“In nothing do men more nearly approach God, than in giving health to men”

*Cicero 106 BC - 43 BC*

A comparative study of the systemic inflammatory response to hepatic resection, microwave ablation, cryotherapy and radiofrequency ablation. Which is safest and how much can we safely ablate?

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

Fateh Ahmad BSc(Hons) MBBS(London) MRCS(Eng)

Department of Surgery

University of Leicester

September 2009
### Contents

Table of Contents i-iv  
Statement of Originality v  
Acknowledgements vi-vii  
List of Abbreviations viii-ix  
Prizes x  
Publications xi  
Oral Presentations xii  
Poster Presentations xiii

**Chapter 1**  
1-22

Introduction (I): History of tumour treatments, including ablation

1.1 Liver Tumours  
1.2 Treatment options  
   1.2.1 Medical Treatment  
   1.2.2 Surgical Resection  
   1.2.3 Ablation

**Chapter 2**  
23-44

Introduction (II): Pathophysiology of ablation-related complications, the systemic inflammatory response syndrome and aims of the study

2.1 Complications of ablation and their Pathophysiology  
   2.1.1 Cryotherapy  
   2.1.2 Radiofrequency Ablation  
   2.1.3 Microwave Tissue Ablation
2.2 The Systemic Inflammatory Response Syndrome

2.2.1 Cytokines and their actions

2.2.2 Organ-specific inflammatory response
   i) The respiratory system
   ii) The renal system
   iii) The liver

2.3 Aims of the study

Chapter 3 45-75

Materials and Methods

3.1 Ethical approval
3.2 Ex-vivo work
3.3 Peri-operative methodology
3.4 Sample Analysis
   3.4.1 Cytokine Analysis
   3.4.2 Heat Shock Protein 70 Analysis
   3.4.3 Urinary Retinol Binding Protein (RBP) Analysis
   3.4.4 Wet/Dry Lung Weight Ratio
   3.4.5 Broncho Alveolar Lavage Protein Quantification
3.5 Statistical analyses

Chapter 4 76-84

Clinical results: Survival, tolerance, and behaviour

4.1 Summary
4.2 Results
4.3 Discussion of results
4.4 Other complications
Chapter 5

Comparison of cytokine response to hepatic radiofrequency ablation, cryotherapy, microwave ablation and surgical resection in a rat model.

5.1 Summary
5.2 Experimental Results
  5.2.1 IL-1β
  5.2.2 IL-6
5.3 Statistical Analysis
  5.3.1 IL-1β
  5.3.2 IL-6
5.4 Discussion

Chapter 6

Urinary retinol binding protein and serum heat shock protein 70 expression following hepatic resection, microwave ablation, radiofrequency ablation and cryotherapy.

6.1 Summary
6.2 Experimental Results
  6.2.1 Retinol Binding Protein (RBP)
  6.2.2 Heat Shock Protein 70 (HSP70)
6.3 Statistical Analysis
  6.3.1 RBP
  6.3.2 HSP70
6.4 Summary of Results
6.5 Discussion
Chapter 7 153-184

A comparative study into the pulmonary effects of hepatic resection, microwave, radiofrequency and cryotherapy ablation at three different volumes of ablation

7.1 Summary
7.2 Experimental Results
  7.2.1 Lung Heat Shock Protein 70 (HSP70)
  7.2.2 Total Protein Content in Bronchoalveolar Lavage Fluid (TPC in BAL)
  7.2.3 Wet/Dry Lung Weight

7.3 Statistical Analysis
  7.3.1 HSP70
  7.3.2 TPC in BAL
  7.3.3 Wet/Dry Lung Weight

7.4 Summary of Results
7.5 Discussion

Chapter 8 185-195

Conclusions

8.1 Conclusions
  8.1.1 Cytokine induction as a marker of systemic inflammation
  8.1.2 Acute renal injury
  8.1.3 Acute lung injury

8.2 Limitations and possible modifications to study
8.3 Future work

Bibliography 196-222
The following thesis submitted for the degree of Doctor of Medicine entitled

\textit{A comparative study of the systemic inflammatory response to hepatic resection, microwave ablation, cryotherapy and radiofrequency ablation. Which is safest and how much can we safely ablate?}

is based on work conducted by me at the Department of Surgery, University of Leicester during the period between February 2003 and February 2005.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

Approximate number of words: 29,000

None of the work has been submitted for another degree at this or any other University.

\textbf{Fateh Ahmad}

\textbf{September 2009}
Acknowledgements

I am hugely indebted to many people who have made this work possible. I was honoured to be trusted by Mr David Lloyd to undertake this project, and to him I am thankful for the opportunity, help and encouragement at many levels. Mr Andy Strickland was an inexhaustible source of ideas and support and I would not have been able to start the work without the skeleton he provided. For that I am and continue to be truly grateful.

The work was performed at the University of Leicester research facilities and I thank the very able veterinary nurses, particularly Sarah Shorne and Debbie Bursnall, for their expert help and guidance with the experiments, and also for looking after my experimental subjects! Much work was done from very early in the morning until late at night, and this was only made possible by their dedication and unwavering desire to help.

I also thank Professor Nick London for providing important scientific guidance when I needed it most, and Dr Michael Festing for giving up his valuable time at many points throughout the study to direct the statistical aspects of the project.

The work was funded by a University of Leicester Research Fellowship and I am indebted to Microsulis Medical Limited for their keen interest and support for this work. In particular I would like to acknowledge the help of Dr Peter Clegg of the Medical Devices Technology Group, Department of Physics, University of Bath for providing the MTA equipment and guiding its use in the initial phase of the work.

I was ably helped by Dr Rizwan Basit in processing the large numbers of ELISAs and also grateful to Mr Ian Beckingham for loan of the RFA equipment, often to the annoyance of his theatre sister!
Finally, and most importantly, I would like to thank my family for putting up with me during one of the busiest periods of my life, resilient in my absence, and loving in my times of greatest need.

I dedicate this most important piece of work of my life so far, to my wife Uzma and daughters Ayla and Aleena.
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALF</td>
<td>Acute liver failure</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho Alveolar Lavage</td>
</tr>
<tr>
<td>CT</td>
<td>Cryotherapy Ablation</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat Shock Protein 70</td>
</tr>
<tr>
<td>IL-1β</td>
<td>The pro-inflammatory cytokine Interleukin 1-β</td>
</tr>
<tr>
<td>IL-6</td>
<td>The pro-inflammatory cytokine Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>The pro-inflammatory cytokine Interleukin 8</td>
</tr>
<tr>
<td>IL-10</td>
<td>The anti-inflammatory cytokine Interleukin 10</td>
</tr>
<tr>
<td>MOF</td>
<td>Multi organ failure</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTA</td>
<td>Microwave Tissue Ablation</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol Binding Protein</td>
</tr>
<tr>
<td>Resection</td>
<td>Surgical Resection</td>
</tr>
<tr>
<td>RFA</td>
<td>Radiofrequency Ablation</td>
</tr>
<tr>
<td>Sham</td>
<td>Sham Laparotomy</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>TNF-α</td>
<td>The pro-inflammatory cytokine Tumour Necrosis Factor-α</td>
</tr>
<tr>
<td>TPC</td>
<td>Total Protein Content</td>
</tr>
</tbody>
</table>
Prizes, Publications and Presentations arising from Thesis

Prizes and Awards


2) Best Poster, European Hepatopancreatobiliary Association Annual Meeting, Heidelberg, Germany, May 2005

3) Poster of Distinction, American Hepatopancreatobiliary Association Annual Meeting, Florida, USA, April 2005

4) Best Paper, Laparoscopic Session, Association of Surgeons of Great Britain and Ireland Annual Meeting, Glasgow (£100), April 2005

5) 2nd Prize. 4th International Meeting on Hepatocellular Carcinoma, Hong Kong (US$500), December 2004

6) Young Investigator Award (100,000 Yen) International Society for Digestive Surgery, Yokohama, Japan, December 2004
Publications


2) Ahmad F, Gravante GP, Bhardwaj N, Strickland AD, Basit R, West K, Sorge R, Dennison AR, Lloyd DM. Renal effects of hepatic microwave ablation compared to radiofrequency, cryotherapy and surgical resection at different volumes of liver treated. Liver Int 2010 Jun 1. [Epub ahead of print]


Oral Presentations


2) Ahmad F, Strickland AD, Lloyd DM. IL-1β, IL-6 and TNF-α production is not accentuated by large volume hepatic ablation with microwave. American Hepatobiliary Association, Miami, March 2006

3) Ahmad F, Strickland AD, Robertson GSM, Lloyd DM. Do Focal Liver Ablative Techniques Result in Renal Damage? 41st World Congress of the International Society of Surgery, Durban, South Africa, 21-25th August 2005

4) Ahmad F, Strickland AD, Robertson GSM, Lloyd DM. Acute Lung Inflammation Following Hepatic Ablation - A Comparison of Different Ablative Modalities. 6th European Congress of the International HPB Association (IHPBA), Heidelberg, Germany, 25-28th May 2005

Poster Presentations


Chapter 1

Introduction (I): History of tumour treatments, including ablation

“That which drug fails to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. If the heat cannot cure, it must be determined to be incurable”

Hippocrates 460 BC - 357 BC

1.1 Liver Tumours

1.2 Treatment options

1.2.1 Medical Treatment

1.2.2 Surgical Resection

1.2.3 Ablation
1.1. Liver Tumours

**Historical Perspective**

Liver cancer, whether primary or secondary, is a common clinical condition which carries a high mortality. Up until the last 50 years or so, patients with these conditions were thought to be untreatable, confined to alternative medicine and with extremely poor survival rates. Surgical resection was in theory a viable option for tumours limited to the liver such as hepatocellular carcinomas and colorectal liver metastases; however this was accompanied by unacceptable morbidity and mortality rates. Newer surgical techniques, combined with anaesthetic and technological advances then allowed a subset of these tumours to be removed more safely, either as a debulking procedure or in its entirety. Attempts to resect larger tumours or malpositioned tumours (i.e. approximating major hepatobiliary structures) continued to be associated with high complication rates. Advances in chemotherapy led to some improvement in survival, although with high morbidity.

Over the last 3 decades, clinicians have sought alternative ways of treating these tumours, partly to increase efficacy of treatment, but also to increase resectability and operability. It was widely recognised that for larger tumours, or tumours involving several segments, there was a need to leave the maximum amount of normal hepatic parenchyma by selectively destroying or removing the tumour-affected portion of the liver only. The 3-dimensional anatomy of the liver segments as described by Couinaud (1952) greatly improved our understanding of what was possible to resect or ablate (Figure 1.1). Thus evolved the concept in *in-situ* ablation whether thermal, chemical or ultrasonic. More recently, neoadjuvant and adjuvant chemotherapy have been used in treating these patients to downstage tumours and...
make them amenable to resection and/or ablation. Percutaneous and minimal access ablation procedures have also made possible multiple visits to treat recurrence in the same areas of the liver, whilst leaving as much normal hepatic parenchyma as possible. These advances have been facilitated by technological breakthroughs in imaging techniques, particularly cross-sectional computed tomography and MRI.

Figure 1.1. Segmental anatomy of the liver. From http://cancer.inha.com/engine/upload/oncology/Segmental_anatomy_of_liver%5B1%5D.jpg
Incidence

Hepatocellular carcinoma (HCC) is one of the most common solid cancers in the world, with an annual incidence estimated to be at least one million new patients (Di Bisceglie et al, 1998). Furthermore, the liver is second only to lymph nodes as a common site of metastasis from other solid cancers (Weiss et al, 1986). It is not uncommon, particularly in patients with colorectal adenocarcinoma, for the liver to be the only site of metastatic disease. Surgical resection of HCC, colorectal cancer hepatic metastases, and carefully selected patients with liver-only metastases from other types of primary tumours can result in significant long-term survival benefit in 20%-35% of patients (Liver Cancer Study Group of Japan, 1990; Nagorney et al, 1989; Fong et al, 1997; Tuttle, 1998). Furthermore, surgical palliation through tumour cytoreduction in patients with symptomatic neuroendocrine tumour (carcinoid, functioning islet cell) liver metastases can ameliorate the symptoms related to excess hormone production and release (Tuttle, 1998). Relief or reduction of symptoms in this patient population can significantly improve quality of life because many of these patients survive for years despite the presence of neuroendocrine tumour liver metastases.

Unfortunately, only 5%-15% of newly diagnosed patients with HCC or colorectal cancer liver metastasis undergo a potentially curative resection (Liver Cancer Study Group of Japan, 1990; Nagorney et al, 1989). Patients with disease confined to the liver may not be candidates for resection because of multifocal disease, proximity of tumour to key vascular or biliary structures that precludes a margin-negative resection, potentially unfavourable biology with the presence of multiple liver metastases, or inadequate functional hepatic reserve related to coexistent cirrhosis. Thus, for the majority of patients with primary or metastatic hepatic
malignancies confined to the liver who are not candidates for surgical resection, novel
treatment approaches to control and potentially cure the liver disease are being developed.

**Natural history of untreated metastatic liver disease**

Without treatment, median survival rarely exceeds 9 months. In a large prospective
study conducted from 1980–1990 and including 484 patients with untreated hepatic metastases
from colorectal cancer, the median survival was 31% at 1 year, 7.9% at 2 years, 2.6% at 3 years
and 0.9% at 4 years. Factors that independently influenced survival were the volume of the
liver involvement, the presence of extrahepatic disease, metastatic lymph nodes in the
mesentery, CEA level and the age of the patient. According to the presence or absence of these
criteria, the median survival varied from 3.8 to 21 months (Stangl et al, 1994). Patients selected
for surgical resection of liver metastases represent a subset with more limited disease. Few
retrospective studies have compared the survival of patients with potentially resectable
metastases that were left untreated with survival of resected patients. Wilson and Adson (1976)
found no 5-year survivors in patients with untreated, but potentially resectable, liver metastases
compared with 28% for those who had undergone resection of their metastases. Wanebo et al
(1978) found that patients with an untreated solitary liver metastasis had a median survival of
19 months with no patient surviving 5 years, while patients with a resected single liver
metastasis had a median survival of 36 months with 25% of patients surviving 5 years. The
prognosis for patients with untreated disease is most closely associated with the extent of liver
involvement. Residual liver function also correlates with survival, since death is usually due to
liver failure. Poor survival is portended by the presence of symptoms such as pain and weight
loss, liver enzyme abnormalities and synthetic dysfunction, and a poor patient performance
status at the time of diagnosis. Healthy patients with adequate hepatic reserve and minimal liver involvement are most likely to be longer-term survivors without treatment.

1.2. Treatment Options

1.2.1. Medical Treatment

i) Neoadjuvant Chemotherapy

Whilst it is recognised that neoadjuvant chemotherapy is not curative, neoadjuvant treatment strategies are aimed at increasing resectability rates. Reports on the use of fluoropyrimidine-based cytotoxic chemotherapy have focused mainly on the treatment of patients with advanced unresectable disease and as an adjuvant treatment following resection of the primary colonic tumour (Petrelli et al, 1989; Moertel et al, 1990; Poon et al, 1991). There are no primary prospective studies of such therapy used specifically as part of a neoadjuvant programme before surgery. What has become increasingly accepted however, is the role of neoadjuvant chemotherapy as part of a multimodality regimen in liver resection.

ii) Adjuvant Chemotherapy

Following resection of liver metastases, 60-70 per cent of patients will develop recurrent disease, probably due to the presence of microscopic disease at time of initial resection. This may happen despite the use of neoadjuvant chemotherapy, so adjuvant post-operative treatment may also be justified. However, early trials of systemic and intraperitoneal chemotherapy after liver resection for colorectal metastases showed no benefit in terms of survival (Lehnert et al, 1997).
iii) **Hepatic Artery Chemoembolisation**

Hepatic artery chemoembolisation (HACE) was developed as a treatment for unresectable non-disseminated liver tumours (Figure 1.2). It increases the tumour response rate compared with systemic administration of cytotoxic agents, although it has not been shown to prolong survival (Lorenze et al, 1993). HACE has been studied most extensively in the treatment of HCCs, but has also been used in metastatic colorectal cancer. Pre-operative neoadjuvant HACE has been abandoned due to significant associated morbidity and lack of evidence that it produces any survival benefit (Lehnert et al, 1996).

![Figure 1.2](http://www.hopkins-gi.org)

**Figure 1.2.** A, B, Hepatic artery chemoembolisation; A’, with corresponding angiogram. From http://www.hopkins-gi.org
1.2.2. Surgical Resection

During the early 1980s, surgeons had become increasingly aware of the segmental anatomy of the liver and the importance of the blood supply to individual hepatic segments (Fortner et al, 2001). Recognition of the segmental basis of liver anatomy led to the evolution of segment-based resection. This has had a particular influence on surgery for colorectal metastases because it allows excision of bilateral or multiple liver lesions that might previously have been deemed unresectable (Bismuth, 1982). As a result the morbidity and mortality from hepatic surgery have greatly decreased during the past 2 decades, accompanied by a concomitant increase in overall and disease-free survival (Fong et al, 1999). During this period of increased interest in hepatic surgery, intraoperative ultrasound (IOUS) was found to be an excellent tool to define liver anatomy for resection and for the presence of occult metastatic disease. IOUS is now widely considered to be the gold standard for imaging of the liver (Takigawa et al, 2001; Bloed et al, 2000; Cervone et al, 2000), but in combination with pre-operative cross-sectional imaging and clinical assessment at the time of surgery.

Results of liver resection

Without treatment, the outlook for patients with metastatic colorectal cancer is very poor, with a median survival time in the region of 1 year (Stangle et al, 1994; Liu et al, 2003). However, surgical resection of metastases can produce long-term survival and cure in some patients. Two large multicentre studies provide data to support this. Nordlinger et al (1992) reported data from the French Association of Surgery study, which analysed 1,118 patients who underwent potentially curative liver resection at 85 centres from 1959 to 1991. Actuarial
survival was 84% at one year, 40% at three years, and 25% at five years. Hughes et al (1988) compiled data from 859 patients treated at 24 centres from 1948 to 1985 in which actuarial five-year patient survival was 33% (excluding perioperative deaths). Other institutions have reported experiences with 100 or more patients with 25% to 37% actuarial five-year survival and 28- to 34-month median survival. In 1984, a series of 252 patients with biopsy proven, unresected hepatic metastases from colorectal cancer was reported by Wagner and colleagues (1984) to achieve 5-year survival rates of 25% and 2% for patients with resected and unresected disease, respectively.

1.2.3. Ablation

History and Principles of ablation

Numerous techniques have been developed for tissue ablation. Techniques to kill tumour cells include heating, freezing, radiation, ultrasound, chemotherapy, ischaemia (e.g. portal vein embolisation), injection of caustic agents directly into the tumour, as well as various combinations of these.

i) Percutaneous Ethanol Injection (PEI)

Bean in 1981 was the first to describe the use of PEI in the treatment of renal cysts. Subsequently, Livraghi (1986) described the use of US guided needle placement into 14 hepatic lesions. All patients were treated with percutaneous injection of 95% ethanol. 3 to 9 sessions were performed for each lesion according to size. This work demonstrated that while PEI could
be used to treat focal colorectal metastatic disease, its benefits were limited by the natural course of the disease. The same author in 1995 demonstrated PEI to be safe, low cost and easily reproducible in patients with HCC, provided the accompanying cirrhosis was at most Child’s A Pugh’s classification (Livraghi et al, 1995).

ii) Laser

A neodymium:yttrium-aluminium-garnet (Nd:YAG) laser system with ceramic rods was initially developed to treat head and neck tumours using a precise method of surgical dissection. Hahl et al in 1990 were the first to use the laser in patients with malignant hepatic tumours during open laparotomy. Even though only limited necrosis was produced by these lasers, they believed that selective destruction of malignant tumours was possible using laser induced hyperthermia. For this procedure, flexible thin optic fibres are placed into the target through percutaneously placed needles using imaging guidance. The laser provides sufficient energy to allow for significant heat deposition around the fibre tip, inducing protein denaturation and cellular death. As with RFA, thermal profiles have been demonstrated to correlate well with the extent of coagulative necrosis observed histopathologically. Again, as with RFA, this form of ablation is limited by the size of achievable ablation.

iii) High-Intensity Focused Ultrasound (HIFU)

HIFU was described by ter Harr et al in 1989 as a method of treating discrete liver tumours. HIFU technology uses a high-intensity convergent ultrasound beam generated by high-power transducers to produce heat. Yang in 1991 demonstrated that HIFU could destroy
targeted deep seated tissue within the liver without causing damage to non-targeted tissue, while at the same time avoiding the need for a laparotomy. Organ movement during respiration has proved a problem with HIFU in ensuring that the targeted lesion remains within the zone of maximum intensity of the ultrasound beam to minimise collateral damage to non-neoplastic tissue. Hence, HIFU has proved to be more successful and popular in the treatment of non-moving targets, such as the prostate.

iv) Microwave Tissue Ablation (MTA)

Compared to other techniques, MTA arrived somewhat late on the scene. Seki (1994) initially described the use of percutaneous MTA in unresectable HCCs of 2cm or less in diameter. A microwave electrode was placed into the target tumour under imaging guidance and treatment was monitored using real-time ultrasound scanning to determine echogenic response. Effective ablation has been demonstrated by several groups, but up until recently ablation size was also its limiting factor (Matsukawa 1997; Shimada, 1998; Shibata, 2000; Morikawa, 2002; Laing, 2003).

In contrast to RFA, in which the inserted electrode functions as the active heat source, with MTA the inserted probes function as antennae for externally applied energy at 1000 to 2450mHz. The microwave energy applied to the tissue results in rotation of polar molecules that are opposed by frictional forces. As a result, there is conversion of rotational energy into heat (Figure 1.9). Ex-vivo studies have shown greater tissue penetration and larger zones of coagulation necrosis with MTA than with RFA or laser. This, together with a drastic reduction in ablation time, signifies much promise for this particular form of ablation.
**Figure 1.9.** Large Oxygen and smaller hydrogen molecules (a) aligning with the microwave wave (b). They will all oscillate at 2 - 5 billion times a second. They all align symmetrically and oscillate together which gives intense heat (c).

MTA has predominantly been used in the Far East to treat small sized HCCs and has largely been considered in the West to be obsolete owing to the limitations of existing technology and increasing popularity of RFA. However, recent advances in MTA technology have allowed rapid large volume tissue ablation in porcine liver (Strickland, 2002) and clinical trials. Tumours of up to 7cm have been treated effectively at a single session in less than 5 minutes, with little or no morbidity (Strickland, 2005) (Figure 1.10).
Figure 1.10. Interstitial MTA (a) with resulting zone of coagulative necrosis surrounding tumour (b). Surface ablation for superficial tumours (c), with multiple ablations (d).

Local recurrence and survival rates:

Several series have compared MTA and RFA and found MTA to have a lower recurrence rate of 7-11.8% compared with 12.8-20.9% with RFA (Xu et al, 2004; Lu et al, 2005). In addition, overall survival at 1, 2, 3 and 4 years for MTA (81%, 61%, 50% and 36%, respectively) was better than for RFA (71%, 47%, 37% and 24%, respectively). However the overall major complication rate was higher with microwave (8.2% versus 5.7%) (Lu et al, 2005). While generally results were superior for microwave treatment, some series found little difference and concluded that both modalities had equivalent therapeutic effects, complication
rates and rates of residual foci of untreated disease (Shibata et al, 2002). One large series of 288 patients with no microwave treatment-related complications reported a local recurrence rate of 8% and 1-, 2-, 3-, 4- and 5-year survival rates of 93%, 82%, 72%, 63% and 51%, respectively. Survival was worst in patients with the largest tumours, those with the greatest number and also where there was a higher Child–Pugh classification (Liang et al, 2005). Another large series with no major complications reported equally encouraging results in 234 patients undergoing ablation of 339 nodules. Survival at 1, 2, 3, 4 and 5 years was 92%, 81%, 72%, 66% and 56%, respectively, and 90% of patients analysed post ablation, either by biopsy or cross-sectional imaging, showed no evidence of residual tumour (Dong et al, 2003). In terms of 1-, 2-, 3- and 4-year survival rates (94%, 78%, 78% and 62%, respectively), this is comparable to other series (Itamoto et al, 1999).

v) **Cryotherapy (CT)**

In 1987, Ravikumar et al reported their surgical experience with hepatic cryosurgery for metastatic colorectal tumours in the liver. Their development of 8-12mm diameter liquid nitrogen-cooled probes, suitable for surgical placement within the liver, established the advent of interstitial hepatic cryosurgery (Figure 1.8). Real-time ultrasound was used to verify the progression of treatment and to measure the size of the increasing iceball that was formed by freezing. Freezing and thawing for 3 cycles was an effective means of ensuring tumour necrosis. Disadvantages of this technique were the need for open surgery and that fairly large probes had to be placed into the lesions. However, more recently, smaller probes have been developed that may be placed percutaneously under MRI guidance. Several mechanisms have been identified through which cryoablation induces tissue injury. First, cryoablation induces
direct cellular injury through tissue cooling. At low cooling rates, freezing primarily propagates extracellularly, which draws water from the cell and induces osmotic dehydration. The intracellular high solute concentration that develops then leads to damage to enzymatic systems and proteins, and injury to cellular membranes (Bischof and Rubinsky, 1993; Weber et al, 1997).

A second hypothesised mechanism of cryoablative tissue destruction is that of vascular injury, manifested as mechanical injury to the vessel wall from intravascular ice formation resulting in increased vessel permeability, reduced plasma oncotic pressure and perivascular oedema. Endothelial injury further results in exposure of underlying connective tissue and subsequent thrombus formation. Reperfusion injury results from the release of vasoactive factors after the tissue thaws, which leads to vasodilation and increased blood flow into treated areas. Subsequent high oxygen delivery and neutrophil migration into the damaged area result in increased free radical formation, and ultimately, through peroxidation of membrane lipids, further endothelial damage. The degree to which either of these mechanisms plays a role in cell destruction depends on the freezing and thawing rate, coldest temperature reached, and the duration that the temperature is maintained.
Local recurrence and survival rates:

The rate of local recurrence following CT is estimated to be between 9% and 44% (Onik et al, 1991; Adam et al, 1997; Mala et al, 2004). One of the few trials comparing RFA and CT estimated the recurrence rate for CT to be 13.6%, with two-thirds of the recurrence occurring in contact with or adjacent to a major hepatic vessel. Even though technically this is not due to a heat sink effect it is postulated that in-flowing warm blood has a protective effect and prevents adequate freezing and destruction of tumour cells directly adjacent to major blood vessels. In these series the combined rate of major and minor complications was 40.7% (Pearson et al, 1999). In the literature, reports of 3- and 5-year survival rates following CT are uncommon, and ablation is frequently combined with other treatments including chemotherapy and surgery. In one large study, the majority of patients treated with cryoablation also underwent intra-arterial chemotherapy and had a recurrence rate of 33% (Seifert and Morris, 1999). In another study,
although 3- and 5-year survival rates of 40% and 27%, respectively, were reported, the majority of patients were again treated with multimodal therapies including intra-arterial chemotherapy and hepatic artery ligation (Zhou and Tang, 1998). Other studies quote similar figures with a 3-year survival of 48% and 5-year survivals of 28% (Kerkar et al, 2004). These studies illustrate the difficulties encountered when trying to compare the results of CT with surgery or other ablative techniques, as most authors report series where a variety of different treatment modalities are used in conjunction with CT.

vi) Radiofrequency Ablation (RFA)

While percutaneous techniques of radiofrequency ablation (RFA) are relatively new, the basic technique for RFA was described over a century ago by D’Arsonval in 1891, who demonstrated that when radiofrequency (RF) waves passed through tissue, they caused an increase in tissue temperature. RF for medical applications was only popularised after the introduction of the Bovie knife in 1928; this instrument could be used either for cauterisation or for cutting tissue by varying the radiofrequency current. A pulsed or dampened current would cauterise tissue, whereas a more continuous current could be used to cut through tissue. This first generation Bovie knife was a monopolar electrode requiring grounding pads, similar to present day RFA techniques.

RFA works by causing ionic agitation of the tissues surrounding the needle. The shaft of the needle does not produce heat. Rather, the heat is produced in the tissues, and that leads to coagulation and cellular necrosis (Figure 1.3). The term radiofrequency refers to the alternating electric current that oscillates in the range of high frequency (200-1200kHz). One Hertz equates to one cycle per second. The patient is part of a closed loop circuit that includes an RF
generator, an electrode needle, and a large dispersive electrode (grounding pad). An alternating field current is created within the tissue of the patient. Because of the relatively high electrical resistance of tissue in comparison with the metal electrodes, there is marked agitation of the ions present in the target tissue that surrounds the electrode, since the tissue ions attempt to follow the changes in direction of alternating electric current. The agitation results in frictional heat around the electrode. The discrepancy between the small surface area of the needle electrode and the large area of the ground pads causes the generated heat to be focused and concentrated around the needle electrode. Fairly rapid application of current leads to a small region of coagulation as well as local tissue charring. Charring acts to inhibit further ionic agitation, thus limiting the amount of surrounding coagulative necrosis. Furthermore, water is driven away from the site and thus removes the essential electrolyte for conduction. Attempts have been made to counteract this size limitation by having several needle electrodes, each producing overlapping zones of coagulation, thus increasing the zone of tissue necrosis (Figure 1.4).
Figure 1.3. An alternating electrical current is passed across the electrode array at the tip of an RFA needle (lower left), resulting in ionic agitation and heating in the tissue surrounding the electrode array. As coagulative necrosis gradually develops in the tissue, tissue impedance rises, leading to reduction and eventual cessation in current flow from the RF generator.

Figure 1.4. Radiofrequency ablation (RFA) in hepatic cancer showing the cluster arrangement of needle electrodes, producing overlapping zones of ablation. From http://www.hopkins-gi.org
Newer RFA applicators have been designed to keep the tip and shaft of the electrode cool in an attempt to reduce the charring effect, thereby resulting in a more efficient and larger zone of tissue destruction (Figure 1.5) (Francica and Marone, 1999). McGahan and colleagues in 1992 showed that ultrasound could be used to monitor the RFA needle placement and assess the echogenic response in the tissue surrounding the RFA needle during ablation. This area of hyperechogenicity was the result of the production of ‘microbubbles’ forming from tissue dessication and roughly corresponded to the volume of tissue ablated. In addition to the development of cluster or array RFA electrodes, devices for use in open, laparoscopic and percutaneous procedures have been produced.

![Figure 1.5. The Cool-tip RF Applicator device. The generator software monitors tissue impedance and adjusts the output accordingly. Pulsed energy delivery allows the target tissue to stabilise, reducing tissue impedance increases that could limit RF output. Typical treatments are completed in a 12-minute cycle. The Cooling Effect: The electrode’s internal circulation of](image)

*The Cooling Effect:* The electrode’s internal circulation of...
water cools the tissue adjacent to the exposed electrode, maintaining low impedance during the treatment cycle. Low impedance permits maximum energy deposition for a larger ablation volume. From: www.valleylab.com/education/poes/images/cooltipglow.jpg. Copyright © 2010 Covidien. All rights reserved. Reprinted with the permission of the Energy Based Devices Division of Covidien.

**Local recurrence and survival rates:**

The local recurrence rate following RFA treatment is variable, with figures ranging from 2% to 43% (Pearson et al, 1999; Curley et al, 1999; Wong et al, 2001; Solbiati et al, 2001) with the higher rates being associated with percutaneous treatments and larger tumour sizes (Livraghi et al, 2000; Wood et al, 2000). Needle tract seeding appears to be related to experience and, although as high as 12.5% in smaller studies (Llovet et al, 2001), it falls to 0–4% in larger studies. Seeding usually occurs in patients treated percutaneously for large subcapsular tumours or in patients who have undergone pre-operative biopsy (Rhim et al, 2003; Livraghi et al, 2003; De Beare et al, 2003). There is a surprising paucity of data available for RFA survival rates, with few studies quoting 3-year figures; these range between 25% and 80%.

**Summary**

In addition to progress in chemotherapeutic regimen and technological advances in conventional surgery, many forms of *in-situ* ablation have been developed over the years in an attempt to treat previously untreatable patients. The emphasis appears to be safe and efficacious destruction of neoplastic tissue whilst preserving maximal amounts of normal hepatic
parenchyma to sustain life. These techniques may involve thermal, chemical, ionizing, vascular starvation or selective chemotherapeautic tumour destruction. Each has been shown to be effective to some extent in a select group of patients, but all are hampered by limitations. It is likely that combinations of one or more of these therapeutic modalities will form the basis of future anti-neoplastic strategies. The question is, which modality is most effective, whilst safest for the patient?
Chapter 2

Introduction (II): Pathophysiology of ablation-related complications, the systemic inflammatory response syndrome and aims of the study

“As to diseases, make a habit of two things - to help, or at least, to do no harm.”

Hippocrates 460 BC - 357 BC

2.1 Complications of ablation and their Pathophysiology

2.1.1 Cryotherapy

2.1.2 Radiofrequency Ablation

2.1.3 Microwave Tissue Ablation

2.2 The Systemic Inflammatory Response Syndrome

2.2.1 Cytokines and their actions

2.2.2 Organ-specific inflammatory response

i) The respiratory system

ii) The renal system

iii) The liver

2.3 Aims of the study
2.1. Complications of ablation and their pathophysiology

Historically, the treatment of liver tumours has been fraught with associated complications. Early surgical resection of liver tumours often met with disastrous consequences, whilst the side effects of medical oncological treatments are well documented. Despite better understanding of liver anatomy from Couinard’s work, and advances in anaesthetics in the modern era, liver resection still remains a major undertaking not least for the patient. Furthermore, initial work on the ablative modalities also encountered unacceptably high complication rates. To date many limitations of ablation are attributable not just to technology, but also patient tolerance and clinical safety. Local effects of ablative treatment including biliary strictures, deranged liver function, tumour seeding, bleeding and abscess formation have all been reported. Release of inflammatory mediators and necrotic material from the liver into the systemic circulation causes havoc further afield, with reports of respiratory distress and renal impairment most common. In more serious cases, a generalised systemic inflammatory response syndrome develops often with mortality as an end result.

2.1.1. Cryotherapy

Heat-inducing ablation techniques cause tissue destruction by creating ionic agitation and heat, which result in tissue boiling and the creation of water vapour. If cytotoxic temperatures are reached, protein denaturation and vascular thrombosis result. In cryoablation, the freezing and thawing process destroys cell membranes and organelles due to the mechanical stresses associated with the phase change from ice formation (Bischof and Rubinsky, 1993;
Weber et al, 1997). Secondary vascular thrombosis can result, adding to the eventual tissue death, but is not as prominent a feature as with the heat-based ablative modalities (Rubinsky et al, 1990). Microscopically, cell membrane destruction and cell death are clearly seen post-ablation (Ravikumar et al, 1991). In contrast, heat based ablation does not show as immediate a response, with subtle changes in nucleus and organelles (Adam et al, 2002). Some authors feel that the rapid destruction of cell membranes and the relative lack of protein denaturation associated with freezing is responsible for a more severe form of systemic inflammatory response (“cryoshock phenomenon”) than is present with tissue destruction by heat (Blackwell et al, 1999). The hypothesis is that intact cellular elements are more readily delivered into the bloodstream by freezing than with heat ablation, thus ‘presenting’ material to the immune system to mount a response. Cryoshock is a syndrome of multiorgan failure and severe coagulopathy without evidence of sepsis. Clinical studies have shown that cryoablation of 30% to 35% of the liver volume is associated with this cryoshock phenomenon, which carries a high mortality rate (18%) (Sarantou et al, 1998; Seifert and Morris, 1999; Sadikot et al, 2002). Seifert et al (1998) have confirmed the release of TNF-α and IL-6 after hepatic cryoablation in a rat model.

The toxic cellular elements released from a tumour during cryoablation may contribute to the postoperative myoglobinuria observed in most patients. Myoglobinuria can be detected after the iceball thaws, usually resolving in 1-3 days. Occasionally cryosurgery-induced myoglobinuria progresses to acute tubular necrosis with impaired renal function. As with postoperative coagulopathy, this phenomenon appears to be related to the volume of tissue frozen and is most pronounced when large lesions are frozen by 2 freezes followed by one complete thaw (Weaver et al, 1995; Sarantou et al, 1998; Bagia et al, 1998). It is also likely that
an exaggerated SIRS response as described above may also lead to a systemic shock type picture, with resulting renal hypoperfusion and consequential renal failure.

Several studies have also demonstrated acute lung injury, including ARDS, following hepatic cryoablation in animal models. In a study by Chapman et al (2000), histologic lung sections after cryoablation showed multiple foci of perivenular inflammation, with activated lymphocytes, foamy macrophages, and neutrophils. Additionally, activation of nuclear factor kappa B (NF-κB) (a transcription factor in cells involved in immune and inflammatory reactions, and exerts its effect by expressing cytokines, chemokines, cell adhesion molecules, growth factors and immunoreceptors) was detected at 1 hour in both liver and lung tissue samples of animals undergoing hepatic cryoablation but not after RFA, and serum cytokine levels were significantly elevated in cryoablation versus RFA animals. Electron microscopy of cryoablation-treated liver tissue demonstrated disruption of the hepatocyte cell membrane with extension of intact hepatocyte organelles into the space of Disse; RFA-treated liver tissue demonstrated coagulative destruction of hepatocyte organelles within an intact cell membrane. These findings correlate the clinical observation of an increased incidence of multisystem injury, including adult respiratory distress syndrome (ARDS) following large volume cryotherapy.

Pleural effusions are one of the most common processes resulting from cryosurgery of hepatic malignancies (Seifert and Morris, 1999). The majority are limited to the right hemithorax and thought to be secondary to irritative process to the liver caused by cryosurgery beneath the diaphragm. Most cases resolve by 14 days but some may require thoracocentesis to drain fluid from the pleural space. If, however, this is accompanied by ARDS as a result of the
cryoshock phenomenon, then the prognosis is poorer. The extreme end of this complication is thankfully rare and has only been seen with larger volumes of hepatic cryoablation.

Haemorrhage, a feared complication associated with any type of liver surgery, is the major morbidity in hepatic cryosurgery. The incidence of operative haemorrhage is approximately 14%, with most bleeding occurring during the passive thaw phase (Seifert and Morris, 1999). Bleeding can reflect damage to the blood vessels during placement of the cryoprobes, disruption of blood vessels secondary to the freeze process, parenchymal fracture of the liver (usually catastrophic) and/or coagulopathy from freezing large volumes or using multiple freeze/thaw cycles (Cozzi et al, 1994; Gage and Baust, 1998). Hypothermia results in an approximately 2 degree Celsius drop in core temperature and is thought to contribute to coagulopathy (Weaver et al, 1998).

It is thought that cryoablation can be performed safely near major blood vessels because the local warming effect of flowing blood protects the vessel wall, the so-called “heat-sink effect” (Jungraithmayr et al, 2004). However, cryotherapy for lesions at the confluence of the right and left hepatic duct systems remains problematic because of the potential for proximal bile duct injury and/or stricture. Biliary fistula can result from direct placement of the cryoprobes, or more commonly, from extending the freeze process to areas contiguous with the major bile ducts. Freezing of the bile duct leads to necrosis of the duct epithelium with a resultant intrahepatic biliary collection or biliary-cutaneous fistula. Bile leak and fistula have been reported in 1-10% of patients. Attempts at intraoperative intraductal warming or placement of prophylactic metal stents have not proved popular and complication data remains largely anecdotal (Silverstein et al, 1997).
Finally, hepatic cryotherapy produces an area of ischaemic necrosis - abscess formation within this area has been reported up to a year following surgery, particularly when cryotherapy has been combined with surgical liver resection (Cozzi et al, 1994).

2.1.2. RFA

Studies have compared the inflammatory response to hepatic RFA with other modalities such as resection or hepatic cryoablation. However, this has only been limited to ablation of up to 40% of total liver volume, even though in a fully functional liver, up to 70% surgical resection can be performed safely. Although the complication rate after RFA is lower than that following cryotherapy in clinical series (Pearson et al, 1999; Adam et al, 2002) there are scarce data in the literature on the systemic effects of hepatic RFA. Chapman et al (2000) studied acute lung injury following 33% RFA or cryotherapy and demonstrated that in contrast to cryotherapy, RFA did not produce significant lung injury. Some authors have postulated that the systemic inflammatory responses of thermal ablative therapies could be related to the presence of necrotic tissue left in the liver remnant. However, there is insufficient scientific evidence to support this hypothesis. Ng et al (2004) investigated the systemic responses of hepatic RFA in comparison to cryotherapy and surgical resection of the same liver volume (30%) using a porcine liver model. The systemic responses of RFA were significantly less severe than those of cryotherapy in their animal model. However, the increase in serum inflammatory markers and pneumonitis after RFA was substantial when compared with hepatectomy. RFA has been shown to induce an earlier and higher inflammatory response as
measured by inflammatory cytokines, compared with liver resection. This was not significant enough to cause major morbidity or mortality (Schell et al, 2002).

Other reported complications arising from hepatic RFA include damage to major hepatic and biliary structures resulting from placement of the needle electrodes (Rhim et al, 2003; Livraghi et al, 2003; Chen et al, 2005; Akahane et al, 2005). Jansen et al (2005) reported a procedure-specific complication rate of almost 10% and an overall complication rate of 20%. A review by Mulier et al (2002) reported an 8.9% complication rate (3670 patients) with a mortality rate of 0.5-1%. Abdominal bleeding, infection, biliary tract damage, liver failure, pulmonary complications, dispersive pad skin burns, hepatic vascular injury, visceral damage, coagulopathy, renal failure, tumour seeding were all low in incidence. It is worth commenting at this point that at the time of publication of this exhaustive review by Mulier, RFA was only capable of producing effective ablation of up to 3cm tumours. So complications following larger volume hepatic RFA had so far not been an issue as it was technically not feasible.

2.1.3. MTA

Previously available MTA technology has largely been ignored in the West owing to its inability to produce large zones of ablation. However, treatment times were significantly less than that seen with RFA for the same size ablation (Seki et al, 1994). Recent advances in MTA technology has markedly improved the efficacy of the MTA electrode, or applicator, allowing less reflection of power back into the applicator. The effect of this has been to vastly increase the field of ablation, with much larger tumours now amenable to treatment (Strickland et al, 2002). The maximum achievable ablation size is dependent on the variable power delivered and
is much larger than all conventional thermal ablation systems and is on a par with cryotherapy. Additionally, ablation is possible within minutes and several tumour ablations can be performed at one sitting with comparatively less anaesthetic time. As with all other thermal ablation techniques, care is taken to avoid major hepatic vessels and biliary structures and indeed some tumours are deemed unablatable on the basis of their proximity to these structures. As heat delivery is fast and largely dependent on generation of a ‘heat field’ rather than purely conduction, the ‘heat-sink’ effect seen with RFA and cryotherapy is not as dramatic with MTA.

In terms of complications, skin burn following percutaneous application, infection, biliary stricture and bleeding have all been reported with MTA, with the incidence of major complications ranging from 0%-11% (Yamanaka et al, 1996; Mitsuzaki et al, 1998; Abe et al, 2000; Dong et al, 2003; Liang et al, 2003). SIRS has never been reported with MTA and no study to date has studied the inflammatory response to MTA.

**Summary**

With available evidence, what is apparent is that although cryotherapy is capable of performing large volume ablations, the associated complications are too significant to be ignored. On the other hand, RFA seems to be safer in terms of morbidity, but is unable to perform effective large volume ablations. The new generation of MTA technology offers the potential for large volume tissue ablation, but there is a dearth of safety data.

The most serious complication seen following large volume hepatic ablation, aside from haemorrhage, which leads to maximum morbidity and mortality remains the SIRS response.
The remainder of this introductory chapter looks at the SIRS response and how these are measured.

### 2.2. The Systemic Inflammatory Response Syndrome (SIRS)

Inflammation is the body’s non-specific response to cellular injury; the end result of highly amplified, yet tightly controlled, humoral and cellular mechanisms aimed principally at limiting tissue damage. Localised inflammation is an appropriate protective physiological response often eliminating the initiating noxious stimulus and restoring homeostasis. Loss of local control or an exaggerated host response can, however, result in a progressive illness, the systemic inflammatory response syndrome (SIRS) that in extreme cases, can lead to multiorgan dysfunction and death (Parker et al, 2000). Multiple organ failure (MOF) remains the leading cause of delayed mortality in surgical intensive care units (Maship et al, 1994). Once established, MOF defies standard critical care supportive measures and mortality exceeds 50% (Partrick et al, 1999). Despite intensive investigation, the pathogenesis of post injury MOF remains unclear. The hypothesis is that post-injury MOF occurs as the result of a dysfunctional inflammatory response. Following major tissue injury and haemorrhagic shock, patients develop an early physiologic state of systemic hyperinflammation, referred to as the systemic inflammatory response syndrome (SIRS). Certain patients appear to be vulnerable such that early secondary insults amplify SIRS, which produces organ dysfunction ultimately culminating in MOF (Bone, 1996).
2.2.1. Cytokines and their actions

Cytokines are released from activated cells (mainly mononuclear phagocytes but also fibroblasts and myofibroblasts) in response to tissue injury, and bind to specific receptors on cell surfaces (or in serum) and induce dimerisation or polymerisation of receptor polypeptides activating intracellular signalling pathways (e.g. kinase cascades), which results in the production of transcription factors. These migrate to the nucleus and bind to enhancer regions of genes induced by that cytokine. These cytokines have pleiotropic effects on many cells of the body. One major part of the acute phase response is focused on the liver, due to the fact that this organ is a prominent source as well as target of cytokines (Moshage, 1997; Ramadori and Christ, 1999). First, macrophages, which after activation are generally considered to be the main source of inflammatory cytokines, are present within the liver; Kupffer cells lining the hepatic sinusoids are the largest population of resident tissue macrophages of the body. Second, hepatocytes are targets for cytokines in the acute phase response (Jones and Summerfield, 1988). They synthesise the majority of plasma proteins (Barle et al, 1997). Cytokines are also released directly from activated Kupffer cells into the blood, and are most likely responsible for the induction of the acute phase response in distant organs. For comparative purposes, in SIRS, tumour necrosis factor-alpha (TNF-α) appears to be the first cytokine released systemically (peak at 2 hours) followed by interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) (peak 4 hours) (Dofferhoff et al, 1992) (Figure 2.1).
**Figure 2.1.** Cytokine induction following insult.

From www.bio.davidson.edu/Courses/Immunology/Students/Spring2003/Sole/acute.gif

Under most circumstances, cytokines are not stored in a preactivated form in macrophages (or other cells). Cytokine production requires active gene transcription of messenger RNA unique to each cytokine, a process closely regulated by the transcription factor complex nuclear factor kappa B (NF-κB) in the cell. NF-κB is a nuclear factor kappa-light-chain-enhancer of activated B cells (Figure 2.2) and is a ubiquitous rapid response transcription factor in cells involved in immune and inflammatory reactions. It exerts its effect by expressing cytokines, chemokines, cell adhesion molecules, growth factors, and immunoreceptors. In this manner, NF-κB contributes to immunologically mediated diseases such as allograft rejection, rheumatoid arthritis, and bronchial asthma (Lee and Burckart, 1998).
Recent studies suggest that NF-κB activation in alveolar macrophages in the lung occurs as an important early step in the development of adult respiratory distress syndrome (ARDS) (Chapman et al, 2000). Activation of the TNF-α regulatory protein NF-κB has been documented in alveolar macrophages from patients with ARDS, however TNF-α levels in trauma patients have not been found to be elevated, even in patients who eventually developed MOF. Similarly, IL-1 is a proinflammatory cytokine that shares many of the proinflammatory properties of TNF-α. Indeed, TNF-α and IL-1 not only stimulate their own release, but also the release of each other (as well as IL-6 and IL-8), thus amplifying the cascade of inflammatory mediators. IL-1 has two bioactive forms: IL-1α and IL-1β. IL-1β is the predominant IL-1 and a major product of activated human monocytes, tissue macrophages, and neutrophils. Local production of IL-1β is significantly increased following trauma as evidenced by increased levels in bronchoalveolar lavage fluid, but it has not been found elevated in the circulation. This raises the possibility that the local release of these proinflammatory cytokines may significantly differ from their blood levels.
Figure 2.2. The activation of NF-κB. Cell signalling pathways induced by growth factors, chemotherapy, or radiotherapy result in the phosphorylation and proteasomal degradation of IκB. The transcription factor NF-κB is thereby released and promotes the expression of cytokines, cell adhesion molecules, and antiapoptotic proteins. From www.medscape.com

TNF-α and IL-1β are responsible for inducing non-hepatic manifestations such as fever, elevated prostaglandin levels, tachycardia and accelerated catabolism. Levels may rise briefly after the injury but may be difficult to detect. IL-6 is primarily responsible for the hepatic component of the response, resulting in the synthesis of acute phase proteins. In humans this usually peaks at 4 to 48 hours (median 8 hours) after surgery (Koj, 1996).
It has previously been demonstrated that systemic levels of the cytokines IL-6 and IL-1β correlate with MOF following severe injury (Lin et al, 2000). The role of the other proinflammatory cytokines TNF-α and interleukin-8 (IL-8) in the systemic inflammatory response syndrome leading to postinjury MOF, however, remains unclear. TNF-α, produced from the monocyte/macrophage lineage, is well established as a pivotal cytokine responsible for the clinical manifestations of shock induced by endotoxin or bacteraemia leading to SIRS following sepsis (Paterson and Webster, 2000). TNF-α, IL-1β and IL-6 are accepted mediators of the acute-phase response in humans (Figure 2.3). The post-operative levels of these cytokines have been found to correlate with the magnitude of the surgery and the presence of complications. They have therefore been accepted as markers of tissue inflammation after surgery (Baigrie et al, 1992; Cruickshank et al, 1990).
Figure 2.3. Stimulation and synthesis of positive acute-phase reactants during inflammation. Inflammation caused by infection or tissue damage stimulates the circulating inflammation-associated cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor (TNF-α). These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including CRP. IL-6 is the major cytokine stimulus for CRP production (from Adv Neonatal Care 2003; 3(1):3-13)
2.2.2 Organ-specific inflammatory response

Whereas serum cytokine levels give an indication of the systemic response to inflammation, various techniques are available to search for organ specific inflammation with resultant damage. As a syndrome, multi-organ dysfunction syndrome (MODS) is defined as altered organ function in the setting of sepsis, septic shock, or systemic inflammatory response syndrome. The affected organ systems involved are: respiratory, cardiovascular, renal, hepatic, gastrointestinal, haematological, endocrine, and central nervous system. For the purposes of this study, we shall be concentrating on the respiratory and renal systems as these are pertinent to the overall study directly, and are also among the first organ systems to be involved.

i) The Respiratory System

Perhaps the most studied system in terms of the response to systemic inflammation, many groups have attempted to find ways of treating and indeed avoiding the occurrence of the acute respiratory distress syndrome (ARDS). The acute lung injury that manifests clinically as ARDS is a major component of MODS of various aetiologies, which is the primary cause of death in these conditions (Bhatia and Moochhala, 2004). Initiated by numerous factors, acute lung injury is marked by epithelial and endothelial perturbation and inflammatory cell influx that leads to surfactant disruption, pulmonary oedema, and atelectasis (Knaus et al, 1985; Martin, 1999). This syndrome has been associated with a myriad of mediators including cytokines, oxidants and growth factors (Park et al, 2001). Many studies have in the past investigated acute
lung injury following major surgery (cardiopulmonary bypass, abdominal aortic aneurysm, cryotherapy) using various markers of inflammation. Essentially, there is proven vascular leakage that leads to an increase in fluid content that is seen clinically as pulmonary oedema and is non-cardiogenic in origin (Parajasingham et al, 1999) (Figure 2.4). This can be indirectly measured using wet versus dry lung weight ratios (O’Donovan et al, 1995). Furthermore, these proteins leak out into the alveolar spaces and are detected by an increase in total protein content in bronchoalveolar lavage (BAL) fluid (Ito et al, 1975; Demling et al, 1993; O’Donovan et al, 1995; Magnotti et al, 1999). The acute inflammatory response means inflammatory cells such as neutrophils and macrophages are increased in number, and this can be seen either microscopically or indirectly by measuring the total protein content. Furthermore, studies of patients with established ARDS have discovered high levels of TNF-α and IL-1β in BAL fluid (Siler et al, 1989; Park et al, 2001). Glasgow et al (2005) suggested that IL-1β is the primary initiator of pulmonary inflammation following liver injury in mice. Routine histology may demonstrate the presence of oedema, alveolar collapse, and red blood cells within alveoli (Washington et al, 2001). Alveolar tissue concentrations of cytokines and the presence of NF-κB have also been shown to increase during ARDS (Chapman et al, 2000).
Inducible heat shock protein 70 (HSP70) is a stress protein whose expression is upregulated when the cell or organism is placed under conditions of stress. HSP70 is essential for cellular recovery, survival and maintenance of normal cellular function. It is also a molecular chaperone that prevents protein aggregation and refolds damaged proteins in response to cellular stress caused by environmental insults, pathogens and disease. Current research is aimed at exploiting HSP70’s cellular protective abilities as a therapeutic strategy against damaging cellular stress (Pockley, 2003).

In most mammals, the expression of inducible HSP70 is strictly stress inducible and can only be detected following a significant stress upon the cell or organism. However, in humans and primates, inducible HSP70 is present at basal levels and is upregulated in response to stress.
The role of HSP70 has been studied in a variety of medically relevant models or conditions such as hyperthermia, hypertension, toxic exposure to chemical agents, hypoxia, ischemia, inflammation, autoimmunity, apoptosis, cancer, organ transplantation and bacterial and viral infections. HSP70 has also been studied in the normal processes of ageing, spermatogenesis, menstruation and physical activity such as exercise (La Thangue and Latchman, 1988; Donati et al, 1990; Fincato et al, 1991; Welch, 1993; Chouchane et al, 1994). Specifically with regards to hepatic ablation, several groups have studied the HSP70 induction following resection (Kimura et al, 2004), laser ablation (Fujitomi et al, 1999) and RFA (Schueller et al, 2004; Rai et al, 2005; Nikfarjam et al, 2005). HSP70 has been implicated in the pathogenesis of both acute renal dysfunction and ARDS.

**Figure 2.5.** Induction and regulation of heat shock protein expression. Physical or chemical stress induces production of unfolded or misfolded proteins. Heat shock factor monomers in the cytoplasm form trimers, are phosphorylated, and translocate into the nucleus. HSP homotrinomers bind to heat shock protein gene promoter regions, leading to induction of the HSP gene transcription. HSP70 gene transcription is downregulated by interaction of HSP70 with the HSF trimers. From www.openlearn.open.ac.uk
ii) The Renal System

Acute renal injury seen as acute tubular necrosis is characterised at an ultrastructural level by glomerular mesangial hypercellularity, vascular endothelial swelling, tubular epithelial necrosis and shedding, acellular segments of tubular basement membrane and an interstitial infiltrate composed of mast cells, eosinophils, lymphocytes and plasma cells (Dunnill, 1974).

Heat shock protein 70 as a marker of cellular stress also increases within tissue lysate in the presence of inflammation (Bravo et al, 2003).

Retinol-binding protein (RBP) is a small (21kD) transport protein for vitamin A which forms a complex with prealbumin in blood but loses its affinity for prealbumin once the vitamin has been delivered to the target cells. The free RBP molecule is rapidly filtered at the glomerulus and catabolised in the renal tubules after resorption by the proximal tubular cells (like other small molecules e.g. β-2 microglobulin). In kidney disease with prevailing tubular changes these proteins are not reabsorbed and appear in the urine. Thus RBP has been increasingly used as a sensitive marker of renal damage (Blaikley et al, 2003).

In proximal tubular dysfunction a large excess of β-2 microglobulin is excreted in the urine and thus increased urine concentrations occur. Grossly raised urine β-2 microglobulin concentrations are seen in heavy metal, aminoglycoside or cytotoxic induced renal tubular damage. Urinary pH of <6.0 is known to degrade β-2 microglobulin in the bladder, therefore the measurement of urinary concentrations of this protein is of limited value. For the diagnosis and management of renal tubular disorders the estimation of α1- microglobulin or retinol-binding protein is to be preferred because of the inherent instability of β-2 microglobulin at low pH (Trost et al, 2006).
iii) The Liver

Acute hepatic injury is expectedly seen following acute trauma including clinical intervention such as ablation. In the majority of acute liver failure (ALF) there is widespread hepatocellular necrosis beginning in the centrizonal distribution and progressing towards portal tracts. Coagulopathy is another cardinal feature of ALF. Liver has central role in synthesis of almost all coagulation factors and some inhibitors of coagulation and fibrinolysis. Hepatocellular necrosis leads to impaired synthesis of many coagulation factors and their inhibitors. The former produces a prolongation in prothrombin time which is widely used to monitor severity of hepatic injury. There is significant platelet dysfunction (with both quantitative and qualitative platelet defects). Progressive thrombocytopenia with loss of larger and more active platelet is almost universal. Thrombocytopenia with or without disseminated intravascular coagulation (DIC) increases risk of intracerebral bleeding (Dhainaut et al, 2001).

Renal failure is common, present in more than 50% of ALF patients, either due to original insult such as paracetamol resulting in acute tubular necrosis or from hyperdynamic circulation leading to hepatorenal syndrome or functional renal failure. Because of impaired production of urea, levels of blood urea do not represent degree of renal impairment. About 60% of all ALF patients fulfil the criteria for systemic inflammatory syndrome irrespective of presence or absence of infection (Maship et al, 1984). This often contributes towards multi organ failure. Impaired host defence mechanism due to impaired opsonisation, chemotaxis and intracellular killing substantially increase risk of sepsis. Bacterial sepsis mostly due to gram positive organisms and fungal sepsis are observed in up to 80% and 30% patients respectively (Gimson, 1996).
Hence, coagulation screen, liver function tests, tissue cytokine assays and HSP70 quantification as well as of course histology have all been used to assess the presence of injury to the liver.

2.3. Aims of the study

The aims of this study were to:

1) Quantify the inflammatory response to ablation/resection of hepatic parenchyma using systemic and organ-specific markers of inflammation in a rat model.

2) Compare the response between 4 treatment groups, namely surgical resection, microwave tissue ablation, radiofrequency ablation and cryotherapy to ascertain which modality of treatment produced the least inflammation in the rat model.

3) Further, compare the responses between the 4 treatment groups at 3 separate, increasing volumes of liver ablation/resection to ascertain the maximum ‘safe’ volume of ablation for each modality of treatment in the rat model.
Chapter 3

Materials and Methods

3.1 Ethical approval

3.2 Ex-vivo work

3.3 Peri-operative methodology

3.4 Sample Analysis
   3.4.1 Cytokine Analysis
   3.4.2 Heat Shock Protein 70 Analysis
   3.4.3 Urinary Retinol Binding Protein (RBP) Analysis
   3.4.4 Wet/Dry Lung Weight Ratio
   3.4.5 Broncho Alveolar Lavage Protein Quantification

3.5 Statistical Analyses
3.1. Ethical approval

Ethical approval was sought in accordance with the Animals (Scientific Procedures) Act 1986 (http://www.archive.official-documents.co.uk/document/hoc/321/321-xa.htm).

3.2. Ex-Vivo Work

In line with good practice as stipulated by Home Office guidelines, several freshly culled animals underwent surgical dissection to understand the anatomy of the rat. The surgical approach for subsequent manipulation of the liver were finalised prior to commencement of in-vivo work. The rat liver was explanted to demonstrate the presence of 2 major lobes, together with a 3rd lobe formed from a collection of smaller sub-lobes (Figure 3.1).

Figure 3.1. The explanted rat liver, showing 2 dominant lobes and a ‘third’ lobe, made of a collection of smaller lobules. Each lobe accounts for roughly 33% of the total liver volume.
The three ‘lobes’ were separated *ex-vivo* and subjected to a volumetric assessment using the water displacement technique. Secondly, the three separate parts of the liver were weighed. Each of the three lobes was deemed to amount to approximately 33% of the liver, both volumetrically and by weight. This was consistently demonstrated in 6 animal livers. We were thus able to semi-quantitatively perform ablations of the liver. Ablation of half of one dominant lobe amounted to roughly 15%, whilst half of 2 dominant lobes amounted to 33%. Destruction of the whole of the 2 dominant lobes whilst sparing the third ‘lobe’ was assumed to amount to 66% ablation.

In order to document the ablation volumes following each treatment, photographs were taken to demonstrate macroscopically the post-ablation liver immediately following treatment and after 48 hours, demonstrating the proportion of each lobe that had been ablated. Furthermore, microscopic appearance of the junction between normal and ablation zones are shown to confirm cell death within the ablated zones (Figure 3.2).
<table>
<thead>
<tr>
<th></th>
<th>Immediately post-ablation</th>
<th>48 hours post-ablation</th>
<th>Microscopic appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTA</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td><strong>CT</strong></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td><strong>RFA</strong></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
</tbody>
</table>

Images of the liver immediately post-ablation, showing darker areas of reactive hyperaemia. The same areas 48 hours following ablation, clearly demonstrating necrotic, ablated hepatic tissue. Microscopic appearance of the ablation zone/normal parenchymal interface. The arrows point to this interface. The pale pink areas all demonstrate a lack of viable hepatocytes with loss of normal liver architecture.

Figure 3.2. The appearance of the rat liver peri-operatively and at post-mortem, followed by histological analysis, to show that the volume ablated at time of surgery corresponded with the actual volume ablated.
3.3. Peri-treatment methodology

Pre-treatment protocol

Adult male Sprague Dawley rats (350g-400g; Charles River Laboratories, Margate, UK) were allowed to acclimatise in the designated establishment for one week. During this time, they underwent a protocol of daily handling and placement into plastic rodent restraining tubes for conditioning. All animals were maintained under identical conditions, at a fixed temperature and humidity. All were fed on the same diet. Finally, prior to selection, all animals were deemed to be in good physical health.

Treatment groups

Animals were randomly allocated to six groups. One group (Control), consisting of 7 rats, did not receive any intervention and was used as a baseline control. Five groups underwent the different interventions: laparotomy (Sham-second control group for comparative purposes), surgical resection (Resection), microwave tissue ablation (MTA), radiofrequency ablation (RFA), and cryotherapy (CT). Animals from each group were further subdivided into the three different volumes of ablation/resection (15%, 33% and 66%). The initial plan had been to study 7 rats in each group, however following interim analysis of the data and death of all animals in the 66% CT and RFA groups, 3 animals each were deemed a sufficient number (in these 2 groups) for statistical analysis after consultation with a statistician and the Home Office Inspector. In total, 16 groups were configured (1 for control, 3 for laparotomy, 3 for surgical resection, 3 for CT, 3 for RFA, 3 for MTA) for a total of 104 animals (14 groups x 7 animals= 98 animals and 2 groups x 3 animals=6 animals, Total 104 animals) (Table 3.1).
<table>
<thead>
<tr>
<th>Volume</th>
<th>Control 1</th>
<th>Control 2: Sham</th>
<th>Resection</th>
<th>CT</th>
<th>MTA</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>33%</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>66%</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3.1.** The random allocation of rats to each treatment group. Note that in the 66% CT and RFA groups there were only 3 animals. This was deemed a sufficient number for statistical analysis.

**Anaesthesia**

Rats were placed into an induction chamber with halothane and oxygen at a rate of 2L per minute of oxygen and 3-4% halothane. Once the animal was fully anaesthetised, it was placed supine onto a heated operating mat. Anaesthesia was maintained by way of a conical mask placed around its head on an anaesthetic circuit, with halothane and oxygen running at 2l per minute of oxygen and 2-3% halothane. A digital thermometer was inserted rectally for monitoring purposes only. 0.7mg of temgesic and 4mls 0.9% saline was administered subcutaneously. The fur covering the abdominal wall was shaved using clippers. The operating setting is shown in Figure 3.3.
Figure 3.3. The operative setting. The liver lobe to be treated is delivered through the laparotomy wound. The applicator (in this case cryotherapy probe) is applied to the liver. Also seen is a rectal temperature probe. The entire procedure is performed in sterile conditions under general anaesthesia.

Operative procedure

Group A: Control 1 (No Procedure): These animals underwent the same pre-conditioning and pre-operative procedures as all the other animals, including anaesthesia, analgesia and shaving. They were then recovered and observed before being culled at 48 hours.
**Group B: Control 2 (Sham Laparotomy):** Here, as with group A, the animals underwent the same pre-operative routine. On the operating table, a midline laparotomy allowed access into the abdominal cavity. The perihepatic ligamentous attachments were released using sharp dissection and the liver mobilised (Figure 3.4). Care was taken to avoid any damage to the liver and surrounding structures. After instilling 2mls of warmed saline intra-peritoneally to maintain normothermia and recover fluid losses, the abdomen was closed using 5’0 prolene for mass muscular closure followed by 4’0 vicryl subcuticular for skin closure.

![Figure 3.4. Following a midline laparotomy, the liver was mobilised by releasing peri-ligamentous attachments and one major lobe delivered through the wound.](image)

**Group C: Surgical resection:** Following laparotomy in the usual manner, half (15%), 2 halves (33%) or 2 whole lobes (66%) were resected. This was done by application of a vascular clamp horizontally across the midline of a single lobe of the liver. A blade was then used to run along the outside of the clamp, leaving a clean edge of the liver on the inner side of the clamp (Figure 3.5). This allowed time to apply a running 5’0 suture along the exposed edge of the hepatic parenchyma. Upon release of the clamp, minimal bleeding was observed. An omental
patch was also used to oversew the exposed edge of the liver to further prevent any blood loss. Closure and recovery was done in the usual way.

**Figure 3.5.** After applying a clamp across the liver hemi-lobe, a blade is used to cut the tissue and a 5'0 prolene suture run along the liver edge for haemostasis.

**Group D: Microwave Tissue Ablation (MTA):** At laparotomy, the liver was mobilised and 15, 33 or 66% of the liver was ablated using microwave energy. This was achieved by inserting a microwave applicator, or probe, into the hepatic parenchyma (Microsulis Medical Ltd, Edinburgh, UK) (Figure 3.6). This was connected to the microwave generator via a coaxial cable. Microwave energy was applied at a frequency of 9.2GHz and a power of 20 watts. For 15% ablation, 3 insertions of 5 seconds each (total treatment time 15 seconds) were required. For 33% ablation, 6 insertions of 5 seconds each (total treatment time 30 seconds) were required. For 66% ablation, the total treatment time was 45 seconds (9 insertions of 5 seconds each). A saline soaked swab was placed between the liver and surrounding organs to prevent inadvertent organ damage from the probe directly or heat conduction from the liver. Intra-
operatively, the body temperature was maintained without any intervention. Any bleeding from the probe insertion point was rapidly controlled by applying the probe to the bleeding point for a further 2-3 seconds, utilising the thermo-coagulative properties of microwaves.

**Figure 3.6.** The microwave generator and *in-vivo* ablation of one major lobe of the liver. Multiple applications of the probe for 5-6 seconds each are required to achieve target ablation volume.

**Group E: Cryoablation:** The cryoprobe was applied to the edge of the liver as shown in Figure 3.7. The temperature was brought down to -180 degrees Celsius for the duration of the treatment. Once sufficient volume of the liver had macroscopically been frozen, the Cryotech LCS 3000 liquid nitrogen system (Spembly, Hampshire, England) was switched off and the probe was allowed to defrost completely before detachment from the liver. This ensured no fracture of the hepatic parenchyma as control of bleeding would have been much more difficult than with thermal systems. Once again, damp swabs ensured minimal dispersal of thermal energy away from the ablation zone. 2 cycles of treatment were performed to imitate clinical
practice, and with each cycle taking between 8 and 14 minutes, hypothermia was avoided by introducing drops of warmed saline into the peritoneal cavity to maintain body temperature. The liver was allowed to completely defrost before its return to the abdominal cavity. Closure and recovery was routine.

**Figure 3.7.** The cryotherapy machine. The applicator can be seen applied to the tip of the lobe. A polystyrene block can be seen protecting the liver from adjacent organs and skin.

**Group F: Radiofrequency Ablation (RFA):** The dorsal surface of the animal was shaved to allow application of a grounding pad. The Cool-tip RF ablation system (Valleylab, Colorado, USA) senses tissue impedance and automatically delivers the optimum amount of radiofrequency energy. The Cool-tip electrode internally circulates chilled water, cooling the tissue adjacent to the exposed electrode. This reduces tissue impedance, allowing for maximal energy deposition during the treatment cycle and a larger, controlled ablation zone (Figure 3.8).
The applicator tip was inserted into the liver parenchyma to achieve the required volume of ablation (Figure 3.9). A power setting of 10 Watts keeping impedance below 1000Ω was optimal. A damp swab was used to protect the surrounding structures from thermal damage. Closure and recovery was routine.
**Post-operative protocol**

At the end of each procedure, the halothane was switched off and the animal recovered using oxygen only. 2mls of warmed subcutaneous saline was instilled subcutaneously for fluid maintenance. The animals were returned to their cages and kept warm by way of a lightly heated mat. A behaviour and feeding chart was kept to subjectively assess tolerance to the procedures and discomfort.

**Blood Sampling**

Animals were placed into an incubator at 38 degrees Celsius for 3 minutes to stimulate peripheral vessel dilation and facilitate blood sampling. A 25 gauge blue needle was inserted into a tail vein and blood was allowed to drip into the collecting tube. This was a 1ml Eppendorf polypropylene tube without anticoagulant. Once 500µl had been collected the sample was allowed to clot at room temperature for 2 hours before being placed into a microcentrifuge and spun at 13000rpm for 10 minutes as per the ELISA manufacturers instructions. Following separation of the plasma and serum, the serum was siphoned using a micropipette and stored in cryovials. This was immediately snap frozen in liquid nitrogen before storage in a freezer at minus 80 degrees Celsius. Blood sampling was performed in this manner pre-operatively (prior to anaesthetic induction) and at 1, 3, 6, 24 and 48 hours post-operatively.
**Behaviour and Survival**

Several clinical parameters were recorded before and after the experimental procedures in an effort to elucidate the tolerance of the animals to the respective procedures. A set template for each animal was filled in at regular intervals (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>Pre-op</th>
<th>0 Hrs</th>
<th>1 Hr</th>
<th>3 Hrs</th>
<th>6 Hrs</th>
<th>24 Hrs</th>
<th>48 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piloerection</td>
<td>Yes/No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Movement</td>
<td>a=normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b=some movement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c=no movement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eating</td>
<td>Yes/No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drinking</td>
<td>Yes/No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>a=normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b=slight increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c=persistent tachypnoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.2.** The proforma filled by veterinary nursing staff to monitor animal wellbeing, using clinical and behavioural parameters. Animals were observed at the various timepoints for a maximum of 2 minutes. During this time, all the various parameters could be recorded.

**48 Hours**

At 48 hours, each animal was anaesthetised in the standard way and placed onto the operating table. The abdominal wound was re-opened and the bladder emptied under direct vision using a 22 gauge needle and syringe. The urine was placed into a collecting tube and
spun down at 13,000 rpm for 3 minutes to remove any sediment. The urine was placed in cryovials and snap frozen in liquid nitrogen before storage in a -80 degrees Celsius freezer.

Following a midline sternotomy, a cardiac puncture allowed collection of blood whilst exsanguinating the animal. This was the 48 hour blood collection which was then processed and stored in the usual way.

The left main bronchus was tied at its origin using a 3’0 vicryl suture and the entire left lung was removed. The larger, lower lobe was separated and weighed accurately before being placed into a plastic bag and snap frozen. This would subsequently be re-weighed to assess the wet/dry lung weight.

Half of the second lobe of the left lung was placed into a cryovial and snap frozen in liquid nitrogen for subsequent HSP70 analysis. The other half of the lobe was placed into 10% formaldehyde for histological analysis.

The trachea was cannulated using a BD Venflon IV Cannula (20Gauge) and 1ml of sterile normal saline gently flushed into the right lung. Following three aspiration/flush cycles, the lavage fluid was emptied into a cryovial and snap frozen for total protein quantification.

A section of the liver and kidney was then removed and fixed in formaldehyde for histological assessment, and a further section from each organ snap frozen in liquid nitrogen and stored for HSP70 analysis.
3.4. Sample analysis

3.4.1. Cytokine Analysis

Enzyme-Linked Immunosorbent Assays (ELISA) (R&D Systems, Abingdon, UK) were used to quantify the presence of the proinflammatory cytokines IL-1β, IL-6 and TNF-α. All samples were allowed to defrost completely at room temperature. ALL SAMPLES WERE MEASURED IN DUPLICATE.

**Principles of the assay:** This assay employed the quantitative sandwich enzyme immunoassay technique. An affinity purified polyclonal antibody specific for the rat cytokine had been pre-coated onto a microplate. Standards, controls, and samples were pipetted into the wells and any rat cytokine present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the rat cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the colour measured was in proportion to the amount of rat cytokine bound in the initial step. The intensity of the colour was measured in a microplate reader at 540nm. Cytokine concentrations from the sample were quantified by interpolating absorbance readings from a standard curve generated with calibrated cytokine standards provided.
**Cytokine Quantification:** The previously frozen serum samples were defrosted at room temperature. IL-1β, IL-6 and TNF-α ELISA trays were used simultaneously with the same serum samples to avoid repeated freeze-thaw cycles. The commercially available ELISA immunoassay kits were all of the same specification manufactured by the same company (R&D Systems, Abingdon, UK). The procedures were all exactly the same and used agents from their own individual kits. Only the pre-procedure protocols differed in terms of sample dilution and standard curve development.

**Sample preparation:** The rat serum was prepared for analysis by first diluting it in calibrator diluent supplied with the ELISA kit. A sterile Nunc 96-well microplate was used to produce all the dilutions for the samples. This was chosen so as to optimise speed of transfer to the ELISA test tray using a multi channel pipette, bearing in mind three parallel cytokines were being tested for with the same samples.

**TNF-α:** R&D’s Quantikine TNF-α immunoassay kit was used. The rat serum required a 2-fold dilution into calibrator diluent (provided). 75µl of both sample and calibrator diluent were added to the tray and placed on a horizontal plate shaker at low speed for 5 minutes. Control samples were not diluted.

**IL-1β:** R&D’s Quantikine M Murine IL-1β immunoassay kit was used. The rat serum required a 3-fold dilution into calibrator diluent (provided). 50µl of sample and 100µl of calibrator diluent were added to the tray and placed on a horizontal plate shaker at low speed for 5 minutes. Control samples were not diluted.
**IL-6:** R&D’s Quantikine M Murine IL-6 immunoassay kit was used. The rat serum required a 2-fold dilution into calibrator diluent (provided). 75µl of both sample and calibrator diluent were added to the tray and placed on a horizontal plate shaker at low speed for 5 minutes. Control samples were not diluted.

**Preparation of standards:** 8 x 500µl Eppendorf polypropylene tubes were placed in a holder and labeled A-H. 200µl of calibrator diluent was pipetted into each tube with the exception of tube A. 200µl of standard solution of known concentration (provided) was added to tube A. 200µl of the same stock solution was also added to tube B. A dilution series was then developed from tube B by mixing then pipetting 200µl into the next tube in the order, stopping at tube G. In this way, tube A always had the highest concentration of the standard, and tube H only contained calibrator diluent and thus served as a zero standard (0pg/ml).

**TNF-α:** The undiluted rat TNF-α served as the high standard (800pg/ml), and subsequent dilutions were 400, 200, 100, 50, 25, 12.5 and 0pg/ml. The minimum detectable dose of rat TNF-α with this kit was typically less than 5pg/ml.

**IL-1β:** The undiluted rat IL-1β served as the high standard (2000pg/ml), and subsequent dilutions were 1000, 500, 250, 125, 62.5, 31.2 and 0pg/ml. The minimum detectable dose of rat IL-1B with this kit was typically less than 5pg/ml.
**IL-6:** The undiluted rat IL-6 served as the high standard (2000pg/ml), and subsequent dilutions were 1000, 500, 250, 125, 62.5, 31.2 and 0pg/ml. The minimum detectable dose of rat IL-6 with this kit was typically less than 10pg/ml.

The 96 well ELISA immunoassay tray allowed sampling of 48 samples in duplicate. The first eight wells were used to measure the standard curve, 4 random wells were used for the control samples of known cytokine concentrations (supplied), leaving us with 36 prepared serum samples.

**Procedure:** 50µl of assay diluent (provided) was added to each well on the tray. 50µl of standard, control or pre-diluted samples were then added to each well, documenting on a template where each sample was. The tray was gently shaken, covered and incubated for 2 hours at room temperature. The plate contents were then tipped out and each well washed out with 400µl of wash buffer (provided) using a multi-channel pipette (Eppendorf 200 Multipipette). After each of a total of five washes, the contents were thoroughly emptied by initially decanting into a sink then blotting gently against paper towels.

100µl of rat IL-6 conjugate was then added to each well and incubated for a further 2 hours. After repeating the washing step described above, 100µl of substrate solution was added to each well and incubated for 30 minutes at room temperature. The trays were kept in a cupboard away from light. 100µl of stop solution was added to each well and the tray gently tapped to ensure thorough mixing. All plates were read using a microplate reader set at 540nm with wavelength correction (Fluostar Optima, BMG LabTechnologies Offenburg, Germany). The
sample concentrations read from the standard curve were then multiplied by the initial dilution factor.

**Intra-Assay Variability:** For quantification of this protein, one sample was measured 3 times on the same tray using randomly allocated wells. All trays used for quantification of this protein were from the same Lot Number. The standard deviation and thus the coefficient of variance were then calculated.

**Inter-Assay Variability:** One sample was tested across 3 separate trays chosen at random, and using randomly allocated wells. The coefficient of variance was then calculated.

**The problem with TNF-α**

We were unable to obtain any results whatsoever for TNF-α induction. The procedure followed was as described in the manufacturers instruction manual and exactly the same as that for IL-1β and IL-6. The following steps were taken in an attempt to solve the problem.

1) The serum samples used for the TNF-α analysis were from the same cryovial as those used in the IL-1β and IL-6 assays. Whereas the latter 2 trays provided readable levels of their respectable cytokines, TNF-α remained blank.

2) Some samples were used in their undiluted form (versus the manufacturers instructions of a 2-fold dilution), and also in a 3, 4, 5 and 6-fold dilution. These continued to give blank TNF-α readings.
3) Several different TNF-α R&D Quantikine immunoassay kits were used, from separate batches. These continued to give blank TNF-α readings.

4) A technician from the tray manufacturers (R&D) came and supervised the entire experimental protocol, finding no faults.

5) Previous groups who looked at TNF-α in animal models were contacted personally for advice (Chapman, from Glasgow et al, 2005 and Ng, from Ng et al, 2004). They seem to have followed the same protocol and were also at a loss to explain why the kit did not work in our case.

*Following discussion with the research supervisor, it was decided to exclude TNF-α analysis from our data owing to ‘technical’ reasons.*

3.4.2. Heat Shock Protein 70 Analysis

**Principles of the assay:** The Stressgen StressXpress (Stressgen, BC, Canada) HSP70 ELISA is a quantitative sandwich immunoassay with a sensitivity of 500pg/ml and a standard curve range of 780-50,000pg/ml. A mouse monoclonal antibody specific for inducible HSP70 is pre-coated on the wells of the HSP70 immunoassay plate. Inducible HSP70 is captured by the immobilised antibody and is detected with a HSP70 specific biotinylated rabbit polyclonal antibody. The biotinylated detector antibody is subsequently bound by an avidin-horseradish peroxidase conjugate. The assay is developed with tetramethylbenzidine substrate and a blue
colour develops in proportion to the amount of captured HSP70. The colour development is stopped with acid stop solution which converts the endpoint colour to yellow. The intensity of the colour is measured in a microplate reader at 450nm. HSP70 concentrations from the sample are quantified by interpolating absorbance readings from a standard curve generated with the calibrated HSP70 protein standard provided.

Heat shock protein is expressed as HSP as a fraction of the total protein content of the sample. Hence protein quantification was also carried out for each of the samples subjected to HSP70 quantification.

**Sample preparation:** A 0.5cm³ piece of lung, liver or kidney was defrosted and placed into a test tube, together with 1ml of 1X HSP70 extraction reagent. The 1X extraction reagent was prepared by diluting 1ml of 5X extraction reagent with 4mls of deionised water. A commercially available protease inhibitor cocktail tablet was added at a final 1X concentration. To do this, 2mls of extraction reagent, 8mls of deionised water and one ‘Complete mini protease’ tablet (Roche Diagnostics Ltd, West Sussex, UK) were mixed together in a sterile container and kept at 4 degrees Celsius. This provided sufficient solution for 8 tissue samples at 1ml per 0.5cm³ of tissue. A tissue homogeniser was used to blend the tissue specimen fully. The liquid was transferred in the test tube on ice and centrifuged at 16,000G for 10 minutes with a brake setting of 10, at 4 degrees Celsius. The supernatant was removed and stored in polypropylene tubes and the residual pellet was discarded. The supernatant collected was the tissue extract and this was stored in polypropylene tubes and snap frozen in liquid nitrogen before storage at minus 80 degrees Celsius, ready for subsequent analysis using the ELISA technique described below.
Samples were diluted 1:25 prior to HSP70 ELISA immunoassay in the following manner. 20µl of defrosted tissue lysate was added to 480µl of sample diluent (provided) in an Eppendorf tube and mixed thoroughly on a vortex mixer. Each ELISA immunoassay tray provided contained 96 wells, of which 16 wells were used for 8 standard curve samples in duplicate. This left 80 wells which were used for 40 tissue lysate samples in duplicate.

**Preparation of standards**: The HSP70 standard was used to generate a standard curve with 8 points ranging from 0.78-50ng/ml. 0.995ml of sample diluent (provided) was added to tube 1, and 0.5ml to tubes 2-8. 5µl of HSP70 standard stock solution (10µg/ml) was added to tube 1 to make up a standard concentration of 50ng/ml. After mixing thoroughly, 0.5ml of tube 1 contents were added to tube 2, and so on until tube 7. Thus, standard concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78ng/ml were prepared. Tube 8 only contained sample diluent and thus served as the zero standard.

**Assay Procedure**: 100µl of prepared standards and samples were aliquotted into the wells. The plate was then sealed and incubated at room temperature for 2 hours. The wells were emptied at this point and washed with 300µl of wash buffer (provided) for a total of 6 washes. 100µl of previously diluted Anti-HSP70 Biotin Conjugate (Biotinylated rabbit polyclonal antibody specific for inducible HSP70) was then added to each well, sealed and incubated at room temperature for 1 hour. After another wash cycle as before, 100µl of previously diluted Avidin-HRP Conjugate (Avidin-horseradish peroxidase conjugate) was added and incubated for a further 1 hour before another wash cycle. 100µl of TBM substrate (Stabilised tetramethylbenzidine substrate) was added to the wells and incubated for 10 minutes. 100µl of
acid stop solution was added to the wells in the same order that the TMB substrate was added. All plates were read using a microplate reader set at 540nm with wavelength correction (Fluostar Optima, BMG LabTechnologies Offenburg, Germany). The sample concentrations read from the standard curve were then multiplied by the initial dilution factor.

**Total Protein Quantification:** A commercially available protein quantification assay (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, USA) was used in much the same way as ELISA assays, with the development of a standard curve. A series of dilutions of known concentrations were prepared from the protein and assayed alongside the unknowns before the concentration of each unknown is determined based on the standard curve. The standard curve points ranged from 0-2000 µg/ml. It was unclear at first exactly how much dilution would be necessary for sample concentrations to fall within the standard curve and so surplus tissue lysate from various tissues (lung, liver, kidney) were used as a test run, diluted to varying degrees.

<table>
<thead>
<tr>
<th></th>
<th>100%</th>
<th>50%</th>
<th>25%</th>
<th>12.5%</th>
<th>6.25%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>30µl</td>
<td>15µl</td>
<td>7.5µl</td>
<td>3.75µl</td>
<td>1.875µl</td>
</tr>
<tr>
<td>Diluent</td>
<td>0µl</td>
<td>15µl</td>
<td>22.5µl</td>
<td>26.25µl</td>
<td>28.125µl</td>
</tr>
<tr>
<td>Total</td>
<td>30µl</td>
<td>30µl</td>
<td>30µl</td>
<td>30µl</td>
<td>30µl</td>
</tr>
</tbody>
</table>

Running the assay using the protocol described below suggested that dilution to 6.25 or 1 in 16 would allow all sample concentrations to fall within the standard curve values.
**Sample preparation:** Tissue lysate prepared for HSP70 immunoassay described above were also used for total protein content quantification. All samples were defrosted before diluted with deionised water in a sterile 96 well Nunc microplate. 25µl of sample and 375µl of diluent was used to give a 1 in 16 dilution factor.

**Preparation of Standards:** The manufacturers dilution scheme for microplate procedure was used, with bovine serum albumin (provided) used as the standard. The working range was 20-2000µg/ml.

**Assay Procedure:** 25µl of each standard or sample were pipetted in duplicate into a sterile microplate well. 200µl of working reagent (provided) was added to each well and placed on a horizontal plate shaker for 30 seconds. The plates were covered and incubated at 37 degrees Celsius for 30 minutes exactly. The plates were allowed to reach room temperature and read using a microplate reader set at 562nm with wavelength correction (Fluostar Optima, BMG LabTechnologies Offenburg, Germany). The sample concentrations read from the standard curve were then multiplied by the initial dilution factor. As all tests were performed in duplicate, the average reading was taken.

*All HSP70 data was then expressed per µg of protein.*
### 3.4.3. Urinary Retinol Binding Protein (RBP) Analysis

**Principles of the Assay:** A commercially available RBP immunoassay kit (Immunodiagnostik, Bensheim, Germany) was used in this study to detect the presence of RBP as a marker of early tubular proteinuria. This Enzyme-Linked Immunosorbent Assay (ELISA) can be used for quantitative determination of Retinol-binding protein (RBP) in plasma, serum and urine. In a first incubation step, RBP in the samples is bound to polyclonal rabbit anti RBP antibodies, immobilised on the microtitre plate. A peroxidase-conjugated anti RBP antibody is used for detection and quantification, and tetramethylbenzidine (TMB) as a peroxidase substrate. A dose response curve of absorbance unit (optical density at 450 nm) versus concentration is generated using the values obtained from standard. RBP present in the samples is determined directly from this curve.

**Sample preparation:** Previously collected, processed and frozen urine samples were defrosted and diluted in the following manner. A one in three dilution as recommended by the manufacturer (personal communication) was achieved by mixing 80µl of urine sample and 160µl of sample diluent. The resulting 240µl of diluted sample was sufficient for assay of 100µl of each sample in duplicate.

**Preparation of standards:** 5 pre-prepared standards were supplied in the manufacturers kit, at concentrations of 0, 1.1, 3.3, 11 and 33 µg/L.
**RBP Quantification:** The pre-coated microtitre plate was washed five times in the usual manner using the ELISA wash buffer (provided). All tests were performed in duplicate. 100µl of standards, prediluted samples and controls were added to each well and incubated for 1 hour at room temperature shaking on a horizontal plate shaker. The contents of the wells were then decanted and washed five times with wash buffer. 100µl of diluted conjugate (rabbit anti RBP, peroxidase-labelled, provided) was then added to the wells and incubated for an hour at room temperature on a plate shaker. After decanting and washing as usual, 100µl of TMB (tetramethylbenzidin) substrate solution (provided) was added and incubated for 20 minutes at room temperature. 50µl of stop solution was then added and shaken gently. The plate was then read at 450nm. Sample concentrations were then read off the standard curve and multiplied by the dilution factor (3x).

*All measurements were expressed in µg/L.*

**Intra-Assay Variability:** For quantification of this protein, one sample was measured 3 times on the same tray using randomly allocated wells. All trays used for quantification of this protein were from the same Lot Number. The standard deviation and thus the coefficient of variance were then calculated.

**Inter-Assay Variability:** One sample was tested across 3 separate trays chosen at random, and using randomly allocated wells. The coefficient of variance was then calculated.
3.4.4. Wet/Dry lung weight ratio

The left lower lobe that had been removed from the culled animal at 48 hours and snap frozen was removed from its sealed bag and placed onto a pre-weighed non-stick paper baking cup and placed in a laboratory oven at 37 degrees Celsius for approximately 24 hours. Higher temperatures may have desiccated the tissue. After 24 hours, the lung tissue was removed from the oven, weighed, and returned. Once the weight was found to be constant, it was removed from the oven and the final weight recorded. The difference between the pre- and post-drying weights was recorded and this gave an indication of the water content of the lung, expressed as the wet/dry lung weight ratio.

3.4.5. Broncho Alveolar Lavage Protein Quantification

**Total Protein Quantification:** A commercially available protein quantification assay (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, USA) was used with the development of a standard curve. A series of dilutions of known concentrations were prepared from the protein and assayed alongside the unknowns before the concentration of each unknown was determined based on the standard curve. The standard curve points ranged from 0-2000 µg/ml.

**Preparation of Standards:** The manufacturers dilution scheme for microplate procedure was used, with bovine serum albumin (provided) used as the standard. The working range was 20-2000µg/ml.
**Assay Procedure:** 25µl of each standard or sample were pipetted in duplicate into a sterile microplate well. 200µl of working reagent (provided) was added to each well and placed on a horizontal plate shaker for 30 seconds. The plates were covered and incubated at 37 degrees Celsius for 30 minutes exactly. The plates were allowed to reach room temperature and read using a microplate reader set at 562nm with wavelength correction (Fluostar Optima, BMG LabTechnologies Offenburg, Germany). As all tests were performed in duplicate, the average reading was taken.

*All readings were in µg/ml.*

3.5. **Statistical Analyses**

For analyses of the cytokines, various separate tests were employed, and the rationale for their use are best described in conjunction with the results. This is because there were multiple variables such as treatment groups, volumes and time periods. For the remaining organ systems studied, an analysis of variance (ANOVA) was deemed most appropriate to study differences between out more limited number of variables.

An ANOVA is used to test the null hypothesis that there are no differences among the means of two or more groups against an alternative hypothesis that there are such differences. The F-statistic is the ratio of the variances between group means to the variances within groups, and it is used to estimate the probability that differences among group means as large as or
greater than those observed could have arisen by chance sampling variation. If this probability is low, say less than 0.05, then the differences among the means are presumed to have arisen as a result of the treatment and are said to be “statistically significant” at the 5% level of probability. The statistical software used for generation of the ‘F-statistic’ was Analyse-it (Analyse-it for Microsoft Excel version 2.12).

In this test, the degrees of freedom are calculated in the following manner:

\[ df_a = a - 1 \] (where \( a \) = number of treatment groups)

\[ df_{S/A} = a(n - 1) \] (where \( n \) = number of observations in each group)

\( df_a \) is known as the numerator degrees of freedom, whilst \( df_{S/A} \) is known as the denominator degrees of freedom. With these values, one can find out what the F-test critical statistic (\( F_{\text{crit}}(x,y) \)) is, using a table of F-test critical values (http://www.itl.nist.gov/div898/handbook/eda/section3/eda3673.htm). Note \( F_{\text{crit}}(x, y) \) notation means that there are \( x \) degrees of freedom in the numerator and \( y \) degrees of freedom in the denominator.

If our software-generated F-statistic is greater than the critical value (at the 5% significance level) then the results are significant and we can reject the null hypothesis.

The ANOVA depends on three assumptions. First that the experimental subjects are independent. This is achieved by assigning the rats to the various treatment groups at random, and housing and treating them in such a way that there are no systemic differences between groups apart from the treatment. Second, the ANOVA assumes that the variation within each
group is approximately the same and finally it also assumes that the residuals (deviations of each observation from the group mean) have a normal, Gaussian, distribution. These latter two assumptions were verified by studying plots of the residuals, prior to ANOVA analysis.
Chapter 4

Clinical results: Survival, tolerance, and behaviour

4.1 Summary

4.2 Results

4.3 Discussion of results

4.4 Other complications
4.1. Summary

Observational data were recorded following treatment at set time intervals. Parameters chosen were established signs of distress in rats (piloerection, lack of movement, poor feeding) and shock (tachypnoea, tachycardia, loss of skin turgor, difficulty in venepuncture). The rest of this chapter details the data collected for each animal together with a discussion at the end. The final chapter of this thesis will bring together the observational data in this chapter and the objective data collected from the various organ systems.

4.2. Results

A table was devised for each animal to record behaviour at the end of each operative procedure (Table 4.1). A score was given for each element of behaviour observed and summed up to give the total number of animals exhibiting the behaviour in any particular group at set times over the duration of the 48 hour observation period (Tables 4.2 to 4.6).

<table>
<thead>
<tr>
<th></th>
<th>Pre-op</th>
<th>0 Hrs</th>
<th>1 Hr</th>
<th>3 Hrs</th>
<th>6 Hrs</th>
<th>24 Hrs</th>
<th>48 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piloerection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Movement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eating</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drinking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Behaviour record for each animal.
<table>
<thead>
<tr>
<th>Piloerection</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/7</td>
<td>0</td>
</tr>
<tr>
<td>33%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/7</td>
<td>7/7</td>
<td>2/7</td>
</tr>
<tr>
<td>66%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/7</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

**Table 4.2.** Number of animals in each group demonstrating piloerection at any stage following intervention up until the 48-hour timepoint. There were 7 animals in each group except for the 66% CT and RFA groups (3 animals each).

<table>
<thead>
<tr>
<th>Movement</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>7 a</td>
<td>5 a</td>
<td>5 a</td>
<td>4 a</td>
<td>1 a</td>
<td>4 a</td>
</tr>
<tr>
<td></td>
<td>2 b</td>
<td>2 b</td>
<td>3 b</td>
<td>6 b</td>
<td>3 b</td>
<td></td>
</tr>
<tr>
<td>33%</td>
<td>7 a</td>
<td>6 a</td>
<td>4 a</td>
<td>5 a</td>
<td>1 b</td>
<td>2 a</td>
</tr>
<tr>
<td></td>
<td>1 b</td>
<td>3 b</td>
<td>2 b</td>
<td>6 c</td>
<td>4 b, 1 c</td>
<td></td>
</tr>
<tr>
<td>66%</td>
<td>7 a</td>
<td>6 a</td>
<td>3 a</td>
<td>4 a</td>
<td>3 c</td>
<td>3 c</td>
</tr>
<tr>
<td></td>
<td>1 b</td>
<td>4 b</td>
<td>3 b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.3.** Number of animals in each group demonstrating decrease in movement as a marker of distress. a=normal; b=some movement; c=no movement. There were 7 animals in each group except for the 66% CT and RFA groups (3 animals each).
### Table 4.4.

Number of animals in each group not eating normally at any stage following intervention up until the 48-hour timepoint. There were 7 animals in each group except for the 66% CT and RFA groups (3 animals each).

<table>
<thead>
<tr>
<th>Eating</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>0</td>
<td>1/7</td>
<td>1/7</td>
<td>0</td>
<td>1/7</td>
<td>0</td>
</tr>
<tr>
<td>33%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6/7</td>
<td>3/7</td>
</tr>
<tr>
<td>66%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

### Table 4.5.

Number of animals in each group not drinking normally at any stage following intervention up until the 48-hour timepoint. There were 7 animals in each group except for the 66% CT and RFA groups (3 animals each).

<table>
<thead>
<tr>
<th>Drinking</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1/7</td>
<td>0</td>
</tr>
<tr>
<td>33%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6/7</td>
<td>2/7</td>
</tr>
<tr>
<td>66%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>
Table 4.6. Number of animals in each group demonstrating increase in respiratory rate as a marker of distress. a=normal; b=slight increase, not sustained; c=persistent tachypnoea. There were 7 animals in each group except for the 66% CT and RFA groups (3 animals each).

4.3. Discussion of results

i) 15% Treatment groups

All animals tolerated the procedures well in each group. The respiratory rate was marginally higher in 6 of 7 animals in the CT treatment group, but this settled 6 hours following surgery in all cases. Similarly 6 of 7 animals in the same group appeared to have a decreased level of movement but this normalised within the first 24 hours. Occasionally a further subcutaneous dose of analgesia was administered at the discretion of the veterinary nurses. All animals survived to the 48 hour timepoint.
ii) 33% Treatment groups

Most animals in the control, resection, MTA and RFA groups tolerated the procedures reasonably well, with the occasional need for additional analgesia. However, animals undergoing CT appeared quieter, with decreased movement and piloerection in almost all animals. They appeared to be ‘off’ their appetite as they seemed disinterested in both eating and drinking. Their respiratory rate was high, particularly in the first 6 hours following surgery. Some animals in the 33% RFA group also exhibited these anomalies but not to the same extent as CT. However, these parameters had largely normalised within the first 24 hours. All animals survived to the 48 hour timepoint.

iii) 66% Treatment groups

All animals in the two controls, resection and MTA groups appeared to tolerate this large volume of hepatic ablation. Even in the early postoperative period, there was little evidence of distress, except for the occasional need for an additional dose of analgesia administered subcutaneously. The occasional animal in the resection and MTA groups demonstrated piloerection and some reduced movement, although their feeding was normal. There was also some evidence of increased respiratory rate but this settled within the first 6 hours. All animals in these four groups survived to the 48 hour timepoint.

The most dramatic impact on the animals appeared to follow 66% hepatic ablation with RFA and CT. All animals in these two groups died between 4 and 6 hours following surgery. Death was preceded by complete inactivity within their cage, piloerection, tachypnoea and lack
of feeding. Furthermore, they appeared peripherally ‘shut down’ as judged by loss of skin turgor and difficulty in venepuncture from the otherwise reliable tail vein.

The experimental protocol allowed access into the various body cavities such that it was possible to look for overt causes of death. At post mortem, there were no obvious signs of intraperitoneal injury, bowel damage, faecal leak or haemorrhage. Samples from the ablated liver were taken in the same way as in all the other treatment groups. Bladder puncture to retrieve urine samples were also performed although it was always difficult to obtain a good volume of urine compared with animals from other treatment groups, including 66% MTA and 66% resection. Nonetheless, sufficient sample for RBP analysis was always obtainable. In one RFA and 2 CT animals in the 66% groups, the urine was seen to have a slight pink/brown ‘hue’ suggestive of myoglobinuria or haemoglobinuria although this was not investigated further. This was despite the same perioperative fluid management for all animals. Upon opening the chest cavity to examine the lungs and obtain samples, there was no obvious evidence of injury, although studies were done to assess the wet/dry lung weight amongst others. The macroscopic appearance of the lungs and kidneys did not show further evidence of injury.

Studies of the organ systems in this thesis were all done on post-mortem specimens. Clearly in the 66% groups, this brought in two separate timepoints within the study, in that the control, resection and MTA groups were studied at 48 hours, and the RFA and CT groups at about 6 hours when all the animals died. This potential major flaw in the study was discussed at length with the named veterinary surgeon, the supervisor and the statistician. The overall
consensus was to continue with the experimental protocol as designed, the reasons for which will be discussed in Chapter 8.

4.4. Other Complications

**Surgical resection**

The first *in-vivo* rat that underwent surgical resection unfortunately exsanguinated on the operating table. Clamping, sectioning of the parenchyma and suturing was refined further and no similar episodes occurred thereafter. Following discussion with the named Home Office veterinary surgeon, this animal was excluded from the study and replaced with a new animal.

**MTA**

The first *in-vivo* rat that underwent MTA of 15% of the hepatic volume had a smooth immediate post-operative course. The following morning however, the animal was found dead in its cage having apparently re-opened its laparotomy wound and eating parts of its own bowel. This distressing episode was not seen again in any more rats nor could we explain it. Again following discussion with the named Home Office veterinary surgeon, this animal was excluded from the study and replaced with a new animal.

**Cryotherapy**

The first 2 animals that underwent cryotherapy of 15% volume died intra-operatively. Post mortem revealed significant intra-abdominal ‘collateral’ damage relating to the thermal energy. Following lengthy discussions with the named Home Office veterinary surgeon, and
also a previous group who successfully performed this procedure on rats (Chapman, personal communication), the technique was modified where a polystyrene block was used to separate the liver (which has been externalised by delivery through the wound) from adjacent structures including skin. Furthermore, the ablated lobe of liver was allowed to completely defrost before returning to the abdominal cavity. Following these modifications to our technique, no further incidents occurred. The two ‘test’ animals were excluded from the study and replaced with new animals.

**RFA**

One rat in the 15% group sustained a burn relating to the grounding pad on its back (Figure 4.1). As this may have impacted on any potential inflammatory response, this animal was also removed from the study and replaced with a new one following discussion with the named Home Office veterinary surgeon.

![Figure 4.1. A grounding pad related burn on the back of a rat.](image)
Chapter 5

Comparison of cytokine response to hepatic radiofrequency ablation, cryotherapy, microwave ablation and surgical resection in a rat model.

5.1 Summary

5.2 Experimental Results

5.2.1 IL-1β

5.2.2 IL-6

5.3 Statistical Analysis

5.3.1 IL-1β

5.3.2 IL-6

5.4 Discussion
5.1. Summary

**Objective**: A comparative study of the systemic inflammatory response to varying volumes of hepatic destruction following resection, cryotherapy (CT), microwave tissue ablation (MTA) and radiofrequency ablation (RFA).

**Summary background data**: With increasing interest in thermal ablation as an alternative to surgical resection for hepatic tumours, attention has shifted towards the unwanted effects of the treatments, including the potentially fatal systemic inflammatory response.

**Methods**: Live rat models underwent surgical resection, MTA, CT or RFA of 15%, 33% or 66% of the total hepatic volume. Serum samples taken pre-operatively and at 1, 3, 6, 24 and 48 hours post-operatively were analysed using ELISA for the proinflammatory cytokines IL-1β and IL-6.

**Results**: All animals in the 15% and 33% ablation groups survived to the 48 hour endpoint. However at the 66% level, all animals in the RFA and CT groups died within 6 hours of the procedures. Following 15% and 33% ablation the levels of IL-1β and IL-6 in the CT groups were significantly higher than those in the control group, However, at 66% ablation levels, RFA and CT did not differ (p>0.05), but were significantly higher (p<0.05) than in the MTA, surgical resection and sham laparotomy groups, which themselves did not differ (p>0.05).

**Conclusions**: In the small animal model, it appears that large volume (>66%) CT or RFA results in an overwhelming inflammatory response that carries a high mortality. MTA and surgical resection appear to be better tolerated at these high volumes of ablation.
5.2. Experimental Results

This section of this chapter describes the results in the form of scatter graphs, followed by statistical analysis.

5.2.1. IL-1β

Levels of IL-1β are described in Figures 5.1-5.3. At 15% ablation of total hepatic volume, there appeared to be no significant difference between any of the groups over the 48 hour timepoint. At 33% ablation, CT showed higher levels of IL-1β than MTA and resection at the 1 and 3 hour timepoints before dropping to near comparable levels at 48 hours. The most marked difference appeared at the 66% ablation volume where levels of IL-1β were markedly higher for CT and RFA compared with MTA and resection from 1 hour. These differences were maintained until all animals in the CT and RFA groups died at 6 hours. Thereafter, MTA and resection were comparable in IL-1β expression.

Inter-tray variability: The coefficient of variance (CV) for the IL-1β tray using the sample ‘CT 33% 48 hours’ was 7.7%. The tray manufacturers immunoassays typically have CVs less than 10% across the standard curve for both inter- and intra-assay precision.

Intra-tray variability: The CV using the sample ‘CT 33% 48 hours’ was 9%.
Figure 5.1. (a) Levels of serum IL-1β (pg/ml) following 15% ablation at 0 Hours.

Figure 5.1. (b) Levels of serum IL-1β (pg/ml) following 15% ablation at 1 Hour.
Figure 5.1. (c) Levels of serum IL-1β (pg/ml) following 15% ablation at 3 Hours.

Figure 5.1. (d) Levels of serum IL-1β (pg/ml) following 15% ablation at 6 Hours.
Figure 5.1. (e) Levels of serum IL-1β (pg/ml) following 15% ablation at 24 Hours.

Figure 5.1. (f) Levels of serum IL-1β (pg/ml) following 15% ablation 48 Hours.
Figure 5.2. (a) Levels of serum IL-1β (pg/ml) following 33% ablation at 0 Hours.

Figure 5.2. (b) Levels of serum IL-1β (pg/ml) following 33% ablation at 1 Hour.
Figure 5.2. (c) Levels of serum IL-1β (pg/ml) following 33% ablation at 3 Hours.

Figure 5.2. (d) Levels of serum IL-1β (pg/ml) following 33% ablation at 6 Hours.
Figure 5.2. (e) Levels of serum IL-1β (pg/ml) following 33% ablation at 24 Hours.

Figure 5.2. (f) Levels of serum IL-1β (pg/ml) following 33% ablation at 48 Hours.
Figure 5.3. (a) Levels of serum IL-1β (pg/ml) following 66% ablation at 0 Hours.

Figure 5.3. (b) Levels of serum IL-1β (pg/ml) following 66% ablation at 1 Hour.
Figure 5.3. (c) Levels of serum IL-1β (pg/ml) following 66% ablation at 3 Hours.

Figure 5.3. (d) Levels of serum IL-1β (pg/ml) following 66% ablation at 6 Hours.
Figure 5.3. (e) Levels of serum IL-1β (pg/ml) following 66% ablation at 24 Hours. Note that animals in the CT/RFA groups all died within 6 hours of surgery.

Figure 5.3. (f) Levels of serum IL-1β (pg/ml) following 66% ablation at 48 hours. Note that animals in the CT/RFA groups all died within 6 hours of surgery.
5.2.2. IL-6

At 15% ablation, the levels of IL-6 were comparable between the main ablation groups (MTA, CT and RFA) at 1, 3 and 6 hours. At 24 hours, CT and RFA showed greater IL-6 expression than MTA or resection; however these returned to near normal values within 48 hours. At 33% ablation, CT resulted in much higher IL-6 levels than all the other modalities and remained so until the 48 hour timepoint. The most marked difference appeared at 66% ablation where CT produced an exceptionally high IL-6 response compared with RFA, which in turn was significantly higher than the remaining modalities. Again at 6 hours, the animals in CT and RFA groups all died, thereafter MTA and resection did not show much difference in their IL-6 response.

Inter-tray variability: The coefficient of variance (CV) for the IL-6 tray using the sample ‘RFA 33% 48 hours’ was 6.4%. The tray manufacturers immunoassays typically have CVs less than 10% across the standard curve for both inter- and intra-assay precision.

Intra-tray variability: The CV using the sample ‘RFA 33% 48 hours’ was 8.2%.
Figure 5.4. (a) Levels of serum IL-6 (pg/ml) following 15% ablation at 0 Hours.

Figure 5.4. (b) Levels of serum IL-6 (pg/ml) following 15% ablation at 1 Hour.
Figure 5.4. (c) Levels of serum IL-6 (pg/ml) following 15% ablation at 3 Hours.

Figure 5.4. (d) Levels of serum IL-6 (pg/ml) following 15% ablation at 6 Hours.
Figure 5.4. (e) Levels of serum IL-6 (pg/ml) following 15% ablation at 24 Hours.

Figure 5.4. (f) Levels of serum IL-6 (pg/ml) following 15% ablation at 48 Hours.
Figure 5.5. (a) Levels of serum IL-6 (pg/ml) following 33% ablation at 0 Hours.

Figure 5.5. (b) Levels of serum IL-6 (pg/ml) following 33% ablation at 1 Hour.
Figure 5.5. (c) Levels of serum IL-6 (pg/ml) following 33% ablation at 3 Hours.

Figure 5.5. (d) Levels of serum IL-6 (pg/ml) following 33% ablation at 6 Hours.
Figure 5.5. (e) Levels of serum IL-6 (pg/ml) following 33% ablation at 24 Hours.

Figure 5.5. (f) Levels of serum IL-6 (pg/ml) following 33% ablation at 48 Hours.
Figure 5.6. (a) Levels of serum IL-6 (pg/ml) following 66% ablation at 0 Hours.

Figure 5.6. (b) Levels of serum IL-6 (pg/ml) following 66% ablation at 1 Hour.
**Figure 5.6. (c)** Levels of serum IL-6 (pg/ml) following 66% ablation at 3 Hours.

**Figure 5.6. (d)** Levels of serum IL-6 (pg/ml) following 66% ablation at 6 Hours.
Figure 5.6. (e) Levels of serum IL-6 (pg/ml) following 66% ablation at 24 Hours. Note that animals in the CT/RFA groups all died within 6 hours of surgery.

Figure 5.6. (f) Levels of serum IL-6 (pg/ml) following 66% ablation at 48 Hours. Note that animals in the CT/RFA groups all died within 6 hours of surgery.
5.3. **Statistical Analysis**

All animals survived the procedure to the 48 hour time-point except those undergoing RFA or CT of 66% of the hepatic volume, which died within 6 hours of surgery. The mean activity of IL-1β and IL-6 at each time point is shown in Figures 5.7 to 5.12. Levels rose steeply in all except the Sham group up to about six hours post-surgery, followed by a more gradual decline, in some cases reaching nearly normal levels at 48 hrs.

![Graph showing IL-1β levels over time](image)

**Figure 5.7.** IL-1β (pg/ml) levels at each time point following 15% ablation. Note that the levels are increased in all groups compared with the sham controls.
Figure 5.8. Mean activities for IL-1β (pg/ml) following 33% ablation.

Figure 5.9. Mean IL-1β (pg/ml) activity levels after 66% ablation. There were no values for RFA or CT after 6 hours as the animals in these groups all died at this point.
Figure 5.10. Mean IL-6 (pg/ml) levels following 15% ablation. All levels were significantly raised in comparison with the sham controls.

Figure 5.11. Mean IL-6 (pg/ml) activity levels following 33% ablation.
Figure 5.12. IL-6 (pg/ml) mean activity levels for laparotomy, MTA and surgery following 66% ablation. Data on CT and RFA have been omitted as they are well off the scale (see Table 5.2)
Following inspection of these graphs, three metrics were considered for further analysis:

1. Peak activity was estimated as the mean of activity at three and six hours post treatment. Although the three hour time point was somewhat short of the peak, it was considered better to average these two time points in order to minimise any inaccurate measurements.

2. Area under the curve (AUC), i.e. the area bounded by each of the observations from zero to 48 hrs.

3. The mean activity across all time points.

These three metrics were found to be highly correlated, with correlation coefficients of over $r>0.9$ between Peak activity and Mean activity, with a slightly lower correlation with AUC of about $r=0.8$ when considering each data set separately (i.e. interleukin type and treatment). However, the AUC was more heavily weighted to the recovery phase after the six hour time point, and it was thought that peak activity may be a more appropriate measure of biological effect. Accordingly, all further analysis was based on peak activity, i.e. the mean of activity at the three and six hour time points.

Mean peak activities for each treatment group, ablation level and interleukin type are shown in Table 5.2, with any statistically significant differences being indicated. Tukey’s Test compared the means of every treatment to the means of every other treatment, and identified where the difference between two means is greater than the standard error would be expected to allow.
<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>15% ablation</th>
<th>33% ablation</th>
<th>66% ablation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>Sig (1)</td>
<td>N</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>84.69</td>
<td>a,b</td>
<td>7</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>72.19</td>
<td>b</td>
<td>7</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>84.49</td>
<td>a,b</td>
<td>7</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>125.06</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>Surgery</td>
<td>7</td>
<td>96.20</td>
<td>a,b</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>19.60</td>
<td>c</td>
<td>-</td>
</tr>
<tr>
<td>Pooled StDev</td>
<td>29.19</td>
<td>69.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>15% ablation</th>
<th>33% ablation</th>
<th>66% ablation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>Sig</td>
<td>N</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>80.67</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>56.37</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>53.19</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>99.05</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>Surgery</td>
<td>7</td>
<td>67.28</td>
<td>a</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>4.24</td>
<td>b</td>
<td>-</td>
</tr>
<tr>
<td>Pooled StDev</td>
<td>42.12</td>
<td>89.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) Means with the same letter are not significantly different at the 5% level using Tukey's test.

(2) Statistical analysis done following a logarithmic transformation.

Table 5.2. Mean peak IL-1β and IL-6 activity (pg/ml), average of activity at 3 and 6 hrs (post-operation) analysed separately in each ablation level and cytokine sub-group.
5.3.1. IL-1 β response

Following 15% and 33% ablation the levels in the CT group were significantly higher than those in the laparotomy group, and the Sham group had lower levels than any of the other groups but otherwise there were no significant differences among groups (p>0.05). However, at the 66% ablation levels, RFA and CT did not differ (p>0.05), but were significantly higher (p<0.05) than in the MTA, Surgery and Laparotomy groups, which did not differ (p>0.05).

A two-way analysis of variance (F-test for each of the hypotheses, and the F-test is the mean square for each main effect and the interaction effect divided by the ‘within’ variance. The numerator degrees of freedom come from each effect, and the denominator degrees of freedom is the degrees of freedom for the within variance in each case) for the three ablation methods of particular interest, CT, MTA and RFA with the two lower levels of ablation, 15% and 33%, showed a statistically non-significant ($F_{1,36} =2.24, p=0.143$) over-all difference in IL-1β levels of 98.1 and 126.7 pg/ml between the 15% and 33% levels of ablation, and a statistically highly significant effect of treatment means (RFA=95.5, MTA= 88.1, CT=126.7, SE= 16.6, $F_{2,36}=4.69, p=0.016$). This was entirely due to higher levels of activity in the CT group. The interaction between ablation level and treatment was not statistically significant (p=0.55). The results are shown graphically in Figure 5.13.
Figure 5.13. Mean peak IL-1β activity (pg/ml), average of activity at 3 and 6 hrs (post-operation) for three ablation methods and three levels of ablation. Note that the two-way analysis of variance indicates that averaged across the 15% and 33% ablation levels activity was significantly higher (p<0.05) in the CT group than in the other two groups although these differences were not significant in the within-ablation level analyses given in Table 5.2. At the 66% level MTA activity was significantly lower than in the CT and RFA (p<0.05) groups, which do not differ (p>0.05).
5.3.2. IL-6 response

At the 15% ablation level the Sham group had significantly lower peak activity levels than any of the other treatment groups, which did not differ significantly. However, at the 33% ablation levels the CT group had significantly higher levels (p<0.05) than any of the other groups, which did not differ. At the 66% ablation levels Laparotomy, Surgery and MTA did not differ significantly whereas extremely high levels were observed in the RFA and CT groups. In this case it was necessary to do the statistical analyses on log-transformed data, although the un-transformed data is presented in Table 5.2.

A two-way analysis of variance for peak activity of the two lowest ablation levels (15% and 33%) including only CT, MTA and RFA gives highly significant (F_{1,35}=10.58, p=0.003) differences in activity between ablation levels (mean 77.6 and 166.6, SE=23.3 for the 15% and 33% respectively), a highly significant main effect for treatment (averaged across ablation levels RFA=95.4, MTA= 69.2 and CT = 201.6 with SE= 24.0) as well as a highly significant interaction between level of ablation and treatment. Thus, while activity in the RFA and MTA group increased by approximately 30 pg/ml between the 15% and 33% ablation levels, in the CT group the increase was 205 pg/ml. The results are shown graphically in Figure 5.14.
Figure 5.14. Mean peak IL-6 activity (pg/ml), average of activity at 3 and 6 hrs (post-operation) for three ablation methods and three levels of ablation. Note that there are no significant differences among methods at the 15% level but at the 33% ablation level CT is significantly different from RFA and MTA (p<0.05), which do not differ, but at the 66% level all three methods of ablation are significantly different from each other (p<0.05). The CT bar at 66% has been truncated at 2000 pg/ml.
5.4. Discussion

From this study, it is evident that MTA does not induce a significant inflammatory response over and above that caused by a laparotomy alone. Microwave ablation showed no significant differences for IL-1β levels at all ablation volumes and only minor changes for IL-6. This would suggest that the intrinsic response to MTA is extremely minimal if the levels caused by a surgical laparotomy are removed. Furthermore, the inflammatory response stimulated by MTA compared with those caused by the other ablative techniques would indicate it is the least stimulating of them all. In fact, significantly higher values were obtained for CT vs. MTA at all ablation volumes for both cytokines, while higher IL-6 values were present for RFA compared to MTA at 33% and 66% volumes. Even more interestingly, both CT and RFA resulted in an intense inflammatory response that killed all animals undergoing 66% ablation volumes, while surgical resection and MTA ablation of 66% of the hepatic parenchyma were very well tolerated with all animals surviving. Although such high volumes may not actually be used in clinical practice, this study demonstrates significant differences in inflammatory response between MTA, CT and RFA. Overall, the results obtained suggest that MTA could be the procedure that elicits the least inflammatory response, even when used for large volumes of ablation. This could possibly open the way to future clinical studies of feasibility for large volumes MTA ablations, without the associated risks of the cryoshock syndrome reported with cryotherapy.

To investigate the effects of ablative treatments several clinical and animal studies have attempted to determine the maximum volume of hepatic parenchyma which could be safely ablated without causing significant morbidity or mortality (Ng et al, 2006; Seifert et al, 1998).
Some investigators have indicated that large tumour sizes and ablated volumes are associated with increased morbidity following RFA treatments and ablation of less than 33% of the total volume was considered tolerable (Bleicher et al, 2003). Additionally, a clinical report suggested that RFA could be safely performed on liver tumours up to 3000mls in volume (Bowles et al, 2001). The increased morbidity and mortality associated with the various modalities of ablations may depend on the generation of inflammatory cytokines. It is known that the volume of ablation usually correlates well with the magnitude of inflammation produced (Bleicher et al, 2003) and that different ablative techniques elicit different inflammatory responses (Ng et al, 2006). In fact, the “cryoshock” phenomenon is a specific manifestation of hepatic cryotherapy (Washington et al, 2001; Seifert and Morris, 1999) which follows the release of intra-cellular cytotoxic substances during the freeze/thaw process (Poston, 2001). As a result, cellular products and necrotic debris are released into the systemic circulation which induce a systemic inflammatory response that may have catastrophic consequences, including multi-organ dysfunction and death. RFA probably produces a reduced immunogenic response compared to cryotherapy because tissue coagulation limits the release of cellular products into the systemic circulation and protein denaturation reduces the recognition and stimulation of an immunogenic response by antigen presenting cells (Chapman et al, 2000). Among the many pro-inflammatory markers which have been studied, serum IL-1β and IL-6 cytokines have been identified as important mediators of this phenomenon (Blackwell et al, 1999; Seifert et al, 1999; Ng et al, 2004; Osada et al, 2007), yet no clinical and immunological studies relating to these cytokines are available for MTA.

Recently, Jensen et al (2008) in a clinical study hypothesised that during RFA, liver enzymes are massively released into the circulation and a necrotic mass remains in-situ
potentially initiating an inflammatory response. In this study the general SIRS response after RFA was comparable to or even higher than after liver resection. This was despite the fact that patients undergoing liver resection are subjected to significantly greater surgical trauma in terms of larger volume of liver parenchyma removed and greater blood loss. However, the moderate IL-6 cytokine elevation found in their study was not associated with major complications after RFA such as ARDS or death. Interestingly, they also were unable to obtain any detectable levels of TNF-α following even large volume (40%) ablation. In our study, median postoperative TNF-α levels in all treatment groups were found to be below the detection limits (5 pg/ml). This is in accordance with a recently published clinical study reporting no significant increase of postoperative TNF-α levels (as well as IL-1β, IL-8 and IL-10) after RFA compared to baseline values (Teague et al, 2004). Earlier studies likewise demonstrated increased release of IL-6 after cryoablation when compared to patients undergoing liver resection (Seifert et al, 1999; de Jong, 2001). Seifert (2002) reported elevated IL-6 levels (up to 3000 pg/ml) after cryoablation up to the level found in sepsis models.

In contrast, hepatic cryoablation in humans or in rats resulted in higher TNF-α levels when compared to baseline values and also when compared to patients or rats undergoing liver resection (Seifert et al, 1999; Seifert et al, 2002; Chapman et al, 2000; Ng et al, 2004; Osada et al, 2007). Although tissue necrosis is the end result of all forms of ablative treatment, a specific shock syndrome has been reported only for cryoablation. In rats undergoing 35 per cent liver ablation using RFA there was no evidence of acute lung inflammation at 24 hours or a raised serum TNF-α concentration within 6 hours (Chapman et al, 2000), whereas in rats after 35 per cent liver cryoablation there was a mortality rate of 45 per cent at 24 hours associated with acute lung injury (Blackwell et al, 1999). Nuclear factor (NF) κB is a transcription complex
factor that regulates transcription of TNF-α, and IL-1, IL-2, IL-6 and IL-8 (Blackwell and Christman, 1997). Cryoablation and acute lung injury were associated with a raised serum level of TNF-α and NF-κB activation in liver and lung (Blackwell et al, 1999; Chapman et al, 2000). However, ablation of 35 per cent of rat liver by RFA was not associated with an increased TNF-α concentration and NF-κB activation in the first 6 hours after ablation. They suggested that early activation of these inflammatory mediators were linked to the ‘thaw’ phase of cryoablation. Electron microscopy of the ablated liver tissue demonstrated a markedly different injury pattern at the ultrastructural level between the two ablation techniques. Cryoablation resulted in plasma membrane disruption and dispersion of intact cellular structures into the space of Disse that communicated with the systemic circulation. In contrast, RFA appeared to induce a coagulative destruction of intracytoplasmic organelles while maintaining the integrity of the cell surface. Ng et al (2004) postulated that following cryotherapy, a form of ischaemia-reperfusion injury occurs which results in a significant increase in plasma levels of cytokines and other inflammatory mediators. These not only activate neutrophils but also promote expression of endothelial cell adhesion molecules (Gryglewski et al, 1986; McCall et al, 1989). This interaction permits close contact between endothelial cell and neutrophil, allowing release of cytotoxic agents and endothelial cell injury.

Our study certainly supports the contention that cryoablation, whether by release of immunogenic material into the systemic circulation, or by preservation of cytokine proteins, results in a significant inflammatory response. This does not appear to be the case with up to 33% RFA, MTA or resection. Why, then, do we get significant cytokine induction with 66% RFA? Perhaps it is not just intracellular material that spills into the circulation, but extracellular debris from the zone of coagulative necrosis resulting in local followed by systemic
inflammation. The larger the zone, the greater the inflammation as inflammatory cells are attracted to begin the process of phagocytosis. Indeed clinical studies have demonstrated that large tumour size and the number of RFA sessions were risk factors for major complications after RFA (Livraghi et al, 2003; Bleicher, 2003). RFA works mainly by heat conduction through tissues and as a result treatment times may be prolonged, particularly for larger tumours. It is possible that this allows time for necrotic and immunogenic material to be transported into the systemic circulation before the vessels become thrombosed. This theory contradicts the contention by Schell et al (2002) that RFA does not produce a significant inflammatory response owing to “immediate thrombosis of small and medium sized blood vessels”.

It has been suggested that local vasculature plays a significant role in defining the ultimate size and shape of the lesions created (Hansen et al, 1999; Sugimori et al, 2002). The slower the treatment, the less predictable the lesion shape and size. This theory was proposed after observation of the so-called ‘heat-sink’ effect, where circulating blood in local blood vessels take some of the heat away from the zone of ablation, thereby reducing the temperature in the areas directly adjacent to the vessels. Clearly this will have an impact on the ablation field, with some areas reaching cytotoxic temperatures while others do not. With RFA, temperatures around the probe reach as high as 70 degrees Celsius. Whilst this is indeed cytotoxic, the heat sink effect reduces this temperature to as low as 40 degrees Celsius, which may not cause the required cellular destruction and protein denaturation.

The patient is part of a closed loop circuit that includes an RF generator, an electrode needle, and a large dispersive electrode (grounding pad). An alternating field current is created within the tissue of the patient. Whilst there is no direct evidence that passing current through
the patient as part of this closed loop causes detrimental effects, what we did notice in our study was a rise in the core temperature in our RFA rats of between 1 and 2 degrees Celsius. This was despite cooling the liver before replacing it in the abdomen, and protecting the hilum from high temperatures. It is possible that a combination of the heat-sink effect and closed loop current may have led to unpredictable lesion shape and size. This was clearly visible on the rat livers, where, macroscopically at least, the margins of ablation at the end of treatment and also at post-mortem appeared to be poorly demarcated and irregular (Figure 5.15).

![Figure 5.15. RFA during treatment, showing the irregular border of the zone of ablation (left), and the same liver at post-mortem (right), again demonstrating the difficulty with lesion predictability.](image)

In direct contrast, MTA with its field effect, results in rapid achievement of cytotoxic temperatures in excess of 95 degrees Celsius. Even when the heat sink phenomenon is taken into account, treatment temperatures probably drop to 65-70 degrees Celsius in areas adjacent to vascular structures, which is still cytotoxic. Several studies have demonstrated that lesion shape is not distorted by proximity to vessels, and indeed results in consistently sized and
shaped lesions, with no heat-sink effect being observed (Meredith et al, 2005; Awad et al, 2007). In our study, we were able to produce consistent lesions in size and shape at a macroscopic level (Figure 5.16). It is worth pointing out though, that inherent in this is MTA’s key disadvantage, namely its inability to differentiate between tissue to be ablated and important vascular and biliary structures to preserve. Hence this treatment modality should be used with great care and only for pre-selected tumours.

**Figure 5.16.** Hepatic MTA, showing the predictable round ablation zones following 2 MTA treatments during (*left*), and 48 hours following treatment (*right*).

Previous work on MTA (Shibata et al, 2000; Ohno et al, 2001; Strickland et al, 2002; Wright et al, 2003; Wright et al, 2005; Hines-Peralta et al, 2006; Awad et al, 2007) has demonstrated the presence of a ‘fibrous capsule’ that appears to form soon after treatment around the zone of ablation. It is possible this prevents spillage of immunogenic material into
the systemic circulation. As tissue destruction is almost instantaneous, including vessel coagulation, there is little chance for necrotic material to be carried away by the circulation. Furthermore, inflammatory cells may be prevented or at least slowed down on their way to the zone of injury by the fibrous barrier, at least in the acute phase. Further support for this theory comes from the group of animals undergoing surgical resection. In the clinical setting, resection of 66% of the liver is considered a major procedure, requiring intensive care or high dependency support. However, the rats in these groups tolerated the procedure extremely well, with no evidence of a significant inflammatory response, compared with the ablation groups. This suggests that high mortality after large volume RFA and CT was probably unrelated to the liver insufficiency after the operation but to the extent of the systemic inflammatory response to the large volume ablated liver tissue. Clearly, CT at 33% and 66%, and RFA at 66% appear to induce the greatest inflammatory response and this is reflected in both the data obtained for IL-1β, IL-6 and indeed survival.

A criticism of this study is that ablation has been carried out on healthy, non-cancerous liver tissue. There is no evidence, however to suggest that the inflammatory response should be any different between the two types of hepatic parenchyma. In fact it may be more relevant in patients who have developed liver tumours such as colorectal metastases which may occur in non-cirrhotic livers with good functional reserve. Further experiments will have done to evaluate whether the results obtained in this study can be replicated in animals with cirrhotic livers. Ng et al (2004) demonstrated that rats safely tolerated RFA up to 40% in normal livers whereas in cirrhotic livers the maximum safest ablation volume was 20%. Notwithstanding this, if the results in cirrhotic liver were to be consistent with our findings, then it makes our
contention even more pertinent, given the need for conservation of maximum hepatic parenchyma in cirrhotic livers.

The present study, however, has demonstrated that MTA is safe and well tolerated even with high volume liver ablation and may prove to be the ablative procedure of choice for large tumour ablation in humans.
Chapter 6

Urinary retinol binding protein and serum heat shock protein 70 expression following hepatic resection, microwave ablation, radiofrequency ablation and cryotherapy.

6.1 Summary

6.2 Experimental Results

6.2.1 Retinol Binding Protein (RBP)

6.2.2 Heat Shock Protein 70 (HSP70)

6.3 Statistical Analysis

6.3.1 RBP

6.3.2 HSP70

6.4 Summary of Results

6.5 Discussion
6.1. Summary

**Introduction:** Previous studies have demonstrated adverse systemic and organ effects of hepatic ablation. Very few studies have investigated the renal injury sustained from these treatments and in particular, no studies have looked at the effect of MTA on the renal system.

**Aims:** The aim of this study was to investigate the effect of surgical resection, MTA, CT and RFA, at 3 increasing volumes of hepatic ablation, on the kidneys, using markers of acute renal injury.

**Methods:** Animals underwent hepatic ablation of 15%, 33% and 66% of total hepatic volume. At 48 hours, urine was analysed for the presence of retinol binding protein (RBP) and kidney tissue analysed for heat shock protein 70 (HSP70).

**Results:** Levels of RBP were highest in the 66% CT and RFA groups, whilst HSP70 was higher in kidney tissue lysate of all the treatment groups compared with controls. These differences were, however, greater in CT and RFA groups compared with resection/MTA.

**Conclusions:** At large volumes of ablation (66%), MTA and resection appear to have limited effect on renal injury, whereas CT of 33% and CT and RFA of 66% hepatic volume appear to result in detectable levels of renal injury.
6.2. Experimental Results

This section of the chapter describes the results in the form of graphs, followed by statistical analysis.

6.2.1. Retinol Binding Protein

Absolute values of RBP at each ablation volume together with mean values are described in the form of tables (Tables 6.2 - 6.4) and scatter graphs (Figures 6.1 - 6.3). Mean values alone for each group are shown in Figure 6.4.

15% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.68</td>
<td>0.29</td>
<td>0.16</td>
<td>0.43</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>0.66</td>
<td>0.32</td>
<td>0.15</td>
<td>0.39</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>0.68</td>
<td>0.40</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>0.67</td>
<td>0.38</td>
<td>0.12</td>
<td>0.32</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>0.79</td>
<td>0.44</td>
<td>0.14</td>
<td>0.37</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>0.65</td>
<td>0.37</td>
<td>0.11</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 6.2. Mean and standard deviation for urinary retinol binding protein at 15% ablation.

There were 7 animals in each treatment group.
Figure 6.1. Levels of urinary retinol binding protein (µg/L) at 15% ablation. There was no significant difference between any of the groups (p>0.05).

### 33% Ablation

<table>
<thead>
<tr>
<th>Urinary RBP (µg/L) by 33% Treatment</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.68</td>
<td>0.29 to 1.08</td>
<td>0.16</td>
<td>0.43</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>0.73</td>
<td>0.44 to 1.02</td>
<td>0.12</td>
<td>0.31</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>0.86</td>
<td>0.69 to 1.04</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>0.79</td>
<td>0.52 to 1.07</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>0.99</td>
<td>0.66 to 1.32</td>
<td>0.13</td>
<td>0.36</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>0.86</td>
<td>0.55 to 1.18</td>
<td>0.13</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 6.3. Mean and standard deviation for urinary retinol binding protein at 33% ablation.

There were 7 animals in each treatment group.
Figure 6.2. Levels of urinary retinol binding protein (µg/L) at 33% ablation. There was no significant difference between any of the groups (p>0.05).

66% Ablation

<table>
<thead>
<tr>
<th>Urinary RBP (µg/L) by 66% Treatment</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.68</td>
<td>0.29 to 1.08</td>
<td>0.16</td>
<td>0.43</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>0.63</td>
<td>0.38 to 0.89</td>
<td>0.10</td>
<td>0.27</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>0.94</td>
<td>0.68 to 1.20</td>
<td>0.11</td>
<td>0.28</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>0.83</td>
<td>0.62 to 1.03</td>
<td>0.08</td>
<td>0.22</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>1.51</td>
<td>1.15 to 1.87</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>1.36</td>
<td>0.83 to 1.90</td>
<td>0.12</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 6.4. Mean and standard deviation for urinary retinol binding protein at 66% ablation. There were 7 animals in each treatment group, except for the 66% CT and RFA groups (3 animals each).
**Figure 6.3.** Levels of urinary retinol binding protein (μg/L) at 66% ablation. There was no significant difference between Resection, MTA and Controls. However, the difference between CT and RFA, compared with Controls and Sham, were greater.

**Figure 6.4.** Mean levels of Retinol Binding Protein for each treatment group, at each volume of ablation. CT and RFA at 66% produced markedly greater amounts of RBP than the other treatment groups and control.
Inter-tray variability: The coefficient of variance (CV) for the RBP tray using the sample ‘CT 66% 6 hours’ was 12%.

Intra-tray variability: The CV using the sample ‘CT 66% 6 hours’ was 10.3%.

6.2.2. Heat Shock Protein 70

Absolute values of RBP at each ablation volume together with mean values are described in the form of tables (Tables 6.5 - 6.7) and box graphs (Figures 6.5 - 6.7). Mean values alone for each group are shown in Figure 6.8.
**15% Ablation**

Kidney HSP70 (ng per µg of Total Protein) by 15%

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>6.35</td>
<td>1.27</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>9.44</td>
<td>7.69</td>
<td>0.71</td>
<td>1.89</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>13.23</td>
<td>10.23</td>
<td>1.23</td>
<td>3.24</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>11.35</td>
<td>9.38</td>
<td>0.81</td>
<td>2.13</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>13.63</td>
<td>10.66</td>
<td>1.21</td>
<td>3.21</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>11.57</td>
<td>9.61</td>
<td>0.80</td>
<td>2.12</td>
</tr>
</tbody>
</table>

Table 6.5. Mean and standard deviation for heat shock protein 70 at 15% ablation. There were 7 animals in each treatment group.

**Figure 6.5.** Levels of kidney heat shock protein 70 (ng per µg of Total Protein) at 15% ablation.
**33% Ablation**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>6.35</td>
<td>1.27</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>11.21</td>
<td>8.41</td>
<td>1.14</td>
<td>3.03</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>16.32</td>
<td>14.55</td>
<td>0.73</td>
<td>1.92</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>15.24</td>
<td>13.30</td>
<td>0.79</td>
<td>2.09</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>22.37</td>
<td>17.60</td>
<td>1.95</td>
<td>5.16</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>20.87</td>
<td>17.59</td>
<td>1.34</td>
<td>3.55</td>
</tr>
</tbody>
</table>

**Table 6.6.** Mean and standard deviation for heat shock protein 70 at 33% ablation. There were 7 animals in each treatment group.

**Figure 6.6.** Levels of kidney heat shock protein 70 (ng per µg of Total Protein) at 33% ablation.
**66% Ablation**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>6.35</td>
<td>1.27</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>12.03</td>
<td>8.90</td>
<td>1.21</td>
<td>2.97</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>21.12</td>
<td>18.40</td>
<td>1.11</td>
<td>2.95</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>22.17</td>
<td>18.91</td>
<td>1.33</td>
<td>3.53</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>42.09</td>
<td>28.30</td>
<td>3.20</td>
<td>5.55</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>44.93</td>
<td>26.56</td>
<td>4.27</td>
<td>7.40</td>
</tr>
</tbody>
</table>

**Table 6.7.** Mean and standard deviation for kidney heat shock protein 70 at 66% ablation.

There were 7 animals in each treatment group, except for the 66% CT and RFA groups (3 animals each).

![Boxplot of kidney HSP70 levels](image)

**Figure 6.7.** Levels of kidney heat shock protein 70 (ng per µg of Total Protein) at 66% ablation.
Figure 6.8. Mean levels of HSP70. Each mean is based on seven rats except that at the 66% level the RFA and CT means are based on only three rats per group. An asterisk indicates that the mean is statistically significantly different from the Sham group mean (p<0.05). The levels of HSP70 in the 66% RFA and CT groups were clearly greater than the other treatment groups.
6.3. Statistical Analysis

6.3.1. Retinol Binding Protein

15% Ablation

\[ df_a = 6 - 1 = 5 \]
\[ df_{SA} = 6(7 - 1) = 36 \]

The \[ F_{crit}(5,36) \] from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 6.8. As the F-statistic generated (0.13) is less than the critical F-value (2.477, \( p<0.05 \)), there is no statistical difference between any of the groups. No further analysis was therefore carried out in this group.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.0813</td>
<td>5</td>
<td>0.0163</td>
<td>0.13</td>
<td>0.9850</td>
</tr>
<tr>
<td>Residual</td>
<td>4.5668</td>
<td>36</td>
<td>0.1269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.6480</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.8. 1-Way ANOVA analysis of all treatment groups in the 15% ablation group.
**33% Ablation**

\[ df_a = 6 - 1 = 5 \]

\[ df_{S/A} = 6(7 - 1) = 36 \]

The \( F_{crit}(5,36) \) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 6.9. As the F-statistic generated (0.77) was less than the critical F-value (2.477, \( p < 0.05 \)), there was no statistical difference between any of the groups. No further analysis was therefore carried out in this group.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.414</td>
<td>5</td>
<td>0.083</td>
<td>0.77</td>
<td>0.5784</td>
</tr>
<tr>
<td>Residual</td>
<td>3.877</td>
<td>36</td>
<td>0.108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.291</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 6.9.* 1-Way ANOVA analysis of all treatment groups in the 33% ablation group.
66% Ablation

\[ df_{a} = 6 - 1 = 5 \]

\[ df_{S/A} = 6(7 - 1) = 36 \]

The \( F_{crit}(5, 36) \) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 6.10. As the F-statistic generated (5.93) was greater than the critical F-value (2.477, \( p < 0.05 \)), the results were statistically significant. Furthermore, the \( p \) value of 0.0007 confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 6.11).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% Treatment Group</td>
<td>2.62</td>
<td>5</td>
<td>0.52</td>
<td>5.93</td>
<td>0.0007</td>
</tr>
<tr>
<td>Residual</td>
<td>2.47</td>
<td>28</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.09</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.10. 1-Way ANOVA analysis of all treatment groups in the 66% ablation group.
<table>
<thead>
<tr>
<th>66%</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection</td>
<td>1.75</td>
<td>4.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTA</td>
<td>0.62</td>
<td>2.10</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>9.90</td>
<td>25.99</td>
<td>10.30</td>
<td>22.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA</td>
<td>6.47</td>
<td>16.46</td>
<td>5.24</td>
<td>12.42</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.11. 1-Way ANOVA analysis of each group versus the other at 66% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 (p<0.05). The numbers in red therefore show statistically significant differences.
6.3.2. Heat Shock Protein 70

15% Ablation

| n  | 42 |

Kidney HSP70 by 15%

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Pooled SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>1.27</td>
<td>1.03</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>9.44</td>
<td>0.71</td>
<td>1.03</td>
<td>1.89</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>11.23</td>
<td>1.23</td>
<td>1.03</td>
<td>3.24</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>11.35</td>
<td>0.81</td>
<td>1.03</td>
<td>2.13</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>13.63</td>
<td>1.21</td>
<td>1.03</td>
<td>3.21</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>11.57</td>
<td>0.80</td>
<td>1.03</td>
<td>2.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Treatment Group</td>
<td>111.72</td>
<td>5.00</td>
<td>22.34</td>
<td>3.00</td>
<td>0.02</td>
</tr>
<tr>
<td>Residual</td>
<td>268.42</td>
<td>36.00</td>
<td>7.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>380.14</td>
<td>41.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.12. 1-Way ANOVA analysis of all treatment groups at 15% ablation. The F-statistic (3.00) was greater than the $F_{crit}(5,36)$ (2.477, p<0.05).
<table>
<thead>
<tr>
<th>15%</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection</td>
<td>4.57</td>
<td>4.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTA</td>
<td>1.58</td>
<td>3.16</td>
<td>1.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>5.63</td>
<td>8.86</td>
<td>0.05</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA</td>
<td>1.98</td>
<td>3.97</td>
<td>1.29</td>
<td>0.04</td>
<td>1.99</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.13.** 1-Way ANOVA analysis of each group versus the other at 15% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences between the relevant groups.
33% Ablation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Pooled SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>1.27</td>
<td>1.27</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>11.21</td>
<td>1.14</td>
<td>1.27</td>
<td>3.03</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>16.32</td>
<td>0.73</td>
<td>1.27</td>
<td>1.92</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>13.24</td>
<td>0.79</td>
<td>1.27</td>
<td>2.09</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>22.37</td>
<td>1.95</td>
<td>1.27</td>
<td>5.16</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>20.87</td>
<td>1.34</td>
<td>1.27</td>
<td>3.55</td>
</tr>
</tbody>
</table>

Source of variation

<table>
<thead>
<tr>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% Treatment Group</td>
<td>914.90</td>
<td>5</td>
<td>182.98</td>
<td>16.21</td>
</tr>
<tr>
<td>Residual</td>
<td>406.47</td>
<td>36</td>
<td>11.29</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1321</td>
<td>41</td>
<td>11.29</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.14. 1-Way ANOVA analysis of all treatment groups at 33% ablation. The F-statistic (16.21) was greater than the $F_{crit}(5,36)$ (2.477, p<0.05), indicating significant differences between the treatment groups.
Table 6.15. 1-Way ANOVA analysis of each group versus the other at 33% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 (p<0.05). The numbers in red therefore show statistically significant differences between the relevant groups.
### Kidney HSP70 by 66% Ablation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Pooled SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>9.46</td>
<td>1.27</td>
<td>1.47</td>
<td>3.36</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>12.03</td>
<td>1.21</td>
<td>1.59</td>
<td>2.97</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>21.12</td>
<td>1.11</td>
<td>1.47</td>
<td>2.95</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>22.17</td>
<td>1.33</td>
<td>1.47</td>
<td>3.53</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>42.09</td>
<td>3.20</td>
<td>2.25</td>
<td>5.55</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>44.93</td>
<td>4.27</td>
<td>2.25</td>
<td>7.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% Treatment Group</td>
<td>4474.70</td>
<td>5.00</td>
<td>894.94</td>
<td>58.96</td>
<td>0.00</td>
</tr>
<tr>
<td>Residual</td>
<td>409.84</td>
<td>27.00</td>
<td>15.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4884.54</td>
<td>32.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.16.** 1-Way ANOVA analysis of all treatment groups at 66% ablation. The F-statistic (16.21) was greater than the $F_{crit}(5,36)$ (2.477, $p<0.05$), indicating significant differences between the treatment groups.
Table 6.17. 1-Way ANOVA analysis of each group versus the other at 66% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences between the relevant groups.

6.4. Summary of results

**Retinol Binding Protein:** At 15% ablation levels, there were no significant differences between any of the treatment groups at the 95% confidence interval ($p=0.99$) (Table 6.8). Following 33% ablation of total hepatic volume, once again, there did not appear to be a substantial difference between the means of the various treatment groups ($p=0.58$) (Table 6.9). However, at 66% ablation, differences between treatment group means became statistically significant ($p=0.0007$) (Table 6.10). RFA and CT showed higher levels of urinary RBP compared with the control, sham, resection and MTA groups. In contrast, there was no statistically significant difference between surgical resection and MTA or between these two groups and control/sham (Table 6.11).
Heat Shock Protein 70: The induction of HSP70 in kidney lysate was significantly higher in the 15% CT group compared with the control and sham groups (p<0.05) (Table 6.13). At 33% ablation, compared with the control group, resection, MTA, CT and RFA all showed higher levels of HSP70 (p<0.05). There was no significant difference between MTA and the sham and resection groups, nor between the CT and RFA groups. CT and RFA both induced higher mean levels of HSP70 than MTA and resection (Table 6.15). 66% ablation showed the most dramatic responses between the various groups. Each group showed statistically different levels of HSP70 compared with each other, except between MTA and resection, and between CT and RFA. The most marked differences were between CT/RFA and MTA/resection (Table 6.17).

6.5. Discussion

With greater appreciation for the role of in-situ ablation of hepatic tumours, one issue that maintains some pessimism relates to safety. Although the efficacy of the various treatments has been demonstrated in numerous clinical and experimental studies, serious complications have been reported including abscess formation, catastrophic haemorrhage, biliary stricture formation, incomplete ablation and the systemic inflammatory response syndrome (SIRS) (Seifert and Morris, 1999). This was most evident following large volume cryotherapy, leading to the term ‘cryoshock’. This syndrome of multiorgan dysfunction has previously been demonstrated in the lungs, liver and also the renal system (Weaver et al, 1995; Sarantou et al, 1998; Bagia et al, 1998; Chapman et al, 2000; Wudel et al, 2003).
Renal failure may present as anuria or oliguria. One common cause of acute oliguric renal failure is the group of conditions known as acute tubular necrosis. These follow a period of shock or haemorrhage resulting in renal ischaemia or may be due to direct renal poisons (Dunnill, 1974). As we shall see it is possible that both mechanisms play a role in the pathogenesis of renal impairment following ablation.

This study suggests that significant renal impairment occurs following large volume CT and RFA. Urinary RBP and kidney parenchymal HSP70 levels were significantly higher in the groups undergoing 66% CT or RFA than MTA and resection. Although resection and MTA also resulted in a degree of renal injury, this was not as serious as CT and RFA. This pattern was clearly reflected in the behavioural responses where animals in the CT and RFA groups, at 66% volumes, were observed to be quiet, lacking movement, tachycardic, tachypnoeic and all animals in these groups died within 6 hours following surgery. In contrast, all animals in the MTA and resection groups seemed to tolerate the procedures well and survived to the 48 hour timepoint.

Bagia et al (1998) have previously demonstrated renal impairment in patients undergoing cryotherapy which appeared to be related to the volume of ablation. Elevated serum ALT levels were also linked to the magnitude of liver injury. Onik et al (1991) treated 18 patients with unresectable metastatic colon cancer confined to the liver using hepatic CT. During the study they incidentally noted that several patients developed myoglobinuria and myoglobin deposition in the renal tubules. Three patients demonstrated clinically significant acute tubular necrosis, with renal impairment. This complication appeared to correlate with the amount of tissue frozen and resolved a few days following surgery. Myoglobinemia and myoglobinuria are reported to cause acute tubular necrosis and acute renal failure after hepatic
cryosurgery (Weaver et al, 1995; Hammad and Neifeld, 1998; Seifert et al, 1998). Although myoglobin can be released from liver cells after an injury, it is not in sufficient quantity to explain the rise in serum levels after liver cryosurgery. Hence, it is postulated that muscle injury occurs during cryosurgery, but the mechanism is not known. The release of a cytokine, interleukin-6 (IL-6) (speculated to be the cause of sarcolemma injury), has been shown with concomitant rise of serum myoglobin during liver cryosurgery, although no consistent correlation between the two has been found (Hammad and Neifeld, 1998). Shankar et al (2002) reported 2 cases with myoglobinaemia following radiofrequency ablation although the precise mechanism of its induction again remains unclear.

Previous groups including ourselves have clearly demonstrated the induction of proinflammatory cytokines resulting in a SIRS response (Chapman et al, 2000; Ng et al, 2004). This leads to hypovolaemic shock that if profound, will lead to hypoperfusion of the kidneys, and is a recognised cause of pre-renal renal failure (Dunnill, 1974).

Ng et al (2004) demonstrated in porcine models that CT resulted in greater renal injury than RFA and in a further study (2006), that ablation of more than 40% of the hepatic volume (with RFA or CT) was potentially detrimental. These conclusions were drawn mainly on the basis of serum creatinine levels. Previous studies into renal failure from other surgical procedures suggest glomerular and tubular components (Leurs et al, 1989; Hashimoto et al, 1992). Traditional tests of renal function such as serum creatinine and creatinine clearance used in clinical practice focus on glomerular filtration and are relatively insensitive indicators of early damage because of the existence of a large ‘functional reserve’ (Bosch et al, 1983). Urinary excretion of retinol binding protein has been validated as a sensitive marker of early tubular dysfunction. Several studies have confirmed urinary RBP and retinol levels to be more
reliable markers of renal dysfunction than elevated serum creatinine (Smith et al, 1994; Gavrilov et al, 2006). RBP is freely filtered through the glomeruli and normally fully absorbed by the proximal tubules so that only trace amounts appear in the urine even in the presence of massive proteinuria. Consequently RBP is considered an excellent marker of early tubular damage and has been shown to be a more sensitive and accurate index than other tubular enzymes because of its greater stability in acidic urine (Bernard et al, 1987). In this study, urine samples were collected when the animals were culled at 48 hours following surgery, and in the case of those that died sooner, within 6 hours. Hence it is possible that the functional reserve of the kidneys may well have masked detection of abnormal renal function. However, we were able to quantify renal insult using RBP as a highly sensitive and early marker of acute proximal tubular injury. At the time of the study, RBP appeared to be the most sensitive, affordable, rat-specific test kit available on the market and chosen on those criteria. Subsequent markers of acute renal injury have been developed which are even more sensitive and pick up renal injury as quickly as 2 hours following insult. One such marker is the Neutrophil Gelatinase-Associated Lipocalin (NGAL) that has been measured in patients with renal injury following cardiac surgery (Mishra et al, 2005). However, to date, no study has been performed using this as a marker of inflammation-induced renal injury in rats. Another highly sensitive marker of renal injury that has recently been developed for use in rats is the Kidney injury molecule-1 (Kim-1) (Parikh and Devarajan, 2008). Both these markers would probably be better than RBP if the study was to be performed again, based on current evidence.

Heat shock proteins are constitutively expressed and are localised in various intracellular compartments. In physiological conditions some of these proteins function as intracellular molecular chaperones. Their intracellular concentrations can be increased 2 or 3
times by insults such as raised temperature, oxidative stress, infections and ischaemia (Welch, 1993; Chouchane et al, 1994; La Thangue and Latchman, 1988; Morimoto, 1998; Parsell and Lindquist, 1993).

Together, RBP and HSP70 provide sensitive and quantitative data on actual renal damage. This study is the first, to our knowledge, to compare the renal response to resection, MTA, CT and RFA, at increasing volumes of ablation using these markers.

Not a great deal is known about the inflammatory response to MTA. Up until recently, large volume MTA was not technically possible and the morbidity and mortality associated with this procedure was mainly limited to localised problems of incomplete ablation, abscess formation, biliary strictures and bleeding (Erce and Parks, 2003; Winter et al, 2006). Technological advances have allowed the achievement of larger zones of ablation with shorter durations of treatment compared with CT and RFA (Strickland et al, 2002). This theoretically prevents the prolonged heating or cooling obtained with RFA or CT respectively, possibly preventing the immune system from being fully activated. Moreover Kupffer cells within the ablated area, involved in the release of systemic cytokines, would be subjected to thermal injury by MTA and rendered inactive almost instantly rather than slowly through a process of tissue heat conduction as with RFA, and this could explain the lesser inflammatory response from MTA compared with RFA (Simon et al, 2005).

Although it is unclear what the precise aetiology of the renal injury is, my contention is that a profound systemic and ultimately organ-specific inflammatory response is stimulated that is closely related to observed behavioural and clinical changes. The SIRS response induced produces a hypovolaemic insult on the kidneys, and together with toxinaemia resulting from necrotic debris spilling into the systemic circulation, ends in substantial acute renal injury.
MTA has at least two advantages over CT and RFA. Firstly, the SIRS response seen is not as substantial with MTA and this has been discussed in Chapter 5. Secondly, the fibrous capsule that forms around the ablation zone (Bhardwaj et al, 2008) prevents mechanical spillage of immunogenic necrotic debris into the systemic circulation, including myoglobin, which is highly nephrotoxic (Heyman et al, 1996). Myoglobin induces renal injury by mechanisms that remain incompletely defined.
Chapter 7

A comparative study into the pulmonary effects of hepatic resection, microwave, radiofrequency and cryotherapy ablation at three different volumes of ablation

7.1 Summary

7.2 Experimental Results

7.2.1 Lung Heat Shock Protein 70 (HSP70)

7.2.2 Total Protein Content in Bronchoalveolar Lavage Fluid (TPC in BAL)

7.2.3 Wet/Dry Lung Weight

7.3 Statistical Analysis

7.3.1 HSP70

7.3.2 TPC in BAL

7.3.3 Wet/Dry Lung Weight

7.4 Summary of Results

7.5 Discussion
7.1. Summary

Introduction: Previous studies have demonstrated adverse systemic and distant organ effects of hepatic ablation. Very few studies have investigated the pulmonary injury sustained from these treatments and in particular, no studies have looked at the effect of MTA on the lungs.

Aims: The aim of this study was to investigate the effect of surgical resection, MTA, CT and RFA, at 3 increasing volumes of hepatic ablation, on the lungs, using markers of acute pulmonary injury.

Methods: Animals underwent hepatic ablation of 15%, 33% and 66% of total hepatic volume. At 48 hours, lung parenchyma was analysed for the presence of heat shock protein 70, a bronchoalveolar lavage was performed and analysed for total protein content and one lung was removed to quantify the wet/dry lung weight ratio.

Results: Levels of HSP70 and TPC in BAL were significantly higher in the 66% CT and RFA groups. There was no statistically significant difference wet/dry lung ratio between the various treatment groups.

Conclusions: At large volumes of ablation (66%), MTA and resection appear to have limited effect on lung injury, whereas CT of 33% and CT and RFA of 66% hepatic volume appear to result in significant levels of acute lung injury.
7.2. Experimental Results

This section of the chapter describes the results in the form of tables and graphs, followed by statistical analysis.

7.2.1. Heat Shock Protein 70 (HSP70)

15% Ablation

Lung HSP70 (ng per µg of Total Protein) by 15% Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>15.62</td>
<td>13.47</td>
<td>17.77</td>
<td>0.88</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>15.89</td>
<td>13.82</td>
<td>17.97</td>
<td>0.81</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>18.08</td>
<td>15.68</td>
<td>20.47</td>
<td>0.98</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>16.98</td>
<td>15.77</td>
<td>18.19</td>
<td>0.49</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>24.46</td>
<td>21.49</td>
<td>27.44</td>
<td>1.21</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>18.90</td>
<td>17.17</td>
<td>20.63</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Table 7.2. Mean and standard deviation for heat shock protein 70 at 15% ablation.
Figure 7.1. Levels of lung heat shock protein 70 (ng per µg of Total Protein) at 15% ablation.

33% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>15.62</td>
<td>13.47</td>
<td>17.77</td>
<td>0.88</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>14.95</td>
<td>12.71</td>
<td>17.18</td>
<td>0.91</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>26.35</td>
<td>23.07</td>
<td>29.64</td>
<td>1.34</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>19.09</td>
<td>15.74</td>
<td>22.43</td>
<td>1.37</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>58.07</td>
<td>49.22</td>
<td>66.92</td>
<td>3.62</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>40.24</td>
<td>35.63</td>
<td>44.84</td>
<td>1.88</td>
</tr>
</tbody>
</table>

Table 7.3. Mean and standard deviation for heat shock protein 70 at 33% ablation.
Figure 7.2. Levels of lung heat shock protein 70 (ng per µg of Total Protein) at 33% ablation.

66% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>15.62</td>
<td>13.47</td>
<td>17.77</td>
<td>0.88</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>17.08</td>
<td>14.67</td>
<td>19.49</td>
<td>0.94</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>29.81</td>
<td>25.79</td>
<td>33.82</td>
<td>1.64</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>40.52</td>
<td>31.99</td>
<td>49.06</td>
<td>3.49</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>95.71</td>
<td>72.11</td>
<td>119.32</td>
<td>5.49</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>75.90</td>
<td>33.76</td>
<td>118.04</td>
<td>9.79</td>
</tr>
</tbody>
</table>

Table 7.4. Mean and standard deviation for lung heat shock protein 70 at 66% ablation. There were 7 animals in each treatment group, except for the 66% CT and RFA groups (3 animals each), where the differences were statistically significant enough to not require any further data from animals.
Figure 7.3. Levels of lung heat shock protein 70 (ng per µg of Total Protein) at 66% ablation.

Figure 7.4. Mean levels of HSP70 in lung tissue lysate following ablation with the various modalities at increasing volumes of ablation.
7.2.2. Total Protein Content in Bronchoalveolar Lavage Fluid

15% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>430.0</td>
<td>353.4 to 506.6</td>
<td>29.80</td>
<td>72.99</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>436.4</td>
<td>368.1 to 504.8</td>
<td>27.94</td>
<td>73.91</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>438.2</td>
<td>332.2 to 544.1</td>
<td>41.22</td>
<td>100.97</td>
</tr>
<tr>
<td>MTA</td>
<td>6</td>
<td>457.7</td>
<td>337.7 to 577.6</td>
<td>46.66</td>
<td>114.30</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>460.1</td>
<td>374.5 to 545.8</td>
<td>35.01</td>
<td>92.61</td>
</tr>
<tr>
<td>RFA</td>
<td>6</td>
<td>433.8</td>
<td>292.2 to 575.4</td>
<td>55.08</td>
<td>134.92</td>
</tr>
</tbody>
</table>

Table 7.5. Mean and standard deviation for total protein content (TPC) in bronchoalveolar lavage (BAL) fluid at 15% ablation.

Figure 7.5. Levels of total protein content (TPC) in bronchoalveolar lavage fluid (BAL) (µg/ml) at 15% ablation.
### 33% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>430.0</td>
<td>353.4 to 506.6</td>
<td>29.80</td>
<td>72.99</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>424.2</td>
<td>334.8 to 513.5</td>
<td>34.75</td>
<td>85.12</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>482.0</td>
<td>369.0 to 595.0</td>
<td>46.20</td>
<td>122.23</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>507.1</td>
<td>460.8 to 553.4</td>
<td>18.92</td>
<td>50.06</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>593.1</td>
<td>530.2 to 656.1</td>
<td>25.72</td>
<td>68.05</td>
</tr>
<tr>
<td>RFA</td>
<td>6</td>
<td>502.5</td>
<td>419.2 to 585.8</td>
<td>32.41</td>
<td>79.38</td>
</tr>
</tbody>
</table>

**Table 7.6.** Mean and standard deviation for total protein content (TPC) in bronchoalveolar lavage (BAL) fluid at 33% ablation.

**Figure 7.6.** Levels of total protein content (TPC) in bronchoalveolar lavage fluid (BAL) (µg/ml) at 33% ablation.
66% Ablation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>430.0</td>
<td>353.4 to 506.6</td>
<td>29.80</td>
<td>72.99</td>
</tr>
<tr>
<td>Sham</td>
<td>7</td>
<td>447.4</td>
<td>389.3 to 505.5</td>
<td>23.75</td>
<td>62.84</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>451.0</td>
<td>350.6 to 551.4</td>
<td>41.05</td>
<td>108.61</td>
</tr>
<tr>
<td>MTA</td>
<td>7</td>
<td>531.3</td>
<td>434.1 to 628.4</td>
<td>39.70</td>
<td>105.03</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>931.7</td>
<td>666.9 to 1196.4</td>
<td>61.54</td>
<td>106.59</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>889.7</td>
<td>600.7 to 1178.7</td>
<td>67.17</td>
<td>116.34</td>
</tr>
</tbody>
</table>

Table 7.7. Mean and standard deviation for total protein content (TPC) in bronchoalveolar lavage (BAL) fluid at 66% ablation. There were 7 animals in each treatment group, except for the 66% CT and RFA groups (3 animals each), where the differences were statistically significant enough to not require any further data from animals.

![Figure 7.7](image-url) Levels of total protein content (TPC) in bronchoalveolar lavage fluid (BAL) (µg/ml) at 66% ablation.
Figure 7.8. Mean levels of TPC in BAL following ablation with the various modalities at increasing volumes of ablation.

7.2.3. Wet/Dry Lung Weight Ratio

15% Ablation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.7</td>
<td>4.2</td>
<td>5.1</td>
<td>0.18</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>4.8</td>
<td>4.3</td>
<td>5.3</td>
<td>0.18</td>
</tr>
<tr>
<td>Resection</td>
<td>5</td>
<td>5.5</td>
<td>4.9</td>
<td>6.1</td>
<td>0.20</td>
</tr>
<tr>
<td>MTA</td>
<td>5</td>
<td>4.7</td>
<td>4.5</td>
<td>4.9</td>
<td>0.08</td>
</tr>
<tr>
<td>CT</td>
<td>6</td>
<td>4.8</td>
<td>4.1</td>
<td>5.4</td>
<td>0.24</td>
</tr>
<tr>
<td>RFA</td>
<td>6</td>
<td>5.3</td>
<td>3.9</td>
<td>6.7</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 7.8. Mean and standard deviation for wet/dry lung weight ratio at 15% ablation.
Figure 7.9. Wet/Dry Lung Weight Ratio at 15% ablation.

### 33% Ablation

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.7</td>
<td>4.2</td>
<td>0.18</td>
<td>0.45</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>4.9</td>
<td>4.5</td>
<td>0.16</td>
<td>0.39</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>5.1</td>
<td>4.7</td>
<td>0.16</td>
<td>0.42</td>
</tr>
<tr>
<td>MTA</td>
<td>6</td>
<td>5.2</td>
<td>4.2</td>
<td>0.40</td>
<td>0.99</td>
</tr>
<tr>
<td>CT</td>
<td>7</td>
<td>5.6</td>
<td>5.1</td>
<td>0.21</td>
<td>0.55</td>
</tr>
<tr>
<td>RFA</td>
<td>7</td>
<td>4.8</td>
<td>4.5</td>
<td>0.11</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 7.9. Mean and standard deviation for wet/dry lung weight ratio at 33% ablation.
**Figure 7.10.** Wet/Dry Lung Weight Ratio at 33% ablation.

**66% Ablation**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>4.7</td>
<td>4.2</td>
<td>0.18</td>
<td>0.45</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>4.9</td>
<td>4.4</td>
<td>0.18</td>
<td>0.44</td>
</tr>
<tr>
<td>Resection</td>
<td>7</td>
<td>4.8</td>
<td>4.2</td>
<td>0.24</td>
<td>0.65</td>
</tr>
<tr>
<td>MTA</td>
<td>6</td>
<td>5.3</td>
<td>4.12</td>
<td>0.47</td>
<td>1.14</td>
</tr>
<tr>
<td>CT</td>
<td>3</td>
<td>6.1</td>
<td>4.0</td>
<td>0.50</td>
<td>0.86</td>
</tr>
<tr>
<td>RFA</td>
<td>3</td>
<td>5.4</td>
<td>3.7</td>
<td>0.38</td>
<td>0.66</td>
</tr>
</tbody>
</table>

**Table 7.10.** Mean and standard deviation for wet/dry lung weight ratio at 66% ablation.
7.3. Statistical Analysis

The rationale for the statistical tests employed in these analyses is detailed in section 3.5.

7.3.1. Lung HSP70

15% Ablation

\[ df_a = 6 - 1 = 5 \]

\[ df_{SSA} = 6(7-1) = 36 \]

The F_{crit}(5,36) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.11. As the F-statistic generated (13.8) was greater than the critical F-value (2.477, p<0.05), the results were statistically significant.
Furthermore, the $p$ value of $<0.0001$ confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 7.12).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>365.78</td>
<td>5</td>
<td>73.16</td>
<td>13.80</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Residual</td>
<td>185.50</td>
<td>35</td>
<td>5.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>551.28</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.11.** Lung HSP70: 1-Way ANOVA analysis of all treatment groups at 15% ablation.

<table>
<thead>
<tr>
<th>15%</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection</td>
<td>3.47</td>
<td>2.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTA</td>
<td>1.82</td>
<td>1.41</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td><strong>34.81</strong></td>
<td><strong>32.03</strong></td>
<td><strong>16.76</strong></td>
<td><strong>32.63</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA</td>
<td><strong>8.44</strong></td>
<td><strong>7.91</strong></td>
<td>0.46</td>
<td><strong>4.95</strong></td>
<td><strong>15.69</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.12.** Lung HSP70: 1-Way ANOVA analysis of each group versus the other at 15% ablation. The $F_{\text{crit}}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences.
**33% Ablation**

\[ df_a = 6 - 1 = 5 \]

\[ df_{S/A} = 6(7-1) = 36 \]

The \( F_{\text{crit}}(5,36) \) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.13. As the F-statistic generated (79.63) was greater than the critical F-value (2.477, \( p < 0.05 \)), the results were statistically significant. Furthermore, the \( p \) value of <0.0001 confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 7.14).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>10172.38</td>
<td>5</td>
<td>2034.48</td>
<td>79.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>919.81</td>
<td>36</td>
<td>25.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11092.19</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 7.13.* Lung HSP70: 1-Way ANOVA analysis of all treatment groups at 33% ablation.
Table 7.14. Lung HSP70: 1-Way ANOVA analysis of each group versus the other at 33% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences.

66% Ablation

$df_a=6-1=5$

$df_{S/A}=6(7-1)=36$

The $F_{crit}(5,36)$ from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.15. As the F-statistic generated (78.49) was greater than the critical F-value (2.477, $p<0.05$), the results were statistically significant. Furthermore, the p value of $<0.0001$ confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 7.16).
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>20909.03</td>
<td>5</td>
<td>4181.81</td>
<td>78.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>1438.52</td>
<td>27</td>
<td>53.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22347.55</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.15.** Lung HSP70: 1-Way ANOVA analysis of all treatment groups at 66% ablation.

<table>
<thead>
<tr>
<th>66%</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection</td>
<td>58.11</td>
<td>41.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTA</td>
<td>47.96</td>
<td>36.37</td>
<td>7.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>505.88</td>
<td>418.13</td>
<td>248.5</td>
<td>74.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA</td>
<td>101.41</td>
<td>80.48</td>
<td>51.84</td>
<td>19.36</td>
<td>3.12</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.16.** Lung HSP70: 1-Way ANOVA analysis of each group versus the other at 66% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences between the various groups.
7.3.2. Total Protein Content in Bronchoalveolar Lavage Fluid

15% Ablation

\[ df_a = 6 - 1 = 5 \]

\[ df_{S/A} = 6(7 - 1) = 36 \]

The \( F_{crit}(5,36) \) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.17. As the F-statistic generated (0.11) is less than the critical F-value (2.477, \( p < 0.05 \)), there is no statistical difference between any of the groups. No further analysis was therefore carried out in this group.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>5308.4</td>
<td>5</td>
<td>1061.7</td>
<td>0.11</td>
<td>0.9900</td>
</tr>
<tr>
<td>Residual</td>
<td>318205.6</td>
<td>32</td>
<td>9943.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>323514.0</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.17. TPC in BAL: 1-Way ANOVA analysis of all treatment groups at 15% ablation.
33% Ablation

\[ df_a = 6 - 1 = 5 \]
\[ df_{SA} = 6(7-1) = 36 \]

The \( F_{crit}(5,36) \) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.18. As the F-statistic generated (3.64) was greater than the critical F-value (2.477, \( p<0.05 \)), the results were statistically significant. Furthermore, the p value of 0.0098 confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 7.19).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% Treatment Group</td>
<td>125227.8</td>
<td>5</td>
<td>25045.6</td>
<td>3.64</td>
<td>0.0098</td>
</tr>
<tr>
<td>Residual</td>
<td>226830.0</td>
<td>33</td>
<td>6873.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>352057.9</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.18. TPC in BAL: 1-Way ANOVA analysis of all treatment groups at 33% ablation.
Table 7.19. TPC in BAL: 1-Way ANOVA analysis of each group versus the other at 33% ablation. The $F_{\text{crit}}(1,12)$ between any 2 groups was 4.474 (p<0.05). The numbers in red therefore show statistically significant differences between the various groups.

**66% Ablation**

$df_a=6-1=5$

$df_{S/A}=6(7-1)=36$

The $F_{\text{crit}}(5,36)$ from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.20. As the F-statistic generated (23.09) was greater than the critical F-value (2.477, p<0.05), the results were statistically significant. Furthermore, the p value of <0.0001 confirmed that there was indeed a statistically significant difference between the groups. Therefore, further analysis was carried out to compare differences between individual groups (Table 7.21).
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% Treatment Group</td>
<td>1013942.5</td>
<td>5</td>
<td>202788.5</td>
<td>23.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>237090.5</td>
<td>27</td>
<td>8781.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1251033.0</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.20.** TPC in BAL: 1-Way ANOVA analysis of all treatment groups at 66% ablation.

<table>
<thead>
<tr>
<th>66%</th>
<th>Control</th>
<th>Sham</th>
<th>Resection</th>
<th>MTA</th>
<th>CT</th>
<th>RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection</td>
<td>0.16</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTA</td>
<td>3.93</td>
<td>3.29</td>
<td>1.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>71.38</td>
<td>84.87</td>
<td>41.51</td>
<td>30.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA</td>
<td>55.08</td>
<td>64.73</td>
<td>33.04</td>
<td>23.14</td>
<td>0.21</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.21.** TPC in BAL: 1-Way ANOVA analysis of each group versus the other at 33% ablation. The $F_{crit}(1,12)$ between any 2 groups was 4.474 ($p<0.05$). The numbers in red therefore show statistically significant differences between the various groups.
7.3.3. Wet/Dry Lung Weight Ratio

15% Ablation

df_a=6-1=5

df_{S/A}=6(7-1)=36

The F_{crit}(5,36) from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.22. As the F-statistic generated (1.51) is less than the critical F-value (2.477, p<0.05), there is no statistical difference between any of the groups. No further analysis was therefore carried out in this group.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% Treatment Group</td>
<td>3.59</td>
<td>5</td>
<td>0.72</td>
<td>1.51</td>
<td>0.2192</td>
</tr>
<tr>
<td>Residual</td>
<td>13.33</td>
<td>28</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16.92</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.22. Wet/Dry Lung Weight Ratio: 1-Way ANOVA analysis of all treatment groups at 15% ablation.

33% Ablation

df_a=6-1=5

df_{S/A}=6(7-1)=36
The $F_{\text{crit}}(5,36)$ from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.23. As the F-statistic generated (2.28) is less than the critical F-value (2.477, $p<0.05$), there is no statistical difference between any of the groups. No further analysis was therefore carried out in this group.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% Treatment Group</td>
<td>3.44</td>
<td>5</td>
<td>0.69</td>
<td>2.28</td>
<td>0.0693</td>
</tr>
<tr>
<td>Residual</td>
<td>9.96</td>
<td>33</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>13.41</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.23.** Wet/Dry Lung Weight Ratio: 1-Way ANOVA analysis of all treatment groups at 33% ablation.

**66% Ablation**

$df_a=6-1=5$

$df_{S/A}=6(7-1)=36$

The $F_{\text{crit}}(5,36)$ from the table of critical values is 2.477.

The 1-Way ANOVA analysis is shown in Table 7.24. As the F-statistic generated (2.09) is less than the critical F-value (2.477, $p<0.05$), there is no statistical difference between any of the groups. No further analysis was therefore carried out in this group.
Table 7.24. Wet/Dry Lung Weight Ratio: 1-Way ANOVA analysis of all treatment groups at 66% ablation.

7.4. Summary of Results

Heat Shock Protein 70: At 15% ablation, neither MTA nor resection groups had significantly higher levels of HSP70 than controls. In contrast, 15% CT and RFA were significantly higher than controls (Table 7.12). At 33%, all treatment groups had higher HSP70 than controls, although these differences were much greater between CT/RFA and controls (Table 7.14). This trend was also seen at 66% ablation levels (Table 7.16).

TPC in BAL: At 15% ablation, there was no significant difference between any of the treatment groups and controls (Table 7.17). At 33% ablation, CT and MTA showed significantly higher levels of protein in lavage fluid than controls, although this difference was more apparent with CT (Table 7.19). At 66% ablation, significant differences between treatment group and controls were found with CT and RFA only. Furthermore, the differences between CT/RFA were also significant when compared with resection/MTA (Table 7.21).
Wet/Dry Lung Weight Ratio: There were no statistically significant differences between any of the treatment groups and controls at any volume of ablation. It was noted, however, that at 66% ablation, CT resulted in a higher mean than all the other treatment groups (Figure 7.11).

7.5. Discussion

Although the efficacy of hepatic ablation has been demonstrated in numerous clinical and experimental studies, serious complications have been reported including abscess formation, catastrophic haemorrhage, biliary stricture formation, incomplete ablation and the systemic inflammatory response syndrome (SIRS) (Seifert and Morris, 1999).

In Chapter 5, we have shown proinflammatory cytokine induction following hepatic ablation or resection. This was more significant in the larger volume ablations, particularly RFA and CT. This appeared to correlate with animal survival, where all animals in the 66% RFA and CT groups died within 6 hours of the procedure. In Chapter 6, we demonstrated evidence of renal injury in these groups. This present study looked at the pulmonary effects of the various treatments.

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), is a sudden, severe lung inflammation that results in hypoxemia and loss of lung compliance. ARDS is a manifestation of the systemic inflammatory response syndrome (SIRS) and an early marker of multiple organ failure (MOF) (Bone et al, 1992). Mortality from this potentially preventable disease has decreased since its initial description, but remains unacceptably high (30 to 40 percent) (Bernard et al, 1994). The syndrome has diverse
aetiologies involving either direct or indirect insult to the lung. ALI is most often seen as part of a systemic inflammatory process, particularly systemic sepsis, where the lung manifestations parallel those of other tissues (Ware and Matthay, 2000).

ALI/ARDS is thought to develop when pulmonary or systemic inflammation leads to systemic release of cytokines and other proinflammatory molecules (Sorkine et al, 1995; Gilmont et al, 1996; Koksoy et al, 2001; Yang et al, 2007). The cytokines activate alveolar macrophages and recruit neutrophils to the lungs, which in turn release leukotrienes, oxidants, platelet-activating factor, and proteases. These substances damage capillary endothelium and alveolar epithelium, disrupting the barriers between capillaries and airspaces. Oedema fluid, protein, and cellular debris flood the airspaces and interstitium, causing disruption of surfactant, airspace collapse, ventilation-perfusion mismatch, shunting, stiffening of the lungs with decreased compliance, and pulmonary hypertension. Histopathologically, diffuse alveolar damage results with intra-alveolar neutrophils, red blood cells, and cellular debris and denuded epithelial basement membranes with subsequent formation of hyaline membranes (Figure 7.5). Two of the key features of this pathology, extravasation of protein rich fluid and interstitial oedema/fluid filled airspaces were assessed in this study, together with heat shock protein 70 (HSP70), as markers of acute lung injury.
Figure 7.5. The Pathophysiology of Acute Respiratory Distress Syndrome. (From Critical Care Medicine Tutorials - www.ccmtutorials.com)
Although the precise mechanism whereby ALI follows an intense inflammatory response remains unclear, what is certain is that multiple factors are involved and are interlinked in complex ways. We know that increased levels of proinflammatory cytokines have been found in lung tissue lysate following ALI (Goldblum et al, 1989; Ferrari-Baliviera et al, 1989). Elevated levels of HSP70 have also been found in patients with ALI. The highly inducible 70-kDa stress protein is perhaps the best characterised and studied of all the stress proteins identified to date (Wheeler, 2007).

Over the last several years, there has been a growing body of evidence linking the increased expression of HSP70 with cytoprotection in acute lung injury, both in-vitro and in-vivo (Wong and Wispe, 1997; Wheeler and Wong, 2007). Weiss et al (2000) demonstrated that polymicrobial sepsis induced via caecal ligation and puncture impairs lung HSP70 expression in rats and surmised that this intrinsic failure of the normal stress response contributes to lung inflammation and injury. These findings were further corroborated in a translational study in which HSP70 induction in peripheral blood monocytes of patients with acute respiratory distress syndrome was significantly decreased compared with patients without acute respiratory distress syndrome (Durand et al, 2000). Restoration of HSP70 expression in the respiratory epithelium, via adenoviral delivery of mature HSP70, greatly attenuated lung injury and improved survival after caecal ligation and puncture (Weiss et al, 2002). Collectively, these studies suggest that elevated HSP70 expression is an important physiological response in patients with acute lung injury and acute respiratory distress syndrome. Elevated levels of HSP70 in the large volume RFA and CT groups appear to confirm the presence of ALI even as early as 6 hours following surgery.
The mechanisms by which HSP70 and other stress proteins confer such broad cytoprotection are not completely understood. Several in-vitro and in-vivo studies have shown that HSP70 inhibits activation of NF-κB, a pluripotent transcription factor that regulates the expression of many genes associated with ALI, including proinflammatory cytokines (Wong and Wispe, 1997; Malhotra and Wong, 2002; Wheeler and Wong, 2007). What is the nature of the complex interaction between HSP70, NF-κB and inflammatory cytokines? Chapman et al (2000) demonstrated lung activation of NF-κB at 1 hour following 35% cryoablation of rat liver, together with elevated proinflammatory cytokines. This observation was not seen with RFA. They suggested that early activation of these inflammatory mediators were linked to the ‘thaw’ phase of cryoablation. Electron microscopy of the ablated liver tissue demonstrated a markedly different injury pattern at the ultrastructural level between the two ablation techniques. Cryoablation resulted in plasma membrane disruption and dispersion of intact cellular structures into the space of Disse that communicated with the systemic circulation. In contrast, RFA appeared to induce a coagulative destruction of intracytoplasmic organelles while maintaining the integrity of the cell surface. Ng et al (2004) postulated that following cryotherapy, a form of ischaemia-reperfusion injury occurs which results in a significant increase in plasma levels of cytokines and other inflammatory mediators. These not only activate neutrophils but also promote expression of endothelial cell adhesion molecules (Gryglewski et al, 1986; McCall et al, 1989). This interaction permits close contact between endothelial cell and neutrophil, allowing release of cytotoxic agents and endothelial cell injury. This theory received some support by Shields et al (2003) who showed significant ALI following ischaemia reperfusion as measured by cytokine levels, neutrophil infiltration
(myeloperoxidase activity) and endothelial permeability (BAL protein content and wet-dry lung weight ratios).

Our study certainly supports the contention that cryoablation, whether by release of immunogenic material into the systemic circulation, or by preservation of cytokine proteins results in a significant inflammatory response that affects the lungs markedly. This does not appear to be the case with up to 33% RFA, MTA or resection. Why, then, do we get significant ALI with 66% RFA? Perhaps it is not just intracellular material that spills into the circulation, but actual dead cells from the zone of coagulative necrosis resulting in local followed by systemic inflammation. The larger the zone, the greater the inflammation as inflammatory cells are attracted to begin the process of phagocytosis. Indeed clinical studies have demonstrated that large tumour size and the number of RFA sessions were the risk factors for major complications after RFA (Livraghi et al, 2003; Bleicher, 2003). RFA works mainly by heat conduction through tissues and as a result treatment times may be prolonged, particularly for larger tumours. It is possible that this allows time for necrotic and immunogenic material to be transported into the systemic circulation before the vessels become thrombosed. This contradicts the contention by Schell et al (2002) that RFA does not produce a significant inflammatory response owing to “immediate thrombosis of small and medium sized blood vessels”.

Previous work on MTA (Shibata et al, 2000; Ohno et al, 2001; Strickland et al, 2002; Wright et al, 2003; Wright et al, 2005; Hines-Peralta et al, 2006; Awad et al, 2007) has demonstrated the presence of a ‘fibrous capsule’ that appears to form quite quickly around the zone of ablation. It is possible this prevents spillage of immunogenic material into the systemic circulation. As tissue destruction is almost instantaneous, including vessel coagulation, there is
little chance for necrotic material to be carried away by the circulation. Furthermore, inflammatory cells may be prevented or at least slowed down on their way to the zone of injury by the fibrous barrier, at least in the acute phase. Further support for this theory comes from the group of animals undergoing surgical resection. In the clinical setting, resection of 66% of the liver is considered a major procedure, requiring intensive care or high dependency support. However, the rats in these groups tolerated the procedure extremely well, with no evidence of a significant inflammatory response, compared with the ablation groups. This suggests that high mortality after large volume RFA and CT was probably unrelated to the liver insufficiency after the operation but to the extent of the systemic inflammatory response to the large volume ablated liver tissue. Clearly, CT at 33% and 66%, and RFA at 66% appear to induce the greatest inflammatory response and this is reflected in both the data obtained for lung injury and indeed survival.

The pathophysiological mechanism leading to the vascular leak syndrome seen in ALI suggests a central role for neutrophils, demonstrating neutrophil activation and neutrophil-endothelial cell adherence associated with microvascular leakage. Clinical studies support these findings with evidence of neutrophil activation and a direct correlation between this and markers of vascular leakage, such as weight gain and increased protein content in BAL fluid (Welbourn et al, 1991; Edwards et al, 1992; Baars et al, 1992). Nitric oxide, in addition to acting as a local vasodilator, may also affect the interactions between neutrophils and vascular endothelium (O’Donovan et al, 1995). Activated neutrophils demonstrate increased adherence to the vascular endothelium and produce superoxide – key steps in the leukocyte dependent microvascular dysfunction that is observed at the onset of inflammation. Although BAL protein concentrations were used as a measure of pulmonary vascular leakage, we did not perform a
neutrophil count in BAL fluid as a measure of neutrophil influx. However, previous studies on ALI secondary to inflammation have provided evidence to suggest a direct correlation between increased protein content and neutrophilia in BAL samples (Glauser et al, 1988; O’Donovan et al, 1995; Shields et al, 2003). The increased protein content of BAL seen following large volume RFA and CT appear to suggest not only pulmonary vascular leakage, but also neutrophil sequestration. Indeed previous studies following large volume RFA (Ng et al, 2006) and CT (Chapman et al, 2000) have demonstrated this histologically.

Further evidence of increased pulmonary vascular leakage comes from data on the wet/dry lung weight ratios. Once again, most significant were the data obtained from 33% CT and 66% CT and RFA, confirming earlier findings. Although not the most accurate way of measuring pulmonary oedema, this technique has been used as indicators of increased permeability (Fairman et al, 1987).
Chapter 8

8.1 Conclusions

8.1.1 Cytokine induction as a marker of systemic inflammation

8.1.2 Acute renal injury

8.1.3 Acute lung injury

8.2 Limitations and possible modifications to study

8.3 Future work
8.1. Conclusions

8.1.1. Cytokine induction as a marker of systemic inflammation

Serum quantification of the proinflammatory cytokines IL-1β and IL-6 in our animal models demonstrated that at small volumes of ablation, all modalities of treatment were well tolerated with mild to moderate induction of inflammatory cytokines. In most instances these rises were transient and settled within 24 hours of surgery. At 33% ablation, it appeared that cryotherapy resulted in the greatest induction of the cytokines, particularly IL-6, compared with the other modalities. The most obvious differences were seen in the large volume (66%) ablation groups. Here, both CT and RFA resulted in huge increases in both serum IL-1β and IL-6. The corresponding SIRS resulted in death of all animals in these 2 groups within 6 hours of surgery. In comparison, all animals undergoing 66% surgical resection or MTA appeared to tolerate the procedures well and survived to the 48 hour timepoint. In conclusion, large volume MTA and resection appear to produce the least proinflammatory cytokine response compared with CT and RFA.

8.1.2. Acute renal injury

Large volume CT and RFA appeared to result in the most significant acute renal injury as shown by the largest rises in the markers RBP and HSP70. Compared with MTA and
resection, these differences were statistically significant and again these findings tallied with the observed behaviour and survival of the animals, with all animals in the 66% CT and RFA groups dying within 6 hours of ablation.

8.1.3. Acute lung injury

All animals in the 66% CT and RFA groups died within 6 hours of surgery. There was evidence of tachypnoea, pulmonary oedema, increased HSP70 and capillary leakage in these groups, compared with MTA and resection. It appeared the ALI resulted from the overwhelming inflammatory response following these various ablations. MTA and resection at large volumes appeared to induce some lung injury, although not sufficient to cause clinical compromise.

8.2. Limitations and possible modifications to study

Comparisons at 2 separate time-points: For the 15% and 33% groups, all samples were collected were at similar times and we were therefore able to compare directly the differences between treatments at the same volumes (‘intra-volume’) and also between treatments at separate volumes (‘inter-volume’). This was possible as all animals survived to the 48 hour timepoint, at which time they were sacrificed. One criticism highlighted during the study was that tissue analysis in the 66% ablation groups were not comparable directly as samples were
obtained from animals in the control, resection and MTA groups at 48 hours, whilst samples from the RFA and CT groups were obtained and analysed at 6 hours when these animals died.

Clearly their death was an unexpected event and hence a decision had to be made as to whether we ought to change the experimental protocol so that all animals in all the treatment groups were sacrificed at 6 hours. Following consultation with the named veterinary surgeon, the research supervisor and the statistician, it was felt that the best course of action was to proceed with the experimental protocol as designed. The reasons were as follows:

1) As one animal from each treatment group was studied simultaneously i.e. each day, 1 control, 1 resection, 1 MTA, 1 CT and 1 RFA were studied, we would have needed more animals to ‘replace’ the ones from the control, resection and MTA groups that had survived to the 48 hour timepoint. This would have required a re-application to the Home Office animal licensing authority who would have rejected the use of even more animals.

2) The organ systems we were studying looked at injury following a presumed inflammatory response. The evidence from the literature suggested that once these acute injuries occurred, the evidence persisted for longer than 48 hours. Indeed, both MTA and resection of 66% induced lung and renal injury in samples taken at 48 hours, although as demonstrated, not as much as in the CT and RFA groups. Further, it is possible that samples at 6 hours (in the CT and RFA groups) picked up ‘evolving injury’ and not the peak. One can also argue that 48 hour sampling would have demonstrated greater injury as more time had passed, allowing the SIRS response to cause a greater insult. In effect, had the CT and RFA animals survived to 48 hours, it is
probable that the differences seen would have been even more significant. It is very possible, based on available evidence on generation of the SIRS response and its effect on the various organ systems, that there would be more systemic damage evident at 48 hours than at 6 hours. Notwithstanding all the above, the fact remains that there was no direct comparative data, and some of the conclusions drawn were based on an extrapolation of logical assumptions.

3) The advisors also held the view that in addition to the study of various markers of inflammation, the overall effect on the animal subjects was of crucial importance. The tolerance and survival data would be an important aspect of the study, particularly when related to the SIRS induced.

4) Further, the most active phase of cytokine induction (rather than any of the other markers studied) was the first 3-6 hours. Hence much of the cytokine analysis has been based on the first 6 hours of response and we are therefore able to perform direct comparisons of at least this facet of the study.

5) Finally, in future studies it would be ideal to perform a directly comparable experiment with sacrifice of all animals at 6 hours.

Systemic response: It is unclear why exactly TNF-α did not work with our samples. It may have been better to abandon its use early on in the study and use an alternative inflammatory marker, such as the anti-inflammatory cytokine IL-10 or even C-reactive protein. The problem with this was that the Home Office Act only allowed collection of 500 microlitres of blood at each sample time, which after component separation, left 250 microlitres. As each sample was analysed in duplicate, and there were already 3 cytokines being analysed, this left little room
for maneuver as we had already decided to study the 3 most reliable and established inflammatory markers. We had to use at least 3 samples from each group (including all the samples from the 66% CT and RFA groups) to look for TNF-α, in case the higher volume ablations did, in fact produce a measurable response.

Additionally, we could perhaps had tested for the presence of TNF-α at times less than 1 hour (the first post-operative sample) and after 48 hours (the last sample time). This would have excluded the possibility that this cytokine peaked too early and we ‘missed the boat’ at 1 hour, or that this cytokine was induced late. It must be said, however, that the literature strongly suggests neither of these two scenarios to be correct.

The kidneys: Although RBP is a very sensitive marker of acute renal injury, it does not give us specific information on renal function, something perhaps better described by changes in serum creatinine and urea. However, we know that the kidneys have a reasonable amount of ‘functional reserve’ before differences are seen following injury. Hence it was, in our opinion, better to use RBP as an early marker of renal injury and HSP70 as an early marker of inflammation. As such we were able to pick up even the slightest sign of injury, early. On current evidence, it seems β2-microglobulin is as sensitive as RBP, although the commercially available kit for it was not designed for rats at time of this research being performed. More recently of course, newer and even more sensitive markers of acute renal injury such as Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney injury molecule-1 (Kim-1) have been developed which would replace RBP.
Furthermore, as alluded to in the discussion in Chapter 6, that a theory behind what may have caused renal injury (in addition to pre-renal failure caused by SIRS-mediated hypotension) was the elevation of serum myoglobin and its excretion in the urine. It would have been possible to test this theory and indeed we had sufficient urine sample to test for the presence of this protein.

**The lungs:** Specimens were collected (but not included in this thesis) for histopathological study. This may have provided further evidence of the organ injury that may have resulted. Certainly, microscopic appearances of neutrophilia, oedema, haemorrhaging and lung parenchymal collapse have been demonstrated following hepatic cryoablation, following the development of the ‘cryoshock’ syndrome.

Although we were able to demonstrate increase in total protein content in BAL fluid, what we do not know is the nature of this. It would be presumptive to suggest this is entirely due to leakage of neutrophils out of the capillaries into alveoli. BAL fluid cellular constituents are often used to characterise the inflammatory cellular population in the lungs. A simple way to do this would have been to perform a differential cell count or use a computerised image analyser. A more objective way would be to measure actual myeloperoxidase activity (as a marker neutrophil activity).

**The liver:** One of the aims of this thesis was to investigate systemic - and by extrapolation - distant organ inflammation following hepatic resection or ablation. It would have been useful to
investigate what ‘local’ effects are within the liver itself. Liver function tests (itself requiring significant amounts of serum which we did not have), histology and HSP70 could all have been used to investigate the extent of local inflammation.

8.3. Future Work

1) The rat model: Although rats have been used extensively to study hepatic ablation and indeed inflammation following other insults, they are not necessarily entirely representative of how human subjects will respond. The experimental model may yield more reliable results if a larger model is used, such as a pig or sheep model. More serum samples can be collected which would allow for all the extra tests (urea, creatinine, liver function tests, CRP etc).

2) The normal liver: The entire experimental protocol is based on treatment of normal hepatic tissue. The assumption is, of course, that this will behave in much the same way as cancerous tissue, in terms of inflammatory response induced. There are conflicting data suggestive of tumour biology enhancing - and by the same measure - dampening the immune response following insult to the liver (see Chapter 2). There do exist larger animal hepatic tumour models and this may be one line of investigation. Furthermore, many patients with non-colorectal liver cancer have a background of hepatic cirrhosis, again perhaps resulting in different physiological responses to those observed in our study. Once again, large animal models with induced liver cirrhosis exist, which may not only corroborate our data, but also
give data on maximum safe ablation volume in the *diseased* liver. Some data addressing this exist with regards to resection and RFA but not CT and MTA.

3) **Survival:** We saw in our study that an exaggerated inflammatory response resulted in 100% mortality in the large volume (66%) RFA and CT groups. All animals in the other groups (66% resection and MTA) survived to 48 hours, and exhibited normal behaviour. These observational data were quite dramatic and provide strong support for our contentions. What we do not know, however, is what would have happened to the animals had we left them for longer (rather than cull them at 48 hours). The 48 hour timepoint was chosen as many previous studies suggested the acute inflammatory response occurred largely in the first 48 hours. Therefore urine, BAL and tissue samples taken at, say, one week may have missed the ‘peak of inflammation’. Notwithstanding this, a simple survival study following these ablations at increasing volumes would be a valuable contribution to the literature.

4) **Clinical Studies:** It would be useful to collect data on the effects of ablation in human subjects. It would be relatively easy to collect blood specimens postoperatively for cytokine analysis, as well as measuring renal, liver function and clotting profiles. The difficulty, however, would be two-fold. One is getting a large enough number of patients to stratify according to volume ablated, and the second would be the direct comparisons between treatment groups. An answer to this would be a multicentre study, although an immediate criticism would be the multiple operators and other variables this would bring in.
5) In practice, many ablation procedures are combined with surgical resection. A hemi-hepatectomy to remove the most tumour-burdened portion of the liver, together with MTA, CT or RFA of smaller tumours in the remaining portion of the liver are commonplace. Having seen that large volume resection appears to be relatively safe, is there a case for using this ‘dual modality’ approach to try and minimise any adverse systemic effect? Can we, for example, perform resection of 50% and CT/RFA of 15%? Is this safe? Is it safer than 66% CT or RFA? Or shall we just ablate with MTA which has been shown in this study to be safe in rats, even at large volumes of ablation.

6) Specifically with regards to MTA, is there a role for intra-ductal cooling to increase the application of MTA? It has been shown that RFA adjacent to major biliary structures may lead to stenosis. Tumours close to these structures therefore are often excluded from treatment. However, catheterisation of the bile duct and cooling with saline may allow some of the heat to be dissipated (the so-called ‘heat sink effect’) thus allowing tumour destruction whilst protecting the bile duct. There is some experimental data to support this. With regards to MTA, no study has looked at this, although my prediction is that the same level of protection will not be possible. This is because RFA is slow and relies mainly on conduction (one of its inherent disadvantages!), whereas MTA relies largely on the heat ‘field’ generated immediately and conduction to a much lesser extent. As such this amount of thermal energy may be too much to be ‘sunk away’. Nonetheless this would be a study worth conducting not least for adding to the literature.
7) Alternative study models: Due to ethical reasons, it would clearly be impractical to replicate this study in human subjects. It would be possible and perhaps desirable to perform this entire experimental study in a larger animal model, such as pigs, or sheep. These models are established animal models for studying effects of various operative procedures, including hepatic surgery. This would allow the quantification of many more parameters such as liver and renal function tests, a wider range of pro- and anti-inflammatory cytokines, coagulation profile etc. Moreover, there would also be capacity to monitor more accurately and invasively the clinical parameters associated with an inflammatory response. However, there are enormous cost implications involved with large animal work.
Bibliography


Ahmad F, Gravante GP, Bhardwaj N, Strickland AD, Basit R, West K, Sorge R, Dennison AR, Lloyd DM. Renal effects of hepatic microwave ablation compared to radiofrequency, cryotherapy and surgical resection at different volumes of liver treated. Liver Int 2010 Jun 1. [Epub ahead of print]


Awad MM, Devgan L, Kamel IR, Torbensen M, Choti MA. Microwave ablation in a hepatic porcine model: correlation of CT and histopathologic findings. HPB (Oxford) 2007;9:357-362


Bhardwaj N, Strickland AD, Ahmad F, West KP, Lloyd DM. A comparative histological evaluation of the ablations produced by microwave, cryotherapy and radiofrequency in the liver. Pathology 2009;41:168-172


Bischof JC, Rubinsky B. Large ice crystals in the nucleus of rapidly frozen liver cells. Cryobiology 1993;30:597–603


Bone RC. Towards a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. Crit Care Med 1996; 24: 163-172


Glasgow SC, Ramachandran S, Csontos KA, Jia J, Mohanakumar T, Chapman WC. Interleukin-1beta is prominent in the early pulmonary inflammatory response after hepatic injury. Surgery 2005 Jul;138:64-70


Hamad GG, Neifield JP. Biochemical, hematologic, and immunologic alterations following hepatic cryotherapy. Semin Surg Oncol 1998;14:122-128


Montcalt-Smith E, Caviness J, Chen Y, McCarron RM. Stress biomarkers in a rat model of decompression sickness. Aviat Space Environ Med 2007 Feb;78:87-93


Ng KK, Lam CM, Poon RT, Shek TW, Ho DW, Fan ST. Safety limit of large-volume hepatic radiofrequency ablation in a rat model. Arch Surg 2006 Mar;141:252-258


Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F 2nd, Park DR, Pugin J, Skerrett SJ, Hudson LD, Martin TR. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 2001 Nov 15;164:1896-1903


Ravikumar TS, Steele G Jr, Kane R, King V. Experimental and clinical observations on hepatic cryosurgery for colorectal metastases. Cancer Res 1991 Dec 1;51:6323-6327


Shibata T, Niinobu T, Ogata N et al. Microwave coagulation therapy for multiple hepatic metastases from colorectal carcinoma. Cancer 2000;89:276-284


Trof RJ, Di Maggio F, Leemreis J, Groeneveld AB. Biomarkers of acute renal injury and renal failure. Shock 2006 Sep;26:245-253


Welch WJ. How cells respond to stress. Sci Am 1993 May;268:56-64


Yang YJ, Chen SH, Ge XR. Role of interleukin-18 in the development of acute pulmonary injury induced by intestinal ischemia/reperfusion and its possible mechanism. J Gastroenterol Hepatol 2007 Feb;22:253-260
