28.1 (± 1.05)	33.1 (± 3.6)	18.5 (± 2.9)
Day 2	34.6 (± 5.6)	30.9 (± 4.2)	33.5 (± 5.2)	30.7 (± 7.06)
Day 5	29.0 (± 4.2)	30.1 (± 2.4)	29.2 (± 3.3)	29.5 (± 5.7)
Day 10	24.6 (± 1.5)	33.2 (± 3.04)	25.2 (± 3.5)	23.2 (± 1.4)

There were no significant differences in the baseline white cell counts between Groups two and four when compared to the Control group (P -values 0.077 and 0.648 respectively) while Group three was significantly greater than the Control group (P -value 0.008). There were no significant differences between Groups two, three and four when compared to the control Group (P -values 0.651, 0.890 and 0.634 respectively) at day 2 or day 5 (P -values 0.861, 0.980 and 0.943 respectively).

At day 10 there were no significant differences between Groups three and four and the Control group (P -values 0.862 and 0.708 respectively) while Group two was significantly greater than the Control group (P -value 0.035).

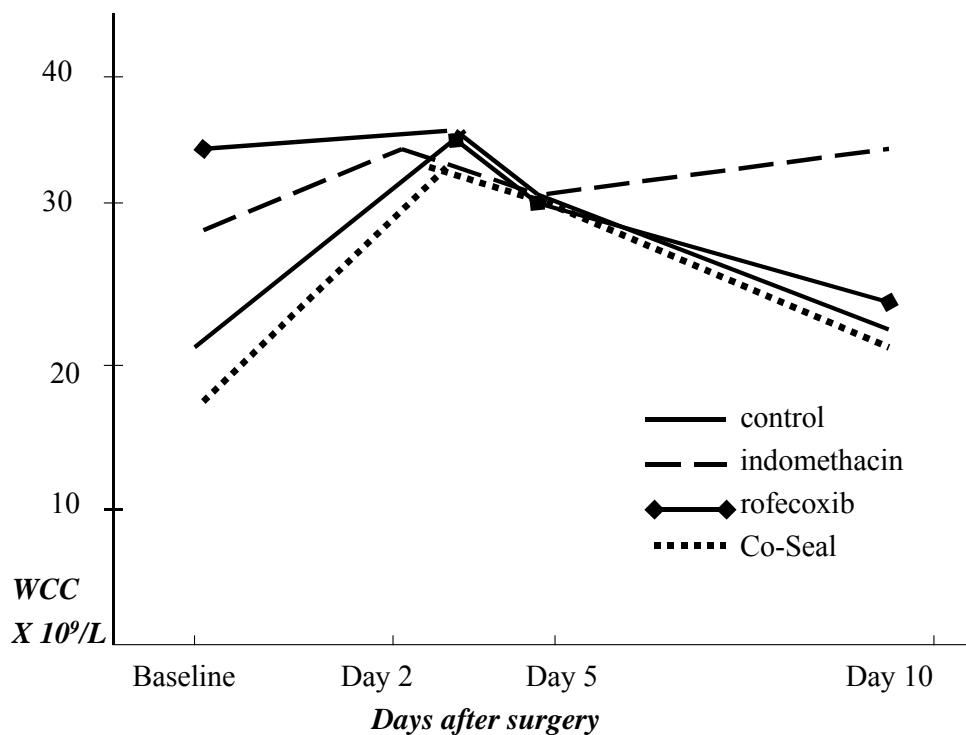


Figure 5.1 Mean total white cell count in each group before and after surgery (N = 4 per group).

5.2.3.2 Erythrocyte sedimentation rate.

The results of the erythrocyte sedimentation rate (ESR) measured before the surgery and also at days 2, 5 and 10 post-operative are presented in Figure 5.2 and Table 5.10. In the Control group, the ESR increased markedly from the pre-operative baseline level at days 2 and 5, followed by a decline at day 10.

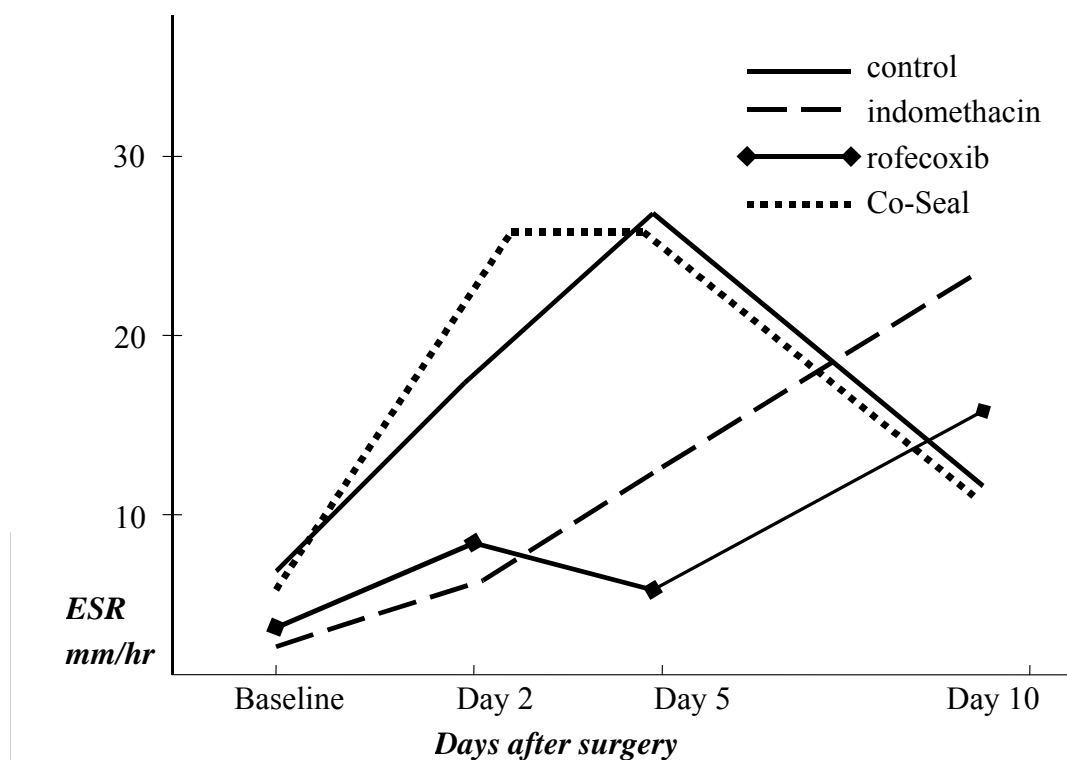


Figure 5.2 Mean erythrocyte sedimentation rate before and after surgery in each group (N = 4 per group).

Table 5.10 Mean (\pm SEM) erythrocyte sedimentation rate (mm/hr) values for the four groups of pigs.

Day of test	Group			
	Control	Indomethacin	Rofecoxib	Co-Seal
Baseline	6.2 (\pm 3.6)	1.2 (\pm 0.2)	1.5 (\pm 0.28)	4.5 (\pm 1.04)
Day 2	17.5 (\pm 11.02)	5.7 (\pm 2.4)	8.0 (\pm 1.2)	25.7 (\pm 13.8)
Day 5	25.7 (\pm 17.1)	12.0 (\pm 1.4)	6.0 (\pm 1.6)	25.0 (\pm 11.2)
Day 10	11.0 (\pm 8.0)	22.2 (\pm 6.1)	14.5 (\pm 4.2)	10.7 (\pm 4.1)

There were no significant differences between Groups two, three and four and the Control group for baseline ESR values (P -values 0.088, 0.103 and 0.527 respectively), day 2 values (P -values 0.371, 0.467 and 0.526 respectively), day 5 values (P -values 0.365, 0.201 and 0.960 respectively) or day 10 values (P -values

0.201, 0.681 and 0.976 respectively). In the indomethacin group, the ESR had increased at day 2 and continued to rise at day 5 and 10 but at a slower rate than the control group. In Group three (rofecoxib), the ESR was increased at day 2 followed by a decline on day 5 then a further increase at day 10. Finally in the Co-Seal group, the ESR had markedly increased from the baseline level at day 2, here behaving similar to the Control group, followed by a plateau at day 5 and a decrease at day 10, again similar to the Control group.

5.2.3.3 C- reactive protein concentrations.

The results of the C- reactive protein (CRP) concentrations measured before the surgery and also at days 2, 5 and 10 post-operative are presented in Figure 5.3 and Table 5.11. All animals tested had a pre-operative CRP concentration below 5mg/L. In all groups, there was a marked increase in CRP concentration at day 2 post-operative, the least increment being in the indomethacin and Co-Seal groups.

There was a plateau in the value at day 5 in the control and indomethacin groups and all groups had a marked decline to concentrations approaching baseline values at day 10. Notably, the rofecoxib group had scored the highest values.

There were no significant differences in C-reactive protein concentrations between Groups two, three and four and the Control group for baseline (*P*-values 0.230, 0.539 and 0.230 respectively), day 2 (*P*-values 0.589, 0.651 and 0.473 respectively) or day 5 values (*P*-values 0.950, 0.153 and 0.577 respectively). At day 10 there were no significant differences between Groups two and three and the Control group (*P*-values 0.825 and 0.941 respectively) while Group four was significantly greater than the Control group (*P*-value 0.040).

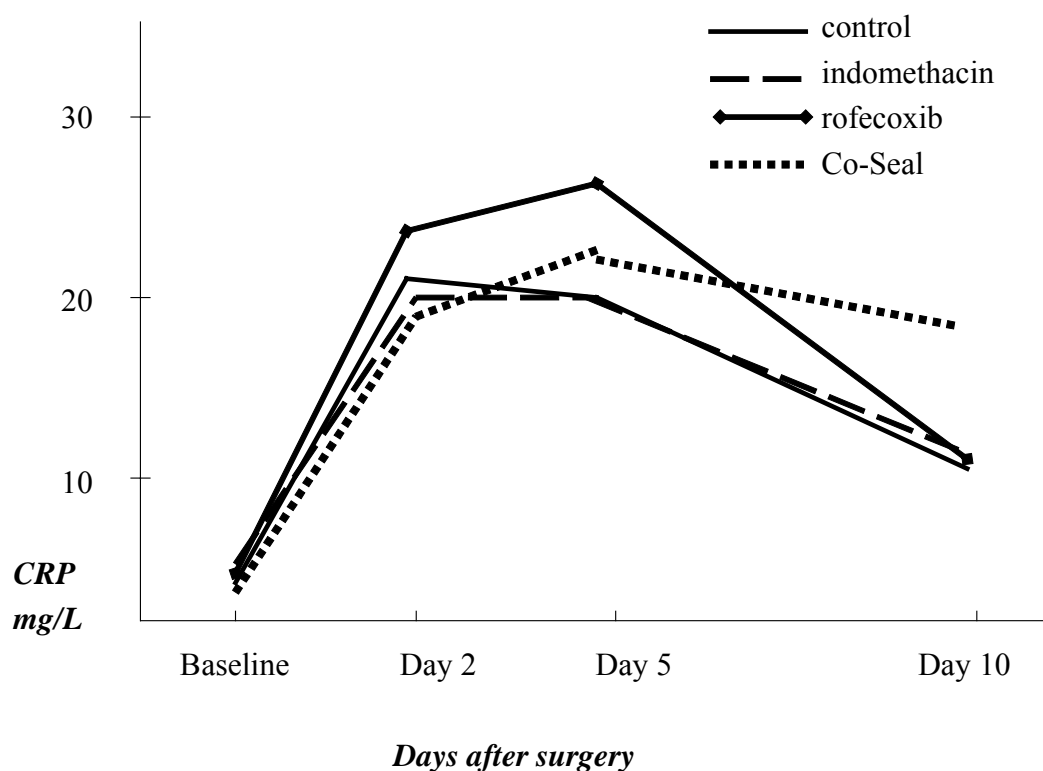


Figure 5.3 Mean C-reactive protein concentration before and after surgery in each group (N = 4 per group).

Table 5.11 Mean (\pm SEM) C-reactive protein (mg/L) concentrations for the four groups of pigs.

Day of test	Group			
	Control	Indomethacin	Rofecoxib	Co-Seal
Baseline	4.5 (\pm 1.5)	3.0 (\pm 0.0)	3.7 (\pm 0.7)	3.0 (\pm 0.0)
Day 2	21.5 (\pm 2.7)	20.0 (\pm 1.8)	22.7 (\pm 1.7)	19.5 (\pm 0.8)
Day 5	20.2 (\pm 3.9)	20.0 (\pm 1.5)	26.3 (\pm 3.0)	22.5 (\pm 1.8)
Day 10	12.0 (\pm 2.8)	12.7 (\pm 1.4)	12.2 (\pm 3.0)	20.3 (\pm 0.3)

5.2.3.4 Prostaglandin E2 concentrations.

With respect to prostaglandin E2 (PGE2) concentrations, there was a gradual increase in PGE2 levels in the control group over the first five days post-operative followed by a small decrease at day 10 (Figure 5.4 and Table 5.12).

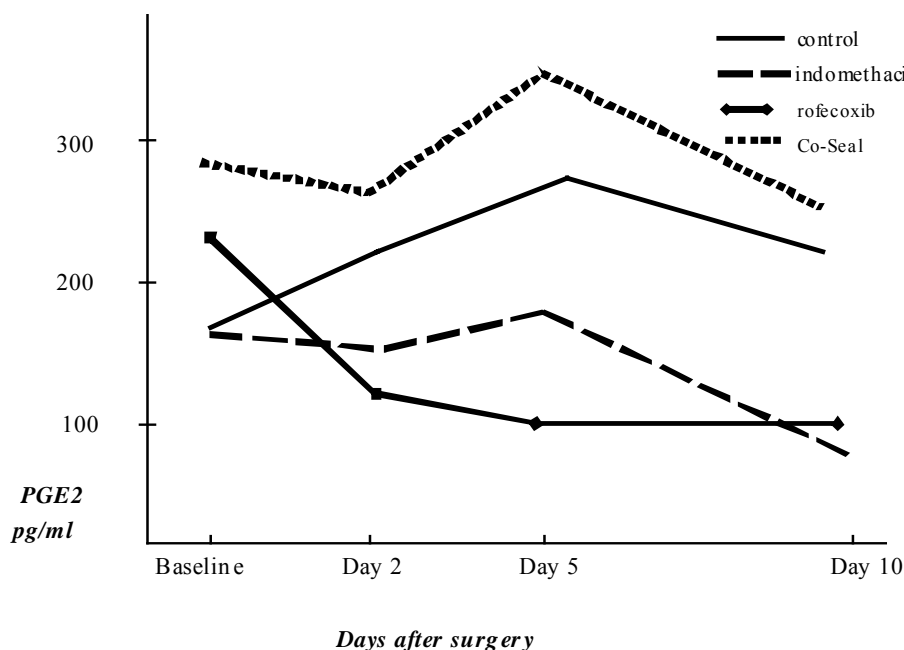


Figure 5.4 Mean prostaglandin E2 concentrations before and after surgery in each group (N = 4 per group).

Table 5.12 Mean (\pm SEM) prostaglandin E2 (pg/ml) values for the four groups of pigs.

Day of test	Group			
	Control	Indomethacin	Rofecoxib	Co-Seal
Baseline	180.0 (\pm 42.4)	172.5 (\pm 40.2)	230 (\pm 23.8)	292.5 (\pm 60.8)
Day 2	212.5 (\pm 62.8)	157.5 (\pm 41.3)	120 (\pm 20.0)	277.5 (\pm 33.2)
Day 5	265.0 (\pm 20.6)	182.5 (\pm 32.2)	108 (\pm 35.5)	345.0 (\pm 26.2)
Day 10	235.0 (\pm 48.5)	95.0 (\pm 20.6)	105 (\pm 35.0)	280.0 (\pm 46.9)

In the indomethacin group, there was a decline at day 2 followed by a return to near baseline concentrations on day 5 then a decrease at day 10. The rofecoxib group showed the most impressive decline in PGE₂ post-operative with concentrations continuing to drop even 10 days after surgery. In regards to the Co-Seal group, there was a decline at day 2 followed by an increment at day 5 and a return to near baseline at day 10.

There were no significant differences between Groups two, three and four and the Control group for baseline (*P*-values 0.906, 0.436 and 0.095 respectively) or day 2 concentrations (*P*-values 0.376, 0.148 and 0.299 respectively). At day 5, there were no significant differences between Groups two and four and the Control group (*P*-values 0.069 and 0.077 respectively) while Group three was significantly less than the Control group (*P*-value 0.003). At day 10, there were no significant differences between Group four and the Control group (*P*-value 0.435) while Groups two and three were significantly less than the Control group (*P*-values 0.027 and 0.038 respectively).

5.2.3.5 Thromboxane B₂ concentrations.

The best inhibition of thromboxane B₂ (TXB₂) was achieved in the indomethacin group which showed a marked inhibition of this inflammatory marker at days 2 and 5 after surgery (Figure 5.5 and Table 5.13). Rofecoxib also inhibited TXB₂ synthesis, while there was an increase in this marker in both the Control group (as seen at day 5) and the Co-Seal group (as seen at day 2) above pre-operative baseline levels.

There were no significant differences between Groups two, three and four and the Control group for baseline (*P*-values 0.853, 0.261 and 0.879 respectively), day 2 (*P*-values 0.055, 0.756 and 0.213 respectively) or day 10 concentrations (*P*-values 0.329, 0.929 and 0.837 respectively). No significant differences between Groups three and four and the Control group were found at day 5 (*P*-values 0.813 and 0.376), while Group two was significantly less than the Control group (*P*-value 0.015).

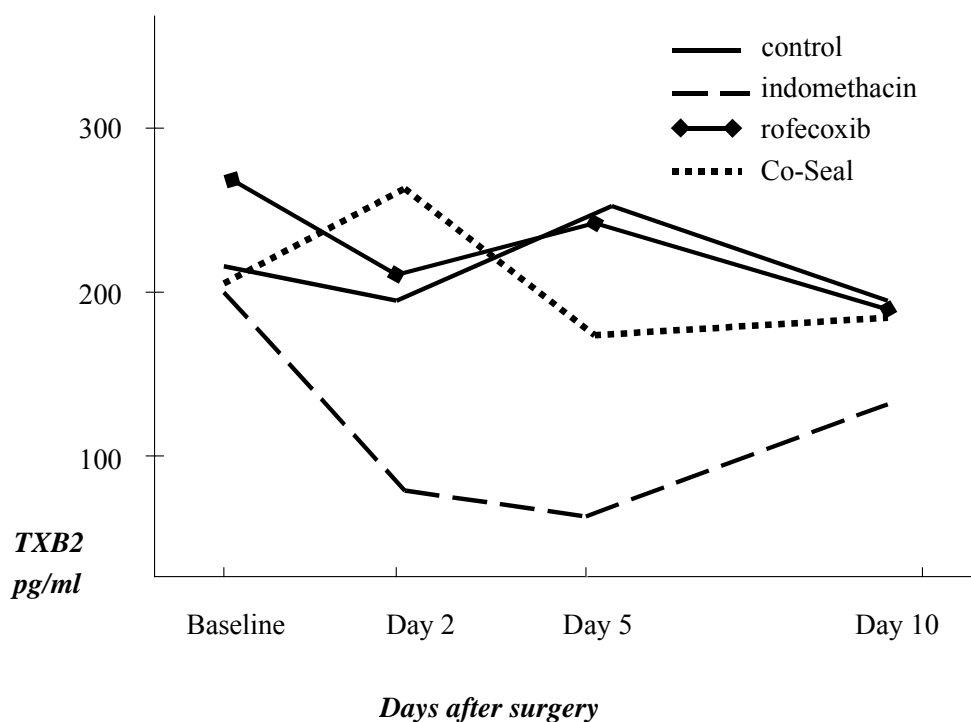


Figure 5.5 Mean thromboxane B2 concentrations before and after surgery in each group (N = 4 per group).

Table 5.13 Mean (\pm SEM) thromboxane B2 (pg/ml) concentrations for the four groups of pigs.

Day of test	Group			
	Control	Indomethacin	Rofecoxib	Co-Seal
Baseline	212.5 (\pm 31.4)	201.2 (\pm 55.2)	282.5 (\pm 39.4)	203.2 (\pm 38.1)
Day 2	199.0 (\pm 37.2)	82.5 (\pm 35.9)	216.5 (\pm 31.4)	271.2 (\pm 48.6)
Day 5	250.0 (\pm 49.8)	60.0 (\pm 12.4)	233.7 (\pm 58.5)	188.2 (\pm 54.2)
Day 10	210.0 (\pm 49.1)	140.0 (\pm 46.0)	203.7 (\pm 38.2)	195.5 (\pm 58.8)

5.3 Discussion

Two non-steroidal anti-inflammatory agents were used in this study, indomethacin and rofecoxib. The aim was to inhibit the acute inflammatory response after major surgery thereby reducing and changing the extent and type of adhesive tissues that

would form. One form of assessment of these adhesive tissues was to grade the adhesions using gross evaluation scales (see Chapter three). The other form was to measure the effect of the NSAIDs on the acute inflammatory response by measuring inflammatory markers in the blood after surgery in the drug groups and comparing the results with the control group as was done in this Chapter.

The initial phase was to establish whether measuring the inflammatory marker levels would give the required results. Two pigs were dosed with indomethacin and rofecoxib and no relationship was found between plasma concentrations of indomethacin and inhibition of the COX pathway as determined by plasma concentrations of PGE2 and TXB2 (see 5.2.1.1). Even when indomethacin concentrations were below the detection level of the assay, inhibition of both PGE2 and TXB2 continued in most samples as compared to the baseline values. The interpretation of these results was that measurement of plasma concentrations of indomethacin would not give an accurate estimate of its effectiveness.

Although a positive relationship was found between plasma concentrations of rofecoxib and inflammatory marker inhibition, on day one when plasma concentrations of rofecoxib were undetectable, there was an inhibition of the inflammatory markers below baseline levels. This indicates that measurement of inflammatory markers rather than plasma concentrations would be more appropriate to determine the effectiveness of both indomethacin and rofecoxib.

Assessment of any anti- or pro-thrombotic effect of both indomethacin and rofecoxib was done by measuring blood plasma Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and International Normalization Ratio (INR). No significant change was found and it was concluded that both anti-inflammatory drugs did not effect coagulation during or after surgery. This correlates well with the clinical findings of no complications from bleeding in any of the animal groups.

The average inflammatory marker results of Groups two, three and four were compared to those of the Control group according to the day of testing. The idea here was to assess whether the anti-inflammatory agents used in this study had achieved significant inflammatory marker inhibition when compared to the Control group,

especially for PGE2 and TXB2. There was no expectation that Co-Seal would cause inhibition of inflammatory markers as it has no anti-inflammatory properties.

With baseline levels, there was no significant difference between the three treatment groups and Group one in any of the inflammatory markers tested apart from the white cell count for Group three which was significantly greater than Group one. The comparison of marker averages on day 2 measurements between Groups two, three, four and Group one revealed no significant difference in any of the inflammatory markers tested.

This was not the case with day 5 measurements as there were some significant differences in PGE2 and TXB2 levels. For PGE2 measurements, Group three was significantly less than Group one and when looking at TXB2, it was found that Group two was significantly less than Group one. There were no significant differences with the other general inflammatory markers (WCC, ESR and CRP) on day 5.

In regards to day 10 measurements, there were mixed results. In the WCC, only Group two showed a significant difference greater than Group 1. For the CRP results, only Group four was significantly greater than the Control group while there were no significant differences in CRP levels between Groups two and three when compared to Group one. When comparing PGE2 averages, there were no significant differences between Group four and the control Group while both Groups two and three were significantly less than Group one. There were no significant differences on day 10 in both ESR and TXB2.

Indomethacin and rofecoxib achieved inhibition of the highly specific markers (PGE2 and TXB2) from day 5 onwards. This is seen in the marked inhibition of TXB2 by indomethacin and of PGE2 by rofecoxib, both on day 5. On day 10, PGE2 was significantly reduced compared to Control group values in both the indomethacin and rofecoxib groups.

Both anti-inflammatory drugs used in this study however, did not achieve significant inhibition of the general inflammatory markers (WCC, ESR and CRP). Another observation with these three inflammatory markers was the inconsistency of levels

expected over the days measured. The expectation would be that these markers would increase over the first week or so after surgery then decline towards day 10 in the control and Co-Seal groups due to the maximal inflammatory response that occurs after surgery within the first five days or so. It was expected that these markers would be inhibited in the anti-inflammatory groups in the first week post-operative while the drugs were being administered and to rise again towards day 10 after the anti-inflammatory drugs had been stopped after day 5. The WCC results met the expectations in both control and Co-Seal groups but not in the rofecoxib group as WCC levels decreased towards day 10.

ESR results better met expectations in control, Co-Seal and rofecoxib groups but not in the indomethacin group as this marker continued to rise after day two. Concentrations of CRP were not effected by either anti-inflammatory drug used. Concentrations of this marker continued to rise during the first 5 days in Groups two and three and declined towards day 10, opposite to expected.

This inconsistency may be due to the generality of these markers and their fluctuating levels with associated problems other than inflammation post-operatively. For example any condition that elevates fibrinogen (e.g., pregnancy, diabetes mellitus, end-stage renal failure, heart disease, collagen vascular diseases, malignancy) may also elevate the ESR (Sox and Liang 1986). In anaemia, with the hematocrit reduced, the velocity of the upward flow of plasma is altered so that red blood cell aggregates fall faster. Macrocytic red cells with a smaller surface-to-volume ratio also settle more rapidly (Malcolm 1999). In the animals tested in this study however, none were anaemic or had an elevated fibrinogen level post-operatively. Due to the variation of results with the inflammatory markers, a larger number of animals would need to be tested to yield statistically significant differences.

5.4 Summary

Indomethacin and rofecoxib were used as anti-inflammatory agents with the aim of reducing the acute inflammatory response after major surgery. There was little correlation found between the plasma concentrations of indomethacin and the degree of inhibition of the inflammatory response as seen by the results of inflammatory marker measurements but there was some correlation in the rofecoxib group.

Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and International Normalization Ratio (INR) were assessed for significant rate of change after surgery. No significant change was found.

Plasma concentrations of general and highly specific inflammatory (WCC, ESR, CRP and PGE₂, TXB₂ respectively) markers were assessed for inhibition after surgery in four animals in each group. The results of four samples on different dates were compared between Groups two, three and four and Group one to assess any inhibition of these markers in the anti-inflammatory groups when compared to the Control group. The main inflammatory markers were significantly inhibited by indomethacin and rofecoxib were PGE₂ and TXB₂ within the first week after surgery.

CHAPTER SIX

GENERAL DISCUSSION

This research project investigated reducing the extent of adhesion formation following surgical procedures on the heart. Adhesion formation is the formation of abnormal attachments between tissues and organs as the end result of the inflammatory response to surgical and non-surgical tissue trauma. This is part of the normal healing process after injury but the aim of this study was not to prevent adhesions from forming but to reduce and change the tenacity of adhesive bands from thick fibrous tissues to thinner tissues that are easier to separate.

The stimulus for this investigation was that post-operative adhesions are a cause of increased morbidity and mortality in human patients. Abdominal adhesions are associated with more clinical symptoms than adhesions around the pericardium. Some clinical complications include post-operative pain, intestinal obstruction, infertility and prolonged hospital stay. Following surgery on the heart, the problem arises on re-sternotomy as adhesions uniformly form between the epicardium and surrounding structures such as the pericardium, mediastinal fat, pleura and sternum (Vander Salm *et al.* 1986). Those patients requiring re-operations are at risk of injuries to the heart, great vessels, and cardiac grafts during the re-sternotomy. Adhesions can severely complicate re-operations by making re-entry hazardous, impeding orientation and visibility, increasing the amount of blood loss and prolonging operative time (Hendrix *et al.* 2001). Changing the tenacity of adhesive tissues will therefore be associated with less post-operative complications whether they be clinical or on re-opening patients when required.

Most studies undertaken to reduce or totally prevent adhesion formation have been performed targeting peritoneal rather than pericardial adhesions. In many of these studies, research teams have used steroidal and non-steroidal (NSAIDs) anti-inflammatory drugs to antagonize the inflammatory response mainly after surgery. Other groups have used various barrier techniques to separate inflamed tissues.

Steroids have been abandoned as post-operative medications because they have been associated with major wound healing problems such as increase incidence of

dehiscence and infection. This was seen by Vander Salm and his team in 1986 when they used methylprednisone to inhibit pericardial adhesions. Various non-steroidal anti-inflammatory drugs have been investigated to inhibit adhesion formation. They have been associated with less dehiscence and infection rates than steroidal agents and have therefore been a major part of this study.

6.1 The pig model

In this study, pigs were used as research animals mainly because they have been recognized as suitable animal models for human disease based on their comparative anatomy and physiology. They have been increasingly utilized as biomedical research models in the last twenty-five years (Swindle and Smith 2000) and have been used as general surgical models of most organs and systems with particular emphasis for cardiovascular research including atherosclerosis. They have also been used as models in research for the digestive system, and in recent years in transplantation and xenograft research (Swindle and Smith 2000).

Another reason for pigs being explored as models for research is their widespread availability. They are also relatively easy to handle and house, and breed well giving large litters. With this increase in the use of pigs in biomedical research have come technical developments in surgery, anesthesia, husbandry and handling techniques. These technical advancements have made it easier to use this species in research and have also improved the humane care and use of pigs by research institutions worldwide.

Sheep could have been used in the study because they are readily available and are cheaper to purchase and feed than pigs. However, sheep are ruminant animals and have a number of differences in their physiology and anatomy compared to humans. Dogs could be considered as a surgical model as in the past they have been used in a variety of cardiac and other biomedical studies. Vander Salm *et al.* (1986) used dogs as an animal model for prevention of pericardial adhesions, but modern society has considerable reservations about the use of dogs for research purposes and it was deemed that it would have been difficult to obtain approval from an Animal Ethics Committee. In addition, it would have been difficult to obtain dogs of uniform age and genetic background for these studies.

The experience from this study was that pigs can withstand major surgery with minimal complications. There were no complications during induction and maintenance of anaesthesia and minimal complications during the surgical procedure. The tissues do need however, careful handling during surgery, especially the heart as all the arrhythmias that were seen during surgery occurred during handling of the heart especially when it's surface was rubbed as part of the technique to induce adhesions. Adequate fluid therapy during surgery was essential to minimize episodes of hypotension and decreased cardiac output especially when the heart was elevated to access rubbing of the posterior surfaces.

6.2 Selection of drug doses

A literature review did not identify information on therapeutic doses for indomethacin and rofecoxib in pigs. Most pigs are killed at a young age (12-20 weeks) for human consumption, and while some adult breeding pigs may develop arthritic problems, it is not usual to treat these animals with NSAIDs as is the case in aged pet animals such as dogs and cats. Merck Sharp and Dohme who produce both indomethacin and rofecoxib could not assist, as their initial animal trials for both these medications did not use pigs as their model. Research centers in Australia such as The Baker Institute in Melbourne and Herston Research Center in Brisbane (University of Queensland) had not used these drugs in pigs in the same fashion as in this study.

Short-term administration of indomethacin in pigs has been reported in Denmark (Unmack *et al.* 2001). This research group was contacted for advice but they had only used indomethacin for 10-24 hours intravenously (M. Unmack-pers. comm. 2003) and had no experience in the doses required for oral or intramuscular administration for five days as was intended in this study. For these reasons, in this study, human doses were taken into consideration with the assistance of a pharmacological software program to reach an adequate and safe dose for indomethacin and rofecoxib in pigs. This was followed by a trial to find out if these doses were adequate and safe to use, as mentioned in detail in Chapter three, section 3.2.

6.3 Surgical procedures, induction and assessment of cardiac adhesions

The surgical procedures used on the pigs were designed to simulate human cardiac surgery as much as possible. The idea was to expose the pig heart, pericardium and surrounding tissues to the same type of surgical handling as that of the human heart in cardiac surgical units around the world.

From the stages of preparation of the skin and draping the animals before surgery, care was taken to use disinfectants and drapes used in human operating theatres. After induction and maintenance of anaesthesia, the type of incision used to reach the heart was a median sternotomy, identical to most human operations. Once entering the pericardial sac, the same suture material as in human surgery was used on the pig aorta and right atrium as purse-string sutures. Closure of the chest cavity was identical to human procedures, using six sternal wires to approximate the sternal tables followed by closure of the fat layer and skin using the same suture material as in human surgery. The procedures used to induce adhesion formation were quite effective as the pericardial adhesions in the control animals were extensive, very firm and difficult to separate.

This model was calculated in a sense to induce the maximum inflammatory response after surgery, leading to maximum adhesive tissue formation in the pericardial sac and the retrosternal tissues. The amount of adhesions that eventually formed as seen on re-opening the control animals after three and six months showed that this model was extremely effective in adhesion induction. The only aspect that may be changed is the epicardial drying time to be increased from 10 minutes to at least double the time as more extensive drying may enhance the inflammatory response, but the amount and density of adhesions in the control group animals was maximum and closely resembled the type of adhesions seen in re-sternotomy operations on human patients.

The presence of adhesions on re-opening the animals in all groups and visual assessment is the main determinant, and is crucial. In the Adhesion Percentage Scale (APS), three assessors evaluated the anterior, lateral and posterior surfaces of the heart and the percentage of those surfaces covered by adhesions. The type of dissection on re-opening, which ranged from mainly blunt to mainly sharp, formed

an important part of the gross evaluation of adhesive tissue formation. This was the basis for the Adhesive Tissue Tenacity Scale (ATTS). These adhesion assessment scales are described in more detail in Chapter three, section 3.6.

Analysis of the adhesive tissues formed after the initial surgery on the animals was done (Chapter 4 section 4.2.7) to correlate the thickness and type of adhesions with their gross assessment and then find any common relationship between the two methods of assessment in each animal group. The expectation would be to find minimal tissue thickness measurements with thin, easily dissectible adhesions and maximum thickness measurements with thick dense, difficult to dissect tissues and to be predominant in the same group. Special interest was focused on the retrosternal adhesions and epicardial thickening on myocardial biopsies. Retrosternal adhesions represent the first layer of adhesions during re-sternotomy procedures and are the first layer that needs to be dissected in order to reach the underlying structures.

Clinical open-heart surgery adhesions can be a cause for major injury to the heart or aorta on re-opening the sternum. Special interest was also given to the epicardial thickening seen on myocardial biopsies. With increased thickness of the epicardium, visibility of the heart surface and coronary vessels becomes obscured. Inpatients requiring re-operations for coronary bypass surgery, it is imperative that the native coronary arteries on the heart surface are isolated and visualized clearly before grafting. This can prove to be very difficult with thickened and non-transparent epicardium on the heart surface. Retrosternal adhesive tissue thickness was least in the indomethacin group (Group two), this was followed by Group four, Group three and finally, the thickest tissues were found in Group one (control group). Epicardial thickening as examined on the myocardial biopsies was least in Group two, followed by Group one, Group three and finally the thickest epicardium was found in Group four (Co-Seal) (for details, see Chapter four, section 4.2.7). The thinness of adhesive tissue seen in the indomethacin group corresponds with the reduction and changes seen in both the APS and ATTS.

Another form of adhesive tissue assessment is to measure the tension of the adhesive bands that form; this can be done using a tensiometer. This method was not used in this study due to unavailability of equipment.

6.4 Inflammatory markers in peripheral blood

Assessment of systemic inflammatory changes was determined by the measurement of a number of the inflammatory markers in peripheral blood of four animals in each group pre- and post-operatively. Ideally, all pigs in each group should have had inflammatory markers measured as well as each 24 hours during the post-operative period. This was not done due to lack of resources, as inflammatory marker measurement was extremely costly. The inflammatory markers measured were total white cell count and differential count (WCC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), prostaglandin E2 (PGE2) and thromboxane B2 (TXB2).

Measurement of inflammatory markers was to assess the severity of the inflammatory response to major surgery in each group and to assess the degree of inhibition of inflammation by giving NSAIDs in Groups two and three as compared to the control group. Co-Seal had no known anti-inflammatory effect and is purely a barrier. It was therefore not expected that inflammatory markers would be suppressed due to the direct effect of Co-Seal, but it was interesting to see if this barrier would, due to separation of inflamed tissues, cause a reduction in the acute inflammatory response after surgery and result in a significant reduction in inflammatory markers compared to the control group.

Measurement of inflammatory markers to monitor the extent of the inflammatory reaction after surgical procedures or after induction of an infective process has been used by a number of research groups using the pig model. Inflammatory markers measured have varied between groups. For example, in the surgical field, Burpee *et al.* (2002) measured post-operative CRP, interleukin-6, tumor necrosis factor (TNF) and cortisol after open and laparoscopic liver resection in the pig. They found that interleukin-6 and TNF were significantly elevated in the open group as compared to the laparoscopic group while CRP and cortisol showed no significant difference. The inflammatory response to induced infection has also been monitored by some research teams. Heegaard *et al.* (1998) measured CRP in addition to haptoglobin, and major acute phase protein in pigs after inducing *Actinobacillus pleuropneumoniae* infection by aerosol inoculation. They found that the three markers examined showed a marked increase in the first two to five days due to the

acute inflammatory response and showed that these markers were major acute phase reactants.

Most research groups measured inflammatory markers within the first five days after surgery or infection induction. In this study, measurement of markers for the first 10 days would seem adequate to assess the acute post-operative inflammatory response although daily measurements would have given more data for statistical analyses. However, a balance needed to be made between the cost of analyzing blood samples, number of blood samples that could be obtained and the stress that daily blood samples would have posed to the animals. The latter issue may have been partly overcome by the placement of in-dwelling venous catheters to obtain daily samples.

Both indomethacin and rofecoxib produced a significant inhibition of the highly specific markers (PGE2 and TXB2) from day five onwards. This was mainly seen in the marked inhibition of TXB2 and PGE2 by indomethacin on days five and 10 respectively and of PGE2 by rofecoxib on day five. Neither anti-inflammatory had any significant inhibition of the general inflammatory markers (WCC, ESR and CRP). Indomethacin was the most effective inhibitor of the inflammatory markers and this corresponded well with adhesions being the thinnest as assessed by histopathological examination (see Chapter 4 section 4.2.7) and easiest to dissect as seen by both the APS and ATTS in this group (see Chapter 4 section 4.2.5). As expected Co-Seal did not have any effect on the acute systemic inflammatory response but did manage to inhibit adhesion formation and significantly change adhesion tenacity by separating the inflamed tissues from each other.

6.5 Coagulation screen assessment

Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and International Normalization Ratio (INR) were assessed for significant rate of change after surgery. The pre-operative (baseline) values and post-operative values at day 2, 5 and 10 were assessed in each animal group and compared to the control group and each other (details in Chapter 5, section 5.2.2.1).

There was no significant rate of change in any of the coagulation screen values after surgery in any of the groups. This reveals that both indomethacin and rofecoxib have

no significant effect on the coagulation screen and therefore do not have a pro or anti-coagulatory effect after surgery. Clinically, throughout the study, there was no excessive bleeding during or after surgery in any of the animals.

6.6 Future research directions

The results of this study have been promising in terms of reducing and more importantly, changing the texture of adhesions that form after surgery. The agent that provided maximal beneficial effect was indomethacin followed by a PEG barrier (Co-Seal).

This research work described in this thesis could be extended along the following lines of inquiry:

1. Use the same animal groups with cardiopulmonary bypass with or without coronary artery grafting. This would make the experimental work much more similar to clinical work in human cardiac surgery.
2. Combine indomethacin and Co-Seal in one group. As both agents have given good results in terms of adhesive tissue reduction and change in tenacity, combining both agents in one group by administering Co-Seal locally on the heart before closure followed by administration of indomethacin pre and post-operatively as discussed in Chapter 3, section 3.3.3. would theoretically give better results than if each agent was used on its own.
3. Use other barriers on the market such as Tisseel 500 (Baxter Healthcare, NSW) that are competitive in price and effect with Co-Seal.
3. Start human trials for indomethacin and Co-Seal. The recommended doses for indomethacin in humans would be 25-50 mg eight hourly which is the standard daily dose currently used in humans for arthritic problems.

6.7 Conclusion

As mentioned throughout this thesis, the reduction of post-surgical adhesion formation remains a challenge. Many methods have been tested to reduce and inhibit adhesive tissue formation after surgery, whether it is in the abdomen or the chest.

In this study, the use of NSAIDs and a barrier was effective in reducing the amount of adhesions forming in the pericardial sac after surgery. The best results were seen with indomethacin at 1mg/kg/dose two hours pre-operative and eight hourly post-operative for five days. Indomethacin significantly changed the texture of adhesions from dense to thin adhesions requiring mainly blunt dissection on re-sternotomy. These thin adhesions also corresponded with a significant inhibition of inflammatory markers post-operative, especially TXB2. The next agent to give significant changes in tissue tenacity from dense to thin adhesions on gross evaluation was Co-Seal, a PEG barrier. This was followed by rofecoxib, which significantly changed adhesive tissue tenacity but not the amount of adhesions.

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