# Epigallocatechin-3-gallate and recombinant human activated protein C and the modulation of acute pancreatitis

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# *ABBREVIATIONS*

| | |
|---|---|
| AP | Acute pancreatitis |
| APACHE | Acute physiology and chronic health evaluation |
| APTT | Activated partial thromboplastin time |
| AT III | Antithrombin III |
| Bax | Bcl-2 associated X Protein |
| BAD | Bcl-2-associated death promoter |
| BAK | Bcl-2 homologous antagonist killer |
| Bcl-xl | B-cell lymphoma-extra large |
| Bcl-2 | B-cell lymphoma 2 |
| BID | BH3 interacting domain death agonist |
| Ca / $Ca^{2+}$ | Calcium / calcium ions |
| CRP | C-reactive protein |
| $dH_2O$ | Distilled water |
| DIC | Disseminated intravascular coagulation |
| EDTA | Ethylenediaminetetraacetic acid |
| EGCG | Epigallocatechin-3-gallate |
| ELISA | Enzyme-linked immunosorbent assay |
| EPCR | Endothelial protein C receptor |
| ERCP | Endoscopic retrograde cholangiopancreatography |
| GTE | Green tea extracts |
| $H_2O_2$ | Hydrogen peroxide |
| ICAM-1 | Intercellular adhesion molecule -1 |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IκB | I kappa B protein |
| NaCl | Sodium chloride |
| NF-κB | Nuclear factor kappa B |
| MDA | Malondialdehyde |
| MPO | Myeloperoxidase |
| PAI | Plasminogen activator inhibitor |
| PAF | Platelet activating factor |
| PAR | Protease activated receptor |
| PARS | Poly(ADP-ribose) synthetase |
| PBS | Phosphate buffered saline |
| PT-INR | Prothrombin time - international normalised ratio |
| rhAPC | Recombinant human activated protein C |
| sTM | Soluble thrombomodulin |
| SIRS | Systemic inflammatory response syndrome |
| $t_{1/2\alpha}$ | Half life in plasma during initial rapid distribution phase |
| $t_{1/2\beta}$ | Half life in plasma during second slower distribution phase |
| TAFI | Thrombin-activatable fibrinolysis inhibitor |
| TGF-β | Tumour growth factor - beta |
| TNF-α | Tumour necrosis factor alpha |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| VEGF | Vascular endothelial growth factor |

# *Abstract*

Effective management of acute pancreatitis has for centuries eluded mankind. The disease has a wide spectrum of presentation; the milder form is usually a self limiting condition, whereas the severe form presents as a highly morbid and frequently lethal attack. The ability to predict disease progression on admission would aid in the comprehensive and multidisciplinary management of patients.

The perfect predictor of disease progression has been an elusive factor hindering the management of the disease. On systematically reviewing literature and identifying appropriate biochemical markers in predicting progression of acute pancreatitis, the ideal predictor would be a combination of biochemical, clinical and contemporary organ dysfunction scoring systems.

Early prediction of disease progression however, is important in the better management of the disease. The pathophysiological changes of acinar cell injury and death are the earliest events that occur in acute pancreatitis. Identification of potential pharmacological interventions offered through valuable insight in to experimental and clinical acute pancreatitis may lead on to the development of various natural and synthetic potential disease modifiers.

Green Tea Extracts (GTE) consumed in many parts of the world has been examined as a potential therapeutic medication. Experimental results have demonstrated the effect of GTE on the oxidative pathway significantly ameliorating the effects of pancreatic injury. The various green tea catechins especially Epigallocatechin-3- gallate (EGCG) can perhaps be useful lead compounds for new drug discovery.

With no specific targeted therapy for severe acute pancreatitis at present, various medications have been tested. The possibility of targeting initial acinar cell injury may not be a feasible option as patient presentation and management would usually be after this phase. As the disease progresses, severe acute pancreatitis is characterised by inflammation and necrosis. The hypothesis of preserving pancreatic parenchymal microvascular patency and thus ameliorating pancreatic injury through the early administration of recombinant human Activated Protein C (rhAPC) has identified a potential treatment for acute pancreatitis. rhAPC converted from its inactive precursor, protein C, by thrombin acts through fibrinolysis and inhibition of thrombosis. Studies on rhAPC in experimental acute pancreatitis examined the modulation of rhAPC on inflammatory markers, morphology, microvascular thrombosis and apoptosis. The encouraging results from initial experimental work helped set up the Phase 2 clinical trial of administering rhAPC early on in severe acute pancreatitis. Prior to taking this significant step from bench to bed side, the variation in functional protein C levels with the severity of the disease was examined as a precursor to the Phase 2 trial.

# *Declaration*

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

# *Copyright Statement*

# *Dedication*

This work is dedicated to my parents who have made me what I am.

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*"It's the journey, not the destination"*

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# 1 Introduction

## 1.1 A historical perspective of acute pancreatitis

### *1.1.1 Early concepts*

Acute pancreatitis has for centuries challenged scientific minds. History has presented various accounts of the disease over time, one of the early accounts of the disease described being the fatal illness of Alexander the Great 323BC.[1] The first clinical description of the disease was presented by the Dutch anatomist Nicholas Tulp in 1652.[2] Guy Patin (1601-1672), one of the most respected professors at the University of Paris, in 1652, mentions of the term "pancreatic abscess" in his "The magic disease" letter, the same year that Tulp described the disease.[3] 150 years after Tulp's description of the disease, another distinguished Professor of Medicine from Paris describes a patient with peri-pancreatic necrosis as "the pancreas to be immensely large and full of steatomatous concretions. It had coating of the consistency of suet about half an inch thick".[3]

Management of pancreatitis moved from a medically managed disease to the operative era when Werner Kőrte described it to the German Congress of Surgery in 1894, becoming" the father of operative treatment of acute pancreatitis".[3]

Kőrte's description of the indications and surgical technique were not far from that prevailing now. In the 21st century modern open necrosectomy can be performed without the procedure-related deterioration in organ dysfunction associated with major debridement with an emphasis on aggressive multidisciplinary care.[4] With advancement in technology minimally invasive procedures have evolved in managing complications of acute pancreatitis.[5]

### *1.1.2 Evolution of understanding of aetiology and pathophysiology of acute pancreatitis*

Despite various descriptive terminologies and management strategies, many questions still remain unanswered.

The aetiology of pancreatic inflammation has been the subject of intense speculation. Reginal H. Fitz in 1889, at the New York Pathological Society's Middleton-Goldsmith lecture, described the aetiology of pancreatitis to be gallstones, alcohol, perforating gastric ulcer, and trauma.[2] Seven years later, Chiari postulated the underlying pathophysiology of pancreatitis being auto digestion.[6]

In spite of over a century's work in to the mysteries of pancreatic enzyme activation, the key initiating factors and the subsequent mechanisms of glandular damage in acute pancreatitis are still poorly understood. Although controversial, most investigators now believe that acute pancreatitis is caused by an unregulated activation of trypsin within pancreatic acinar cells[7].

Since the first description of acute pancreatitis by Tulp, it is becoming clearer that there are two phases in acute pancreatitis, the early phase i.e. within the first week and the subsequent phase occurring after the first week of onset of the disease[8].

The first phase is essentially the body's systemic inflammatory response to tissue injury and consequent organ failure[7]. A dynamic evolution occurs with the initial stage of inflammation, pancreatic and peripancreatic ischaemia or oedema. The end result is either resolution or irreversible necrosis and liquefaction. This liquefaction could potentially lead on to a collection.

The second phase of the disease is characterised by either one of three outcomes:

1) Resolution of the disease

2) Stabilisation

3) Progression of the disease.

The first outcome of the disease is usually an oedematous pancreas with almost no necrosis which usually resolves with little further change[9]. In the second outcome the disease stabilises but does not normalise. The third outcome is characterised by a progressive and protracted disease period lasting for weeks to months with pathological changes happening at a very slow pace. Here morbidity and mortality are based on whether the necrosis becomes infected leading on to local and systemic infection.[8]

## 1.2 Clinical Acute Pancreatitis.

### *1.2.1 Classification of acute pancreatitis.*

In his landmark paper on acute pancreatitis, Reginald Huber Fitz in 1889 distinguished between the haemorrhagic, suppurative and the gangrenous forms of the disease thereby beginning the first attempt at classifying the disease.[2 10] In spite of the accumulating evidence on pancreatitis, it was not until the middle of the 20th century that an understanding of the differences between acute and chronic forms of the disease was appreciated.[2]

The classification incorporates pathological and morphological description of different local complications of the disease.

### *1.2.2 Atlanta Classification*

The variability in presentation and clinical course of the disease has plagued the study and management of acute pancreatitis since its original clinical description. Various classifications of the disease have emerged over the past five decades.[11] The 1992 Atlanta symposium provided a consensus agreement on a clinically based classification system for acute pancreatitis. The uniformity created by over 40 experts based on the data available, this classification has resulted in being used widely by the pancreatic community (pancreatic gastroenterologists, pancreatic surgeons and radiologists) improving the management of acute pancreatitis and clinical research relating to the condition.[12] Based on the Atlanta

classification, 80% of patients with acute pancreatitis have the milder form of the disease which resolves without serious morbidity.Being a dynamic and evolving disease, the severe form constitutes 20% and is complicated by a mortality of 10-25% despite improvements in critical care.[13]

Unfortunately, validation of the Atlanta classification over the years has not taken place and various terminologies proposed by the consensus have not been accepted or utilised by various clinicians around the world.[11] Since the Atlanta Classification the radiological input in assisting with the diagnosing and staging of the disease has improved. This has brought to the forefront the need for a morphological descriptive system for disease classification.

### *1.2.3 Revisiting the Atlanta Classification*

With increasing knowledge of the patho-biology of necrotizing pancreatitis, improved imaging modalities for the study of the disease and its complications and the development of minimally invasive techniques for its management have made it necessary to revisit the Atlanta classification. The acceptance that acute pancreatitis is a dynamic and evolving process, various issues pertaining to the disease that needed attention were 1) clinical severity assessment 2) terminology in distinguishing degrees of fluid and necrotic areas in the pancreatic and peri pancreatic regions.[8]

### *1.2.4 Prediction of severity, necrosis, organ failure and death*

Early prediction of disease severity would aid clinicians in planning and aggressively managing acute pancreatitis. Much effort has been placed in identifying objective predictive markers for the course of the disease. The 1992 Atlanta classification transformed the approach by which acute pancreatitis is categorised, with more emphasis now being focused on severe acute pancreatitis accounting for 30% of the patients presenting with the disease.[12]

Based on objective clinical and laboratory data, various scoring systems were developed due to the failure of clinical assessment alone, in predicting pancreatic severity.

Although both the Ranson's and Glasgow's prognostic scoring system have been present for some time an inherent flaw is that they are based on data collected over a 24-48 hour time period which may be a missed opportunity in the critical management of these patients.

The meta-analysis of 19 studies involving prediction of severity and the 10 studies on prognosis, have demonstrate Ranson's signs to have poor discriminant power for both predictions.[14]

The APACHE II scoring system although better at predicting severity than the other scoring systems on admission only has 61% sensitivity in predicting a severe attack of acute pancreatitis on admission.[15]

The quest for the perfect biochemical marker in predicting the progression of acute pancreatitis on admission with its wide spectrum of presentation has been elusive. Most of the printed literature deals with an array of markers which the respective authors believe would be the ideal marker, but unfortunately translation to clinical practice has been hindered for a variety of reasons.

The various markers that have been researched in the last ten years have been categorised by grouping them in to four major categories based on the endpoints that the authors have used.

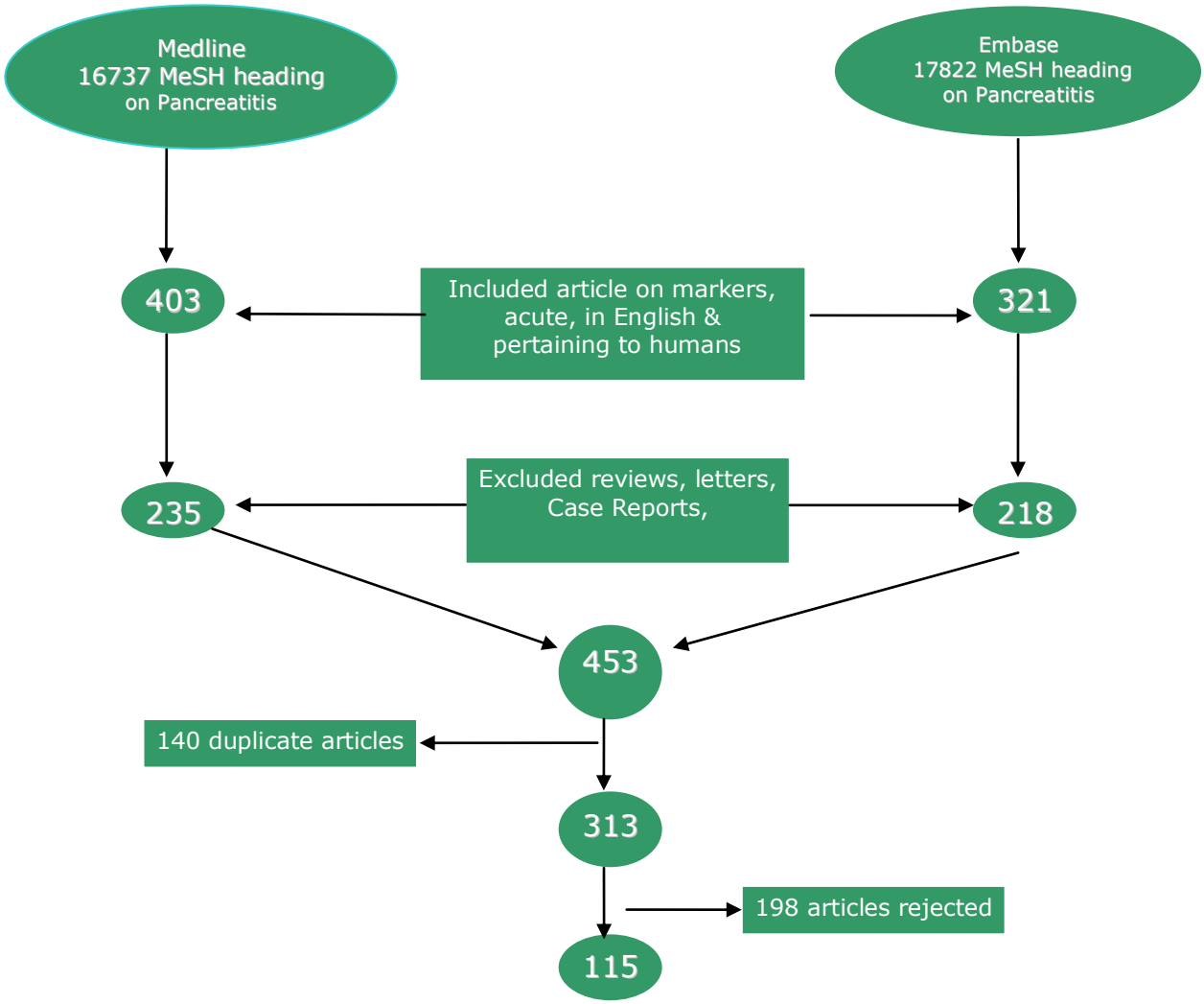
The broad classification that the author used was based on the admission predictability of the marker for severity, necrosis, organ failure and death.

A systematic review on the available literature from January 1995 – June 2007 from the databases EMBASE and Medline using the search engine ADITUS (NHS North West Health Care Libraries Unit, Wigan, UK) was performed. The Medical Subject Heading (MeSH) "Pancreatitis " with thesaurus mapping yielded 16737 articles from Medline database and 17822 articles on EMBASE. With assistance of Boolean operators, subheadings used were " acute " and "markers" in both the databases.

Articles in English and pertaining to humans were only included.

Reviews, letters, case reports and neoplasm were excluded using Boolean Operators.

The MeSH Heading in combination with keyword “markers” generated 1185 hits. On combining this result with another keyword “Acute” resulted in 657 articles. On combining these articles with the above mentioned inclusion and exclusion criteria yielded 235 articles
A similar search when performed on EMBASE with keyword “Markers “generated 849 hits. This result when combined with keyword “Acute” produced a list of 434 articles
This was then combined with the above mentioned inclusion and exclusion criteria yielded 218 articles. The total number of articles from both Medline and EMBASE was 453
There were 140 articles that was duplicated in both the databases which was removed resulting in a total of 313 articles.These articles were downloaded and analysed. A total of 198 articles were rejected on examining the abstracts and finding them to be irrelevant to the study. These articles were abstracts presented at meetings or symposiums, news letters, reports, grey literature, not related to study and some duplicates which were missed on initial computerised extraction of duplicates.These exclusions produced a final study population of 115 non duplicated relevant articles.




*Flow Chart on selection process*

### 1.2.4.1 Predictive marker on admission for severity

Early detection of patients with the potential to develop severe acute pancreatitis is pertinent in current practice as it allows treatment to be delivered in an appropriate “High-Dependency” environment with invasive monitoring.

Although a host of biochemical markers have been evaluated as indicators of severity, as yet, no single individual “gold-standard” marker has been established

Various markers have been proposed, for example non-specific proteins like C Reactive Protein (CRP)> 150mg/l which is widely used, being an inexpensive marker and easy to perform. It has a positive predictive value ranging from 50%-75% on admission. Its sensitivity on admission ranges between 50%-85%. The sensitivity and specificity improves

by 48- 72 hours of onset of pain.[16 17] The markers that have the highest sensitivity on admission for predicting severe acute pancreatitis are Urinary Trypsinogen Activation Peptide[18 19] Serum IL 18[20], plasma malondialdehyde[21] and Resistin.[22]

| Paper | | Marker | Sensitivity |
|---|---|---|---|
| Khan Z | 2002[18] | Urinary trypsinogen activation peptide> 35 nmol/l | 100% |
| Schaffler A | 2007[21] | Resistin | 93.3% |
| Abu HM | 2006[20] | Plasma Malondialdehyde | 93.3% |
| Kylänpää- BM | 2000[19] | Urinary trypsinogen 2>50µg/l | 91.0% |

*Table 1 Markers of severity on admission.*

Markers that show a very high sensitivity within 48 hours of admission are soluble receptors of TNF- α[22] and serum procarboxypeptidase B[23] with more than 90% sensitivity.

| Paper | | Marker | Sensitivity |
|---|---|---|---|
| Heresbach-D | 1998[22] | Soluble receptor for TNF- α | 100% |
| Muller C A | 2002[23] | serum procarboxypeptidase B | 95% |

*Table 2: Markers of severity at 48 hours*

### 1.2.4.2 Predictive marker at 48 hours for necrosis

There is a 30-40% chance of infection occurring in patients with more than 30% of necrosis of the pancreas[24]. 80% of deaths in acute pancreatitis are a result of infection[24].Thus a marker for pancreatic necrosis is pertinent in the management of severe acute pancreatitis. There are not many markers available that would detect pancreatic necrosis at admission. Soluble receptor of tumour necrosis factor Receptor 1, although not a specific marker has a high sensitivity, positive predictive value and area under the curve at admission.[25]
Muller *et al* have demonstrated that immunoreactive carboxypeptidase B activation peptide has a very high sensitivity, specificity and area under the curve.[23] Serum Procalcitonin at 48 hours has been shown to have a higher sensitivity compared to the other markers.[26-28]

### 1.2.4.3 Predictive marker for organ failure

Organ dysfunction can range from a mild impairment to established organ failure. Early mortality is often due to multi organ failure and sepsis supervenes at a later stage. Early

identification of patients “at risk” of developing organ failure would allow efficient allocation of resources.

Remes- Troche *et al* have showed that haematocrit on admission has a high negative predictive value in predicting organ failure.[29]

Interleukins have been proposed to be a predicted marker for organ failure. IL10 and IL 6 have been reported as good predictors of organ failure. [24] Development of organ failure in patients with acute pancreatitis can be predicted within 12 hours of admission with higher diagnostic accuracy by using a combination of IL-10 and calcium than by a single laboratory marker or clinical scoring system.[24] IL-10 reflects the initial activation of systemic inflammation that ultimately leads on to organ failure.

Severe hypoxemia ($P_aO_2$ <60mmHg) on admission is an early, easy-to- perform, predictive marker of pulmonary complications.[30] Procalcitonin has a sensitivity of 86% and a specificity of 92% in assessing patients with acute pancreatitis with severity of associated systemic complications.[27] IL-8 has been shown to have a strong correlation with presence of multiple organ failure, indicated by a constant rise especially during the second week of the disease. Thus, IL-8 provides information during the later stages of acute pancreatitis.[27]

#### 1.2.4.4 Predictive marker for death

Not many researchers have identified a biochemical marker for predicting death.

At present there is no single laboratory indicator predicting the potential lethal course of acute pancreatitis. In the last ten years, only three papers have looked in to this aspect.

Mantke *et al* proposed the use of plasma soluble thrombomodulin (sTM) in predicting lethality in patients with acute pancreatitis. sTM plasma levels (cut-off level, 75ng/ml) have shown a sensitivity of 100% and a specificity of 77% as early as the third day in identifying a potentially lethal course of acute pancreatitis.[31]

Serum Amyloid A and C-reactive protein have been studied in predicting death in patients with C reactive protein being a better predictor in the first week of acute pancreatitis.[32]

Procalcitonin has been shown to be raised in predicting death with a sensitivity of 100% and a specificity of 92%.[27]

### *1.2.5 Conclusion*

The diagnosis and treatment of acute pancreatitis continues to evolve especially with the focus on the severe phase of the disease. The gamut of markers can only help if they can be performed locally and if the results are available in a timely manner. Although various biomarkers have been suggested no single biomarker to date would be able to accurately predict the disease course. A combination of biomarkers along with clinical evaluation and critical care scoring systems would be the way forward in predicting the progression of the disease.

## 1.3 Pathophysiological cascade in acute pancreatitis

The pathophysiological cascade of injury resulting in acute pancreatitis is still being debated. Although a disparate list of causes exists, once initiated the disease usually displays a similar mechanism of injury but with marked variations in inflammatory response and recruitment of other cell types. The effects culminate in inflammation, oedema, necrosis and haemorrhage of the pancreas and surrounding structures.[7 13]

### *1.3.1 Acinar Cell Injury*

The acinar cells of the pancreas are the primary target of injury from alcohol metabolites, bile, hyperlipidaemia, hypercalcaemia, drugs and viral infection.[33] Advances in powerful imaging modalities (e.g. 2-photon confocal microscopy), and innovative fluorescent tracers have provided an in depth knowledge of the physiological and pathological response to stimuli occurring in these acinar cell. Experimental and clinical evidence supports the widely accepted theory of injury to the pancreatic acini resulting in the inappropriate activation of pancreatic enzymes.[33]

The influence of the initiating factors of the disease results in the disruption of the fine balance maintained in the acinar cells. The protective mechanisms that maintains this balance include (1) inhibiting the activation of inactive pro-enzymes or zymogens (eg. trypsinogen, chymotrypsinogen, procarboxypeptidase and pro-elastase), (2) packaging of the zymogens in a condensed form bound to calcium ions, within vesicles (3) the serine protease inhibitor, Kazal type 1 (SPINK1), aiding in the inhibition of enzyme activity (4) any enzyme that gets activated can be de-activated by either through autolysis or the actions of mesotrypsin.[34]

The noxious stimulation of the pancreatic acini upsets the intricate balance resulting initially in an abnormal, sustained release of calcium from the endoplasmic reticulum in to the cytosol initiating intracellular zymogen activation, vacuolisation, cytoskeletal damage and other features characteristic of changes observed in experimental acute pancreatitis.[33] This

leads on to premature, intracellular activation of trypsinogen and other digestive enzymes. In contrast to the apical secretion of zymogen in to the ductal lumen found during the physiological functioning of the acinar cells, there are misdirected basolateral secretions of enzymes in to the interstitial spaces in acute pancreatitis.[9] The effectiveness of the microcirculation in clearing zymogens and/or activating enzymes in the interstitial space could be compromised during pancreatitis and thereby modify their injurious effects.[9]

### *1.3.2 Inflammatory effect*

An important characteristic feature of acute pancreatitis is the associated inflammation. Cytokines, released from tissue macrophages, play a major role in the initiation and progression of the inflammation induced by a "cytokine storm".[35 36] This misdirected effect of the cytokines to normal tissue and its persistence results in systemic complications of acute pancreatitis.[36] Thus the degree of cytokine elevation correlates to the severity and morbidity of the disease.[37] These pro-inflammatory cytokines such as Tumour necrosis alpha and pro-inflammatory interleukins are in turn regulated by pleiotropic transcription factors such as NF- κB.[37 38]

NF- κB is a family of proteins that binds to DNA and is responsible for the activation of a multitude of cellular stress related and early response genes. Their activation results in increased levels of cytokine response, adhesion molecules and acute phase proteins.[37]The cytoplasmic activation of NF- κB proteins is controlled by the inhibitory effect of the IκB family of proteins, masking the nuclear localising signal of NF- κB.[39] Once activated the IκB group of proteins become degraded in to proteosomes allowing NF-κB translocation to the nucleus and activating the transcription process.[39]

Several antioxidants have been known to inhibit the up regulation or binding of this group of proteins preventing the NF- κB transcriptional activation.[40 41] The possibility of potential therapeutic intervention at controlling the transcription factors may influence the release of pro-inflammatory cytokines decreasing the severity of the disease.

### *1.3.3 Mechanisms of cell death in acute pancreatitis*

Although mechanisms of cell death in acute pancreatitis remain unclear, it is believed that the two main types of cell death are apoptosis and necrosis. The severity of the experimental form of the disease is known to directly correlate to the necrosis and inversely to apoptosis.[42]

#### 1.3.3.1 Necrosis and acute pancreatitis.

Necrosis is characterised by swelling of organelles and plasma membrane rupture resulting in the damage to surrounding cells promoting an inflammatory reaction in the pancreas making it the "more dangerous" form of cell death.[42] Although previously considered as a "passive process" as a consequence of acute ATP depletion, necrosis is now known to have an "active component" as well. The cytoplasmic swelling caused due to influx of sodium, calcium and water resulting in membrane rupture and nuclear pyknosis eventually resulting in the release of lysosomal and inflammatory granules.[43] This release of the cellular contents leads on to subsequent inflammation of the surrounding tissue.

#### 1.3.3.2 Apoptosis and acute pancreatitis.

Apoptosis is programmed cell death manifested by cell and chromatin shrinkage, caspase activation, DNA fragmentation and formation of membrane bound apoptotic bodies.[35 44] The maintenance of structural integrity of the membrane differentiates it from necrosis and thereby decreasing the severity of the disease.

The influential role of apoptosis in acute pancreatitis has been examined involving non compartmentalised specific enzymes cysteine proteases (caspases) held under tight control for the normal survival of healthy cells. The activated cellular caspases exercise their effect through two separate pathways, the intrinsic and extrinsic pathways.[42 45 46] The extrinsic pathway is usually triggered by inflammatory cytokines like TNF- α. Caspase -8 is the key initiator of death receptor mediated apoptosis in the extrinsic pathway, an early regulator of the proteolytic cascade.[7] Of the intrinsic mitochondrial pathway Caspase 9 is regarded as

the “canonical Caspase”. The main downstream effector Caspase responsible for cleaving the majority of cellular substrates in apoptotic cells is Caspase 3.[47]

In addition to the caspase family of enzymes the Bcl-2 family of apoptotic regulators are an important functional component in the apoptotic pathway. The balance of the pro and anti-apoptotic genes of the Bcl-2 family of genes determine the fate of the cell. Of the Bcl-2 family, Bax, BID, BAK and BAD are pro- apoptotic whereas Bcl-xl and Bcl-2 are considered as anti-apoptotic.[7]

### *1.3.4 Microvascular Injury*

The blood supply to the pancreas is through a rich plexus of arteries derived from the celiac trunk and superior mesenteric arteries. Over the past few decades, applied basic science research has demonstrated that the architecture of the pancreatic microvasculature is of an “end artery” type. The pancreatic lobule is supplied by a single tree-like intralobular arteriole with no anastomosis to adjacent intralobular arterioles[48] supplying the islets prior to vascularising the acini forming the ‘insulo-acinar portal system’.[49] This anatomical arrangement raises the potential problem of pancreatic lobules being susceptible to embolus, thrombus, ischemic injury, spasm of the intralobular arterioles or compression due to interstitial oedema.[48] The pancreas has auto-regulatory mechanisms such as physiological shunts, hormonal and neural mechanisms that control the flow of blood. The exocrine hormones cholecystokinin, somatostatin and secretin exert a tight control on the blood flow to the normal gland.

Experimental and clinical evidence suggests that various microvascular changes are responsible for progression of the disease during early stages of acute pancreatitis.[48 50] This is considered as a critical event in the development of severe acute pancreatitis.[49]

Progression of disease severity from oedematous to haemorrhagic pancreatitis and necrosis is proportional to the functional microcirculatory dysfunction as assessed by invivo functional microscopy and functional capillary density.[49] This being an important aspect, as no

correlation exists between total pancreatic blood flow to regional microcirculatory flow through the pancreas.[51].

### *1.3.5 Coagulation disturbances*

Recent evidence in experimental research has suggested that derangement in the coagulation cascade is a key component in disease progression.[49] Radenkovic and colleagues have shown noticeable changes in coagulation profiles with demonstrable variations in the levels of Protein C and anti-thrombin, d-dimer and PAI -1 level indicating an exhaustion of fibrinolysis and coagulation inhibitors in patients with poor outcome during the course of the disease.[52]

Protein C is a 60 kilo Dalton, vitamin K-dependent, anticoagulant protein playing an integral role in haemostatic regulation.[53] The Protein C anticoagulant pathway is involved in the control of thrombosis.[54] Thrombin, thrombomodulin, the endothelial cell protein C receptor (EPCR), protein C, and protein S are the essential components of the protein C pathway. The activation of protein C is enhanced dramatically when protein C is bound to the EPCR.[54] [55] Activated protein C has antithrombotic activity through its inactivation of coagulation Factors Va and VIIIa, limiting thrombin generation and profibrinolytic activity.[56] The activated form of the protein (activated protein C), is not only an anticoagulant but also has an important link between coagulation, inflammation, apoptosis and vascular permeability.[57] Lindstrom and colleagues examined the interaction between protein C and activated protein C in 31 patients with multiple organ failure as a result of severe acute pancreatitis.[58] Their study demonstrated wide intra-patient variations in protein C levels at various stages of the disease. Activated protein C levels were found to be lower in patients with severe disease associated with multiple organ failure.[58] These findings raise the prospect of the use of recombinant Human Activated Protein C (rhAPC) early in the disease course of acute pancreatitis.

## 1.4 Recombinant human activated protein C

### *1.4.1 Introduction*

Recombinant human activated protein C (rhAPC), (Drotrecogin Alfa, Eli Lilly, Indianapolis, Indiana, USA) has been approved in many countries for the reduction of morbidity and mortality in adult patients with sepsis. Regulatory authorities have concentrated on the use of this drug in patients with a high risk of death (i.e. Acute Physiological and Chronic Health Evaluation (APACHE) II score ≥ 25).

### *1.4.2 Physical and chemical properties*

rhAPC is a 55kD, 2-chain glycoprotein analogue of vitamin K-dependent endogenous protein C synthesised from genetically engineered human cells.[59] It is homologous to plasma-derived human activated protein C. rhAPC, expressed from the human kidney cell line HEK293, is secreted from the cells and purified by column chromatography resulting in a highly purified form of the drug substance.

### *1.4.3 Pharmacokinetics and pharmacodynamics*

#### 1.4.3.1 Rodents:

Based on available data on the pharmacokinetics in rodents, it was found that the clearance of rhAPC involved three processes 1) inhibition of enzymatic activity of activated protein C by endogenous protease inhibitors 2) clearance by liver and kidney 3) degradation of activated protein C by tissue proteases. The $t_{1/2\alpha}$ which is the half life in plasma during the initial rapid distribution phase is between 3.0 and 3.6 minutes for dose of 0.1& 0.5 mg /kg . The $t_{1/2\beta}$ which is the half life in plasma during the second slower distribution phase is 1.4 & 0.8 hours for dose of 0.1& 0.5 mg /kg.[60-62]

#### 1.4.3.2 Humans:

The pharmacokinetic and pharmacodynamics relationships of rhAPC have been investigated extensively in healthy adults and in patients with severe sepsis.

The elimination of the drug is biphasic with a rapid initial phase ($t_{1/2\alpha}$ = 13 minutes) and a slower second phase ($t_{1/2\beta}$ = 1.6 hours), and 90% steady state plasma concentration is achieved within 2 hours of starting a constant infusion and 90% is eliminated within 2 hours of stopping the infusion. The plasma clearance is lower and steady state plasma concentration is higher in healthy subjects compared to patients with sepsis.[63]

### *1.4.4 Mechanistic studies.*

#### 1.4.4.1 Antithrombotic effect

Through the anticoagulant pathway this vitamin K dependent serine protease plays an important role in maintenance of haemostasis inhibiting thrombin generation and stimulation of fibrinolysis.[59] Various animal models were used in studying the antithrombotic effect of rhAPC. The two main primate models that best demonstrated the antithrombotic activity of rhAPC decreasing at equivalent doses were in the rhesus monkey and baboon models.[64 65] In humans, Dhainaut *et al* demonstrated antithrombotic effect of rhAPC in patients with severe sepsis evidenced by the reduction in D-dimer levels and markers of thrombin generation[66] but these significant changes were not noted in a human model of low- dose endotoxemia.[67]

#### 1.4.4.2 Profibrinolytic effect

In vitro studies have demonstrated that two components are thought to exert a profibrinolytic effect of rhAPC: 1) through the inhibition of plasminogen activator inhibitor-1 (PAI-1)[68] 2) the indirect inhibition of activation of procarboxypeptidase B.[69] Although significant pro-fibrinolytic effects of rhAPC have been demonstrated in *in-vitro* studies, no convincing

changes were demonstrable with PAI-1 levels or procarboxypeptidase B levels in patients with severe sepsis compared with placebo – treated patients.[66]

#### 1.4.4.3 Anti-inflammatory and antiapoptotic effects.

rhAPC has been demonstrated to have an inhibitory effect on chemotaxis of neutrophils, monocytes and eosinophils in humans.[70] Similarly Nick *et al* in 2004 demonstrated significant decrease in infiltration of leukocytes and neutrophils in to bronchoalveolar spaces compared with placebo subjects.[71]

Dhainaut et al demonstrated that no significant reduction of inflammatory cytokines TNF-α, Interleukins β, 8 and 10 were notice in patients treated with rhAPC. [66]

Transcript profiling experiments by Joyce et al in 2001 demonstrated the anti-inflammatory effects of rhAPC via suppression of the NF-κB pathway and the apparent ability to modulate genes in the apoptosis pathway.[72]

### *1.4.5 Safety concerns:*

Being a coagulation modifying medication, there is a potential for bleeding associated with the administration of rhAPC. Experimental studies have demonstrated that the main safety concern was bleeding, associated with surgical procedures[73 74] or when administered in combination with heparin. A synergistic effect and prolongation of APTT was noted when heparin was combined with rhAPC.[73 75]

Various clinical studies have demonstrated that the increased risk of bleeding is directly related to drug infusion period which is consistent with the short half life of rhAPC[74]. Thus bleeding being the most common serious adverse effect of rhAPC, careful evaluation and anticipated benefits have to be weighed against potential risks.

# 2 Objectives

## 2.1 Green Tea Polyphenol extract ameliorates pancreatic injury in cerulein induced murine acute pancreatitis.

Green tea polyphenol extracts are naturally occurring antioxidants acting through pathways that include reactive oxygen species and NF-κB. This study investigates the effect of green tea extracts in a cerulein-induced murine model of acute pancreatitis (AP).

## 2.2 Recombinant human activated protein C (Xigris) attenuates murine cerulein-induced acute pancreatitis via regulation of NF-κB and apoptotic pathways.

The aim of this study is to undertake a detailed evaluation of the mechanisms of action of rhAPC in a well validated experimental model of acute pancreatitis with specific reference to the differential roles of modulation of apoptotic and NF- κB pathways and down regulation of inflammation. This study is a necessary pre-requisite for the evaluation of rhAPC as a specific treatment for acute pancreatitis.

## 2.3 Recombinant human activated protein C modulates the sub-cellular expression of Caspases in L-Arginine induced Acute Pancreatitis

Utilising immunohistochemistry and image analysis of rat pancreatic tissue, our study aims at identifying and quantifying for the first time, the effect of rhAPC on the expression of Caspase 8, 9 and 3 in an L-Arginine induced acute pancreatitis model.

## 2.4 Functional Protein C levels during the early phase of clinical acute pancreatitis.

In experimental acute pancreatitis, our group and others have demonstrated that recombinant human activated protein C attenuates disease severity.[76-78] However little is known about endogenous protein C levels early in the disease course of human acute pancreatitis. This information is an important and necessary precursor to any potential therapeutic intervention study using recombinant human activated protein C. The aim of the present study is to assess whether there is depletion of functional protein C levels early in acute pancreatitis.

## 2.5 Setting up of Phase II study on "A preliminary evaluation of the safety profile of twenty four hour infusion of Recombinant Human activated protein C (Xigris) early in severe acute pancreatitis".

The present study represents a logical progression of our current programme of work (kindly supported by Eli Lilly) and takes the evaluation of recombinant human activated protein C to clinical severe acute pancreatitis. This preliminary clinical assessment has a strong focus on safety ensuring that there is rigorous monitoring for adverse effects, in particular, for treatment-related haemorrhage. There is insufficient evidence of likely benefit from intervention to justify randomisation and more importantly, there is a critical need for safety data.The study is a cohort study: 30 consecutive patients with severe acute pancreatitis (APACHE II ≥ 15, within 24 hours of admission to hospital) will be treated with a 24-hour infusion of recombinant human activated protein C (24 μg/kg/hr for 24 hours by intravenous infusion). Endpoints to be assessed include biochemical and radiological markers of inflammation and rheological indices of haemorrhage. Outcome will also be compared to

results in an age, gender and severity matched historical cohort of patients with acute pancreatitis.

# 3 Epigallocatechin-3-gallate (Green tea polyphenol extract) ameliorates pancreatic injury in cerulein induced murine acute pancreatitis.

## 3.1 Introduction

With a known incidence of 4:10,000, acute pancreatitis (AP) has a wide spectrum of presentation ranging from the mild, self-limiting form in approximately 70% to 80% of episodes to a severe illness characterized by necrosis of the pancreatic parenchyma.[79] Current paradigms of the pathophysiology of acinar cell injury in AP suggest that synergy between pro-inflammatory cytokines and oxidative stress triggers common signal transduction pathways converging on nuclear factor kappa B (NF-κB).[80] Along with its crucial role in promoting inflammation, NF-κB has been also shown to regulate the fine balance between apoptosis and necrosis and the progression of pancreatic injury.[81] There are still no therapies for AP, and treatment remains largely supportive, although many strategies targeting key molecules of the inflammatory cascade have been evaluated. Oxidative stress and antioxidant therapy, in particular, have been the focus of several experimental and clinical studies. Despite promising results in the experimental setting, [82] a recent randomized, double-blind, placebo-controlled trial of intravenous antioxidant (n-acetylcysteine, selenium, and vitamin C) therapy provided no evidence of benefit in patients with severe AP.[83] Much effort is being spent toward finding novel artificial and natural compounds to target both inflammation and oxidative stress through NF-κB down-regulation, and in this regard, polyphenol-rich extracts may play an important role. Green tea (Camellia sinensis) is a bioflavonoid polyphenol rich extract that has been demonstrated to have anti-inflammatory, antioxidant, and antimicrobial properties in various human, animal, and in vitro studies.[84] The main polyphenols or catechins involved are epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG).[85] The galloyl moieties, mainly present in EGCG and epicatechin-3-gallate, along with the 2 triphenolic groups present in

the chemical structure of EGCG may be responsible for the potent antioxidant properties of green tea.[86] In vitro studies demonstrate that Green tea extracts (GTE) dramatically inhibits chemokine-induced neutrophil chemotaxis and induces apoptosis and cell cycle arrest in a wide array of cell lines.[87] Furthermore, it has been shown in animal models that GTE possesses potent anti-inflammatory and antioxidant properties in zymosan-induced nonseptic shock, in experimental colitis, in carrageenan-induced pleurisy, and in ischaemia/reperfusion injury of the gut.[84 85 88 89] Finally, ingestion of green tea significantly increased human plasma antioxidant capacity *in vivo*[89] and diminished cytokine gene expression in human mast cells.[90] To obtain a better insight into the mechanisms of action of GTE, we examined its effects on pancreatic injury, inflammatory cascade, oxidative stress, NF-κB activation, and apoptosis induction in a well-characterized murine model of AP.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Materials*

All compounds were obtained from Sigma-Aldrich Company (Milan, Italy) unless otherwise stated. Primary monoclonal intercellular adhesion molecule 1 (ICAM-1) antibodies (CD54) for immunohistochemistry were purchased from Pharmingen. Reagents and secondary and nonspecific immunoglobulin G (IgG) antibody for immunohistochemical analysis were obtained from Vector Laboratories, Inc. All the chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd, Thetford, Norfolk, United Kingdom).

### *3.2.2 Animals*

Male CD-1® mice between 4 and 5 weeks old with a median weight of 37.7 g (range, 33 -41 g) were purchased from Jackson Laboratories (Charles River, Italy). The study was approved by the University of Messina Review Board for the care of animals. Animals were housed in a controlled environment and provided with standard rodent chow and water ad libitum.

Animal care was in compliance with regulations in Italy (DM 116192), Europe (OJ of EC L 358/1, December 18, 1986), and the United States (Animal Welfare Assurance No. A5594-01, Department of Health and Human Services).

### *3.2.3 Green Tea Extract*

The GTE was a kind gift of Indena (Milan, Italy), and it was defined by the producer as a caffeine-free extract from green tea leaves. The content of GTE was investigated by high performance liquid chromatography and is characterized by a high content (75 ± 5%) of polyphenols, the major constituent being EGCG at 62% and the minor ones being epicatechin-3- gallate, epigallocatechin, and epicatechin (patent application WO 96/28178). Various experiments have been carried out with green tea extracts by Professor Cuzzocrea at the University of Messina. The appropriate dose of 25mg/kg body weight was based on a dose response study and evidence from literature[84].

### *3.2.4 Study Design*

Mice were randomly allocated into the following experimental groups (n = 20 for each group). This standard number of animals was used in this laboratory for interventional protocols to minimise sampling error:

#### **3.2.4.1** Cerulein + saline group**,**

CD mice were treated hourly (x6) with intraperitoneal administration of cerulein (50 µg/kg, suspended in saline solution);

#### 3.2.4.2 Cerulein + GTE group,

Identical to group 1, except for the administration of GTE (25 mg/kg, intraperitoneally) at 1, 3, and 6 hours after the induction of pancreatitis;

#### 3.2.4.3 Sham + Vehicle group,

Sham-treated group in which identical treatments to the cerulein group were performed, except that the saline was administered instead of cerulein;

#### 3.2.4.4 Sham + GTE group,

Identical to the sham + vehicle group, except for the use of GTE.

The mice were sacrificed at 24 hours after induction of pancreatitis under ether anaesthesia. Although acute pancreatitis develops rapidly in rodents therapy may not be started earlier than 6 hours after disease onset. This corresponds to a delay of 24-48 hours after the start of symptoms in human pancreatitis.[91] The pancreatic lobules become insensitive to invitro effects of cerulein after 24 hours.[92]

Blood samples were obtained by direct intracardiac puncture. Pancreata were harvested immediately, frozen in liquid nitrogen, and stored at -80°C until assayed. Portions of this organ were also fixed in formaldehyde for histopathology and immunohistochemistry.

### *3.2.5 Morphological Examination*

Haematoxylin/eosin staining was performed on 5-µm sections from the paraffin-embedded pancreatic samples. The sections were then examined by an experienced histopathologist blinded to the identity of the samples. Acinar cell injury/necrosis was quantified by morphometry as previously described.[93] Ten randomly selected microscopic fields (x125) were examined for each tissue sample, and the extent of acinar cell injury/necrosis was expressed as the percent of the total acinar tissue. The criteria for injury/necrosis were the following:

(1) The presence of acinar cell ghosts or

(2) Vacuolization and swelling of acinar cells and destruction of the architecture of whole or parts of the acini, both of which had to be associated with an inflammatory reaction.

### *3.2.6 Determination of Pancreatic Oedema*

The extent of pancreatic oedema was assayed by measuring tissue water content. A freshly obtained blotted sample of pancreas was weighed on aluminium foil, dried for 12 hours at 95°C, and reweighed. The difference between wet and dry tissue weights was calculated and expressed as a percent of tissue wet weight.

### *3.2.7 Biochemical Assays*

Serum amylase and lipase levels were measured at 24 hours after the induction of pancreatitis by a veterinary laboratory. Results are expressed in international units per litre.

### *3.2.8 Sub cellular Fractionation and Western Blot Analysis for IκB-α, Phospho-NF-κB p65 (Serine 536), NF-κB p65 (Nuclear), Bax, and Bcl-2*

Cytosol and nuclear extracts were prepared as previously described with slight modifications. [94] Briefly, pancreas tissues from each mouse were suspended in extraction buffer A containing 0.2 mmol/L of phenylmethanesulphonylfluoride, 0.15 µmol/L of pepstatin A, 20 µmol/L of leupeptin, and 1 mmol/L of sodium orthovanadate, homogenized at the highest setting for 2 minutes and centrifuged at 1000g for 10 minutes at 4ºC. Supernatants represented the cytosol fraction. The pellets, containing enriched nuclei, were re-suspended in buffer B containing 1% Triton X-100, 150 mmol/L of NaCl, 10 mmol/L of Tris-HCl at pH 7.4, 1 mmol/L of ethyleneglycotetraacetic acid, 1 mmol/L of EDTA, 0.2 mmol/L of phenylmethanesulphonylfluoride, 20 µmol/L, and 0.2 mmol/L of sodium orthovanadate. After centrifugation, 30 minutes at 15,000g at 4ºC, the supernatants containing the nuclear protein were stored at -80ºC for further analysis. The levels of IκB-α, phospho-NF-κB p65 (serine 536), NF-κB p65 (nuclear), Bax, and Bcl-2 were quantified in cytosolic fraction from the pancreas tissue collected at 24 hours after cerulein administration. The filters were blocked with PM for 40 minutes at room temperature and subsequently probed with specific monoclonal antibodies against IκB-α (1:1000; Santa Cruz Biotechnology), phospho-NF-κB p65 (serine 536, 1:1000; Cell Signalling) or NF-κB p65 (nuclear, 1:1000; Cell Signalling), or

Bax (1:1000; Santa Cruz Biotechnology) or Bcl-2 (1:1000; Santa Cruz Biotechnology) in PMT at 4ºC overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch) for 1 hour at room temperature. To ascertain whether blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against β-actin protein (1:10,000; Sigma-Aldrich Company). The relative expression of the protein bands of IκB-α (approximately 37 KD), phospho-NF-κB (75 KD), NF-κB p65 (nuclear, 65 KD), Bax (23 KD), and Bcl-2 (29 KD) was quantified by densitometric scanning of the radiographic films with GS-700 imaging densitometer (Bio-Rad) and analyzed by a computer program (Molecular Analyst; IBM).

### *3.2.9 Immunohistochemical Analysis*

Localization of Fas ligand, Bax, Bcl-2, tumour necrosis factor α (TNF- α), P selectin, ICAM-1, transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and poly-ADP ribose synthase (PARS) was performed by immunohistochemistry. At 24 hours after cerulein administration, the pancreas tissues were fixed in 10% (wt/vol) phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4), and buffered formaldehyde and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (vol/vol) hydrogen peroxide in 60% (vol/vol) methanol for 30 minutes. The sections were permeabilized with 0.1% (wt/vol) Triton X-100 in PBS for 20 minutes. Nonspecific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 minutes. Endogenous biotin and avidin-binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin, respectively (DBA, Milan, Italy). Sections were incubated overnight with anti-Fas ligand antibody (1:500 in PBS, vol/vol), anti-Bax antibody (1:500 in PBS, vol/vol), anti-Bcl-2 antibody (1:500 in PBS, vol/vol), anti-TNF-α antibody (1:500 in PBS, vol/vol), anti-P selectin antibody (1:500 in PBS, vol/vol), mouse anti-rat antibody directed at ICAM-1 (CD54; 1:500 in PBS, vol/vol; DBA), anti-TGF-β

antibody (1:500 in PBS, vol/vol) (DBA), anti-VEGF antibody (1:500 in PBS, vol/vol; DBA), or anti-PARS antibody (1:500 in PBS, vol/vol; DBA). Specific labelling was detected with a biotin-conjugated goat anti-rabbit or goat anti-mouse IgG and avidin-biotin peroxidase complex (DBA). To verify the binding specificity, some sections were also incubated with primary antibody only (no secondary) or with secondary antibody only (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out. Immunohistochemistry photographs (n = 5) were assessed by densitometry. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (Apple Inc, Cupertino, California, USA).

### *3.2.10 Terminal Deoxynucleotidyltransferase-Mediated Uridine 5'-Triphosphate Nick-End Labelling Assay*

Terminal deoxynucleotidyl transferase- mediated deoxy- UTP-biotin end labelling of fragmented DNA (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (ApopTag, horseradish peroxidase kit; DBA). Briefly, sections were incubated with 15 Kg/ml of proteinase K for 15 minutes at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% $H_2O_2$ for 5 minutes at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase buffer containing deoxynucleotidyl transferase and biotinylated deoxyuridine 5-triphosphate in terminal deoxynucleotidyltransferase buffer, incubated in a humid atmosphere at 37ºC for 90 minutes, and then washed with PBS. The sections were incubated at room temperature for 30 minutes with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

### *3.2.11 Measurement of TNF-α*

Tumour necrosis factor *α* level was evaluated in plasma samples collected at 24 hours after cerulein injection. The assay was carried out by using a commercial colorimetric kit

(Diaclone Research Cell Science, Cedex, France) according to the manufacturer's instructions. All TNF-α determinations were performed in duplicate serial dilutions. The enzyme-linked immunosorbent assay detection limit is less than 25 pg/ml.

### *3.2.12 Malondialdehyde Activity*

The pancreatic tissue was analyzed for lipid peroxidation by using malondialdehyde (MDA) levels as an index. The MDA assessment was performed as previously described.[94] Pancreatic tissue collected at 24 hours after induction of pancreatitis was homogenized in 1.15% KCl solution. An aliquot (200 µL) of the homogenate was added to a reaction mixture containing 200 µL of 8.1% sodium dodecyl sulfate, 1500 µL of 20% acetic acid (pH 3.5), 1500 KL of 0.8% thiobarbituric acid, and 600 KL of distilled water. Samples were then boiled for 1 hour at 95°C and were centrifuged at 4000 revolutions per minute for 20 minutes at 4°C. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

### *3.2.13 Myeloperoxidase Activity*

Myeloperoxidase (MPO) activity, an index of PMN accumulation, was determined as previously described.[93] Pancreas tissues, collected at the specified time point, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mmol/L of potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at 20,000g at 4ºC. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mmol/l) and 0.1 mmol/L of $H_2O_2$. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per minute at 37°C and expressed in units per gram weight of wet tissue.

### *3.2.14 Data Analysis*

All values in the figures and text are expressed as mean ± SEM of the mean of n observations. In the *in vivo* studies, "n" represents the number of animals studied. In the

experiments involving histologic examination or immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days. The results were analyzed by 1-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. A $P\ value < 0.05$ was considered significant.

## 3.3 RESULTS

### *3.3.1 Effect of GTE Inhibition on the Degree of Acute Pancreatitis*

Intraperitoneal injection of the secretagogue cerulein was associated with a significant rise in the serum levels of lipase and amylase (*Figure 1.1.A,B*) and resulted in acute necrotizing pancreatitis. The histologic examination of the harvested pancreata that were sectioned at 24 hours after cerulein administration confirmed the findings of acute necrotizing pancreatitis, characterized by inflammatory cell infiltrates and acinar cell necrosis *(Figure1.2 B,C).* An inflammatory response characterized by the accumulation of water in the pancreas, as an indicator of fluid content in the pancreas tissues, was also observed *(Figure 1.1C*). The increase in lipase and amylase (*Figure 1.1A,B*), the extent and severity of the histologic signs of pancreas injury (*Figure1.2D*) and the accumulation of water in the pancreas *(Figure 1.1C*) were markedly reduced in cerulein-treated mice after GTE administration ($P < 0.01$). In sham-saline- (data not shown) and sham- GTE- treated mice *(Figure 1.2A);* the histologic features of the pancreas were typical of normal architecture. Similarly, no elevation in the serum levels of amylase and lipase and no tissue water accumulation were observed in the pancreatic tissue from the sham groups (*Figure 1.1A - C*).

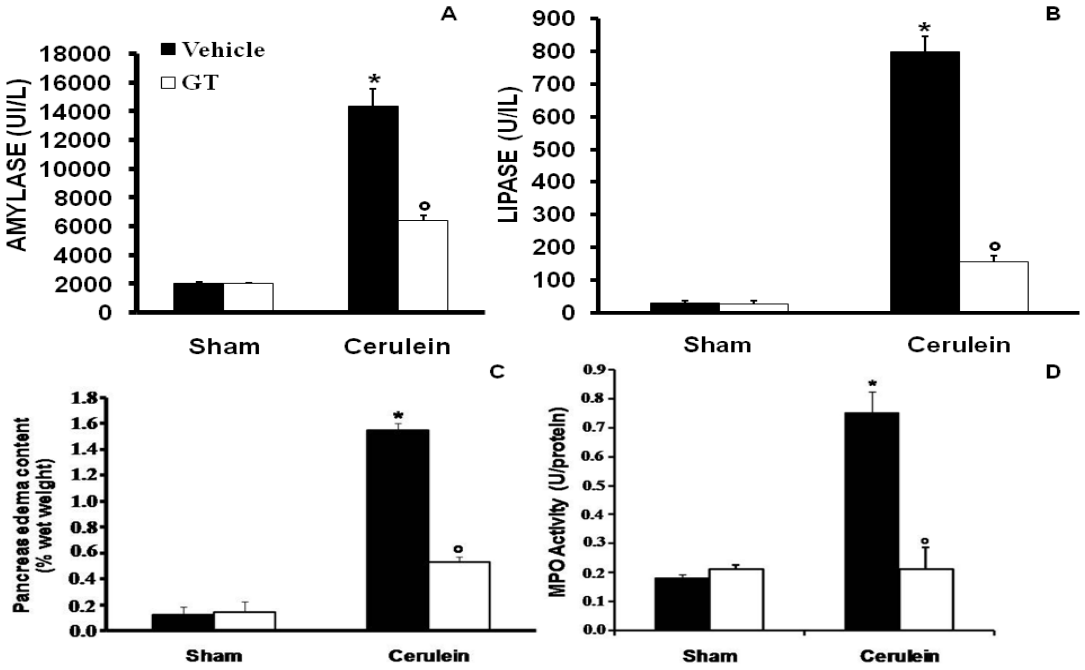



***Figure 1.1 Effects of GTE on amylase and lipase serum levels (U/l); pancreas oedema, and myeloperoxidase activity***.

*The administration of GTE (25 mg/kg) in male CD mice significantly reduced the increase of amylase (A), lipase (B), pancreas oedema (C) and myeloperoxidase activity (D), induced by cerulein. Each value is the mean ± SEM for n = 20. *p < 0.01 versus sham. °p < 0.01 versus cerulein.*

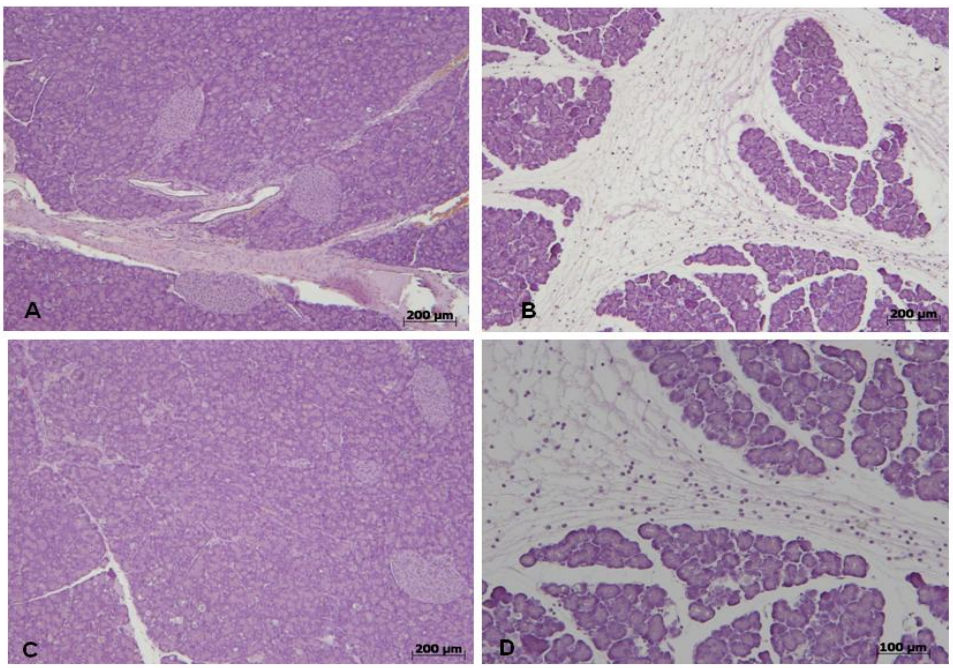



***Figure 1.2 Effects of GTE on morphologic changes of pancreatitis.***

*Representative hematoxylin/eosin-stained sections of pancreas from sham-GTE-treated mice (A) demonstrate that the histologic features of the pancreas were typical of a normal architecture. On the contrary, pancreas sections of cerulein-treated mice (B, C) demonstrate tissue damage characterized by interstitial oedema, acinar cell necrosis and inflammatory cell infiltrates. Pancreas section from mice that had received GTE at the dose of 25 mg/kg 1, 3 and 6 hours after the injection of cerulein (D) showed significantly less histologic alterations. The figure is representative of at least three experiments performed on different experimental days.*

### *3.3.2 Effect of GTE on IkB-α Degradation, Phosphorylation of Ser536 on NF-κB p65 Subunit, and Expression of Nuclear p65 Subunit*

To investigate the cellular mechanisms by which treatment with GTE attenuates cerulein-induced AP, we evaluated IkB- *α* degradation by Western blot analysis, phosphorylation of Ser536 on the NF-κB subunit p65, and total nuclear NF-κB p65 subunit expression. A basal level of IkB-*α* was detected in the pancreatic tissues from the vehicle-treated animals (*Figure 1.3 A; densitometry analysis, Figure 1.3B*), whereas in tissues collected from animals that had received cerulein, IkB-α expression was substantially reduced (*Figure 1.3 A; densitometry analysis, Figure 1.3B*). Green tea polyphenol prevented cerulein-induced IkB-*α* degradation ($P < 0.01$). Significant increases in the phosphorylation of Ser536 and the expression of nuclear p65 NF-κB subunit were observed in the pancreatic tissues collected

at 24 hours after cerulein administration (*Figure 1.3 C, E; densitometry analysis, Figure 1.3 D, F*). Treatment with GTE significantly reduced the phosphorylation of p65 on Ser536 ($P < 0.01$) and the expression of p65 subunit itself in the nucleus ($P < 0.01$;*Figure 1.3 C, E; densitometry analysis,Figure 1.3 D, F*). Phospho-Ser536 and total p65 levels observed in animals treated with GTE were similar to those of the sham groups (*Figure 1.3 C, E; densitometry analysis, Figure 1.3D, F*).

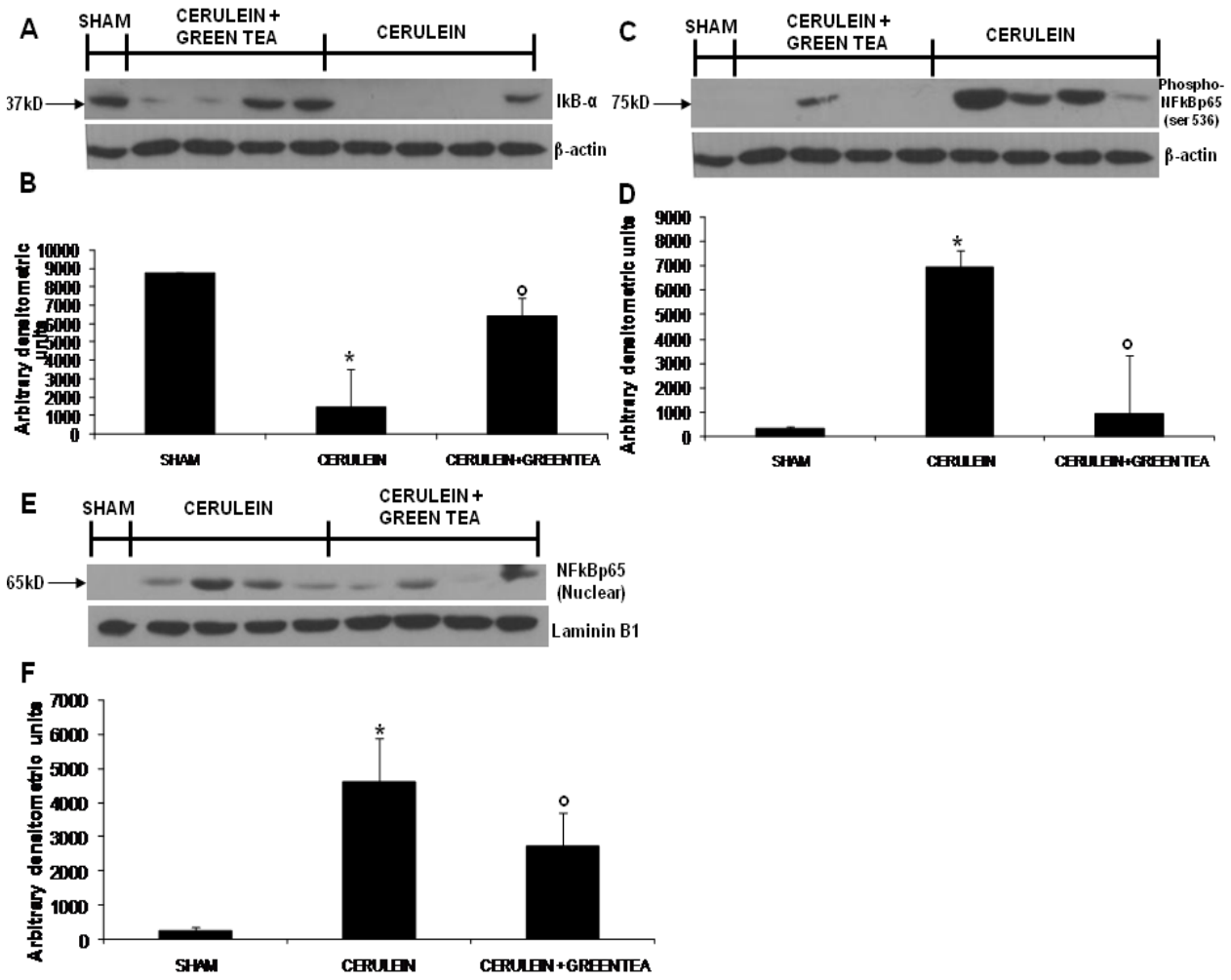



***Figure 1.3 Effects of GTE on IkB-α degradation, phosphorylation of Ser536 on NF-κB subunit p65 and activation of nuclear NF-κB subunit p65.***

*A representative blot of lysates for IkB-α (A), phospho-Ser536 (C) and nuclear p65 subunit (E) obtained from 5 animals per group is shown, and densitometry analyses of all animals for IkB-α (B), phospho-Ser536 (D) and nuclear p65 (F) subunit are reported. GTE prevented cerulein-induced IkB-α degradation and caused a significant decrease in the phosphorylation of p65 on Ser535, as well as a marked decreased expression of the p65 subunit in the nucleus. The results in B, D and F are*

*expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs.cerulein.*

### *3.3.3 Effects of GTE on TNF-α Expression*

To test whether GTE modulates the inflammatory process through the regulation of TNF-α, we measured plasma levels and analyzed the TNF-α expression in the pancreas by immunohistochemistry. A substantial increase of TNF-α was found in the plasma collected from cerulein-treated mice (*Figure 1.4 E*). In contrast, plasma levels of TNF-α were significantly reduced in animals that had received GTE ($P < 0.01$). No elevation in plasma levels of TNF- α was observed in the control groups (*Figure 1.4 E*). In addition, immunostaining for TNF- α showed intense positivity at 24 hours after cerulein administration in mice (*Figure 1.4 B*; densitometry analysis, *Figure 1.4 D*), and this positivity was found significantly reduced in GTE-treated mice ($P < 0.01$; *Figure 1.4 C; D*, densitometry analysis). No positive staining for TNF-α was observed in sham-GTE- (*Figure 1.4 A*; densitometry analysis, *Figure 1.4* D), and sham-vehicle- treated mice.

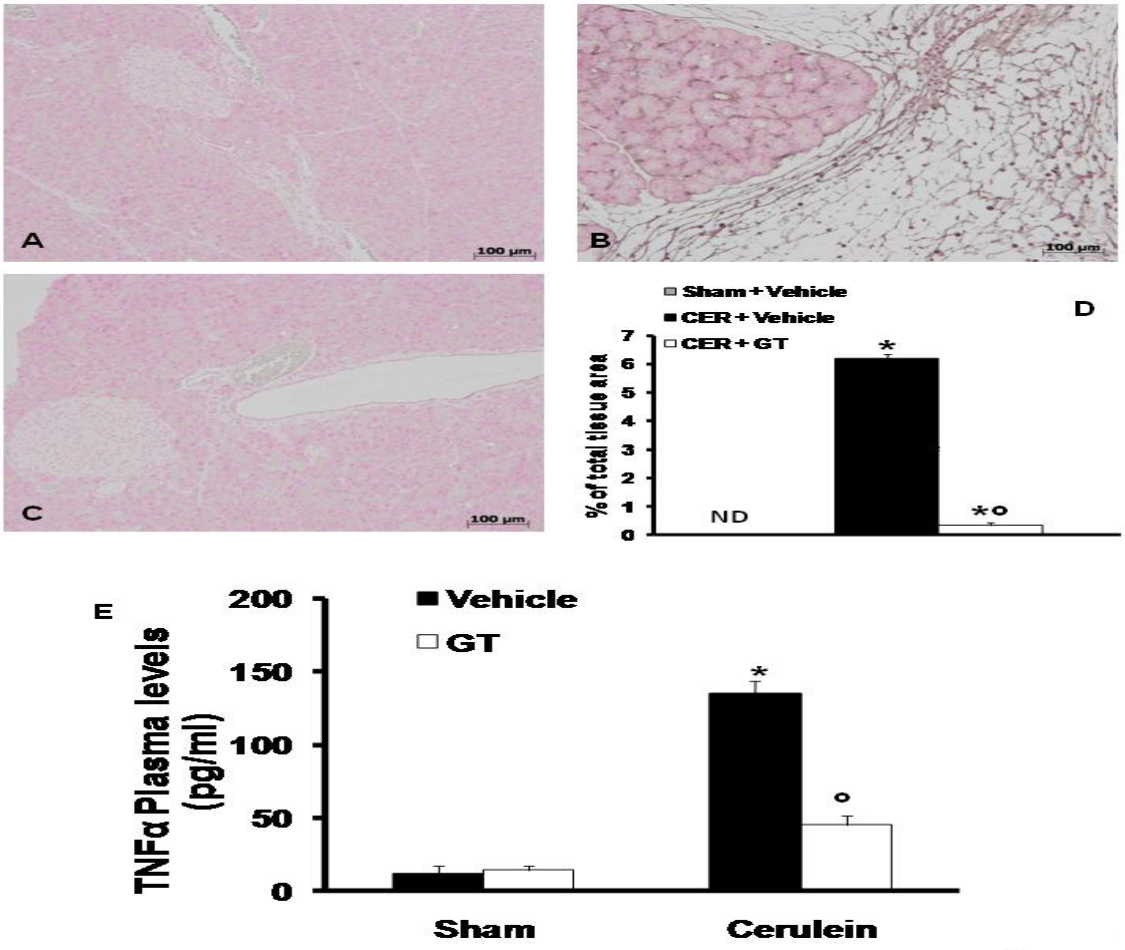



***Figure 1.4 Immunohistochemical localization of TNF-α in the pancreas and effects of GTE on TNF-α plasma levels.***

*No positive staining for TNF-α (A) was observed in pancreas sections from sham-GTE-treated mice. In contrast, intense positive staining for the cytokine (B) was found in pancreas sections from cerulein-treated mice. The intensity of positive staining for TNF-α was markedly reduced after the administration of GTE (C). Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for TNF-α from pancreas tissues was also assessed (D). The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. indicates not detected. Data are expressed as % of total tissue area. *p < 0.01 versus sham. °p < 0.01 versus cerulein. In addition, male CD mice show a significant increase of plasma and levels of TNF-α at 24 hours after cerulein administration. TNF-α plasma levels were significantly reduced after the administration of GTE (E). Data are means ± SEM from n = 20 mice for each group. *p < 0.01 versus sham. °p < 0.01 versus cerulein.*

### 3.3.4 *Effects of GTE on ICAM-1, P Selectin Expression and Neutrophil Infiltration*

The increase of immunohistochemical staining for the adhesion molecules ICAM-1 and P-selectin along the vessel walls of the inflamed pancreas was associated with the increase in MPO activity. Tissue levels of MPO correlate directly with the number of neutrophils in any given tissue. Immunostaining for ICAM-1 and P selectin ( *Figure 1.5 B, F;* densitometry

analysis, ), as well as MPO activity (*Figure 1.1 D*), were substantially enhanced at 24 hours after cerulein administration in mice and significantly reduced in the pancreas from the GTE-treated mice ($P < 0.01$;*Figure 1.1* D and *Figure 1.5 C, G*; densitometry analysis, *Figure 1.5 D, H*). No positive staining for ICAM-1 and P selectin and no MPO activity were observed in pancreas samples obtained from sham-saline and sham-GTE-treated mice (*Figure 1.1 D* and *Figure 1.5 A, E*; densitometry analysis, *Figure 1.5* D, H).

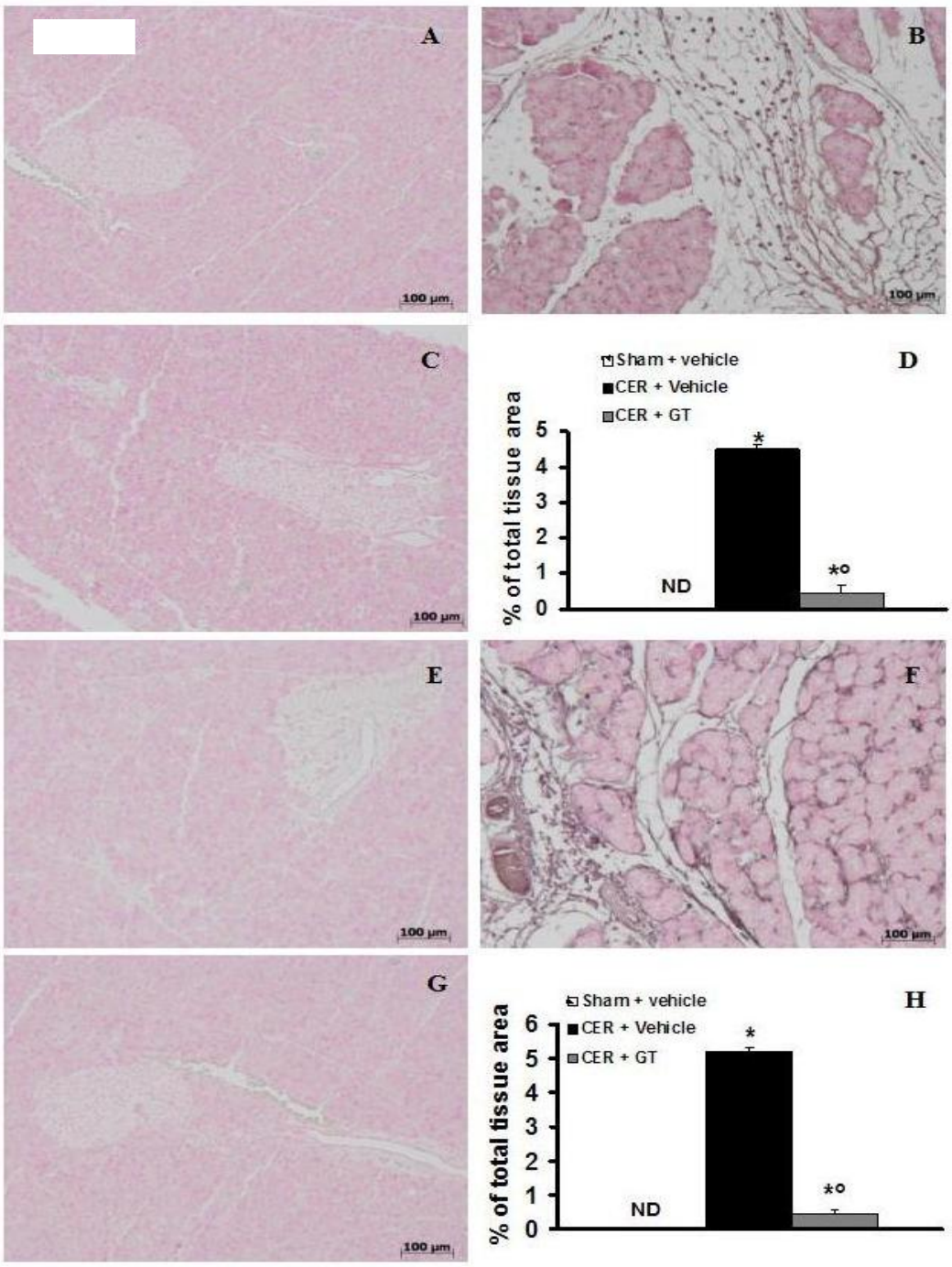



***Figure 1.5 Immunohistochemical localization of ICAM-1 and p-selectin in the pancreas.***

*No positive staining for both ICAM-1 (A) and p-selectin (E) was observed in pancreas sections from sham-GTE-treated mice. In contrast, intense positive staining for the two adhesion molecules was found in pancreas sections from cerulein-treated mice (B and F respectively). The intensity of positive staining for ICAM-1 (C) and p-selectin (G) were markedly reduced after the administration of GTE. Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for ICAM-1 (D) and p-selectin (H) from pancreas tissues was also assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. = not detected. Data are expressed as % of total tissue area. *p < 0.01 versus sham. °p < 0.01 versus cerulein.*

### *3.3.5 Effect of GTE on Lipid Peroxidation, Nitrosative Stress, and Poly-(ADP) - Ribose Synthetase Expression*

The generation of reactive oxygen and nitrogen-derived radicals contributes significantly to the tissue necrosis and dysfunction associated with inflammation.[94] Pancreatic injury in mice was characterized by an increase in the tissue levels of malondialdehyde (MDA), indicative of lipid peroxidation. Tissue MDA levels were markedly reduced by the administration of GTE ($P<0.01$; *Figure 1.6 E*). Furthermore, immunohistochemical analysis for nitrotyrosine, a marker of nitrosative stress, and PARS, an enzyme marker for DNA strand breakage, revealed positive staining in the pancreas tissues from cerulein-treated mice (*Figure 1.6B and 7B*; densitometry analysis, *Figure 1.6D and 1.7D).* A significant reduction in the positivity of Immunohistochemical staining for nitrotyrosine and poly(ADP-ribose) synthetase (PARS) was found after the administration of GTE ($P <0.01$; *Figure 1.6C and 1.7C*; densitometry analysis, *Figure 1.6D and 1.7D*). The present assays were negative in both the sham-vehicle- (data not shown) and sham- GTE- treated mice *(Figure 1.6A and 1.7A*; densitometry analysis, *Figure 1.6D and 1.7D*).

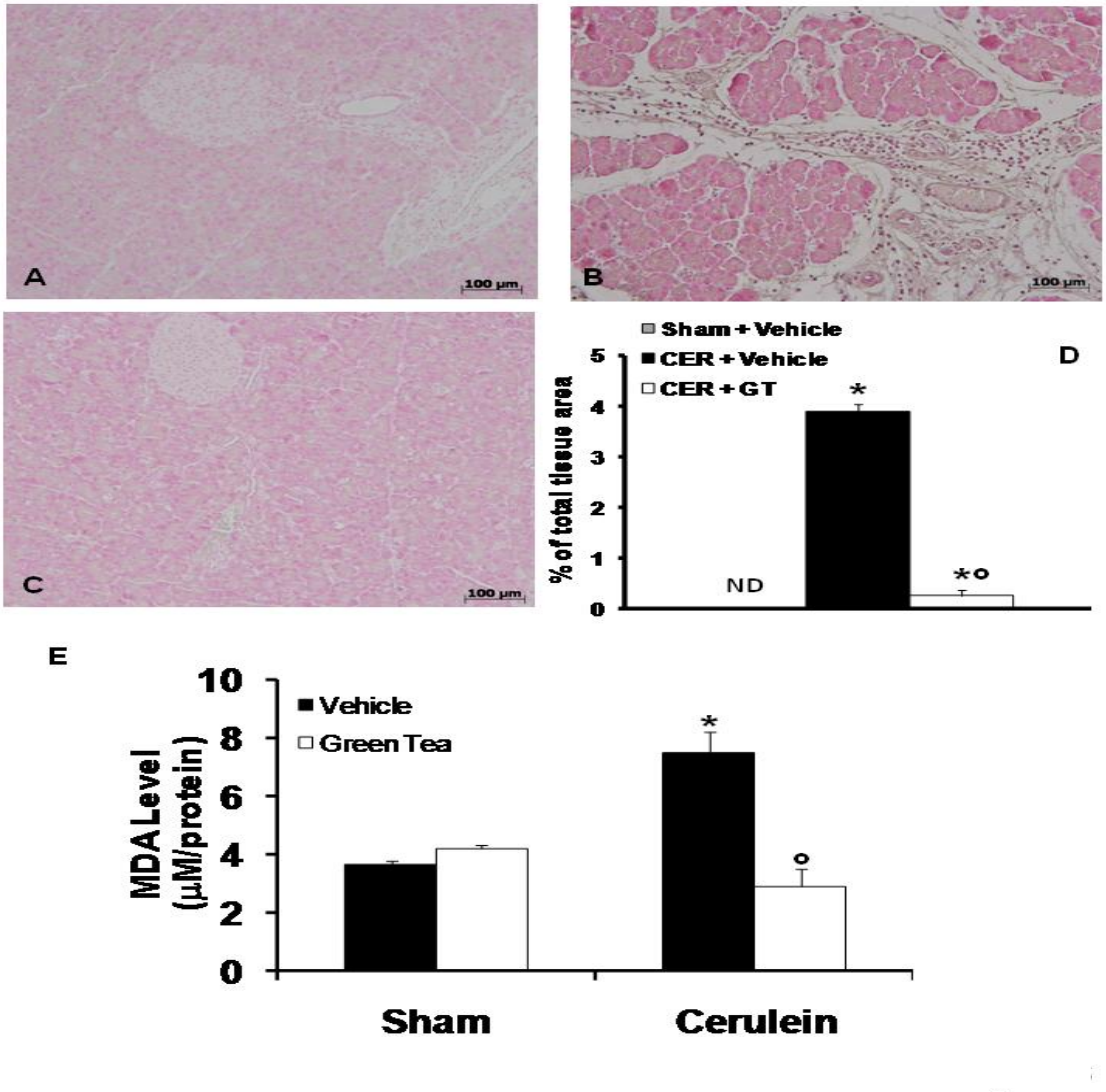



***Figure 1.6 Immunohistochemical localization of nitrotyrosine in the pancreas and effects of GTE on lipid peroxidation.***

*No positive staining for nitrotyrosine (A) was observed in pancreas sections from sham-GTE-treated mice, whereas positive staining was found in pancreas sections from cerulein-treated mice (B). The intensity of positive staining for nitrotyrosine was markedly reduced after the administration of GTE (C).Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for nitrotyrosine (D) from pancreas tissues was also assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. = not detected. Data are expressed as % of total tissue area. In addition, malondialdehyde (MDA) levels, an index of lipid peroxidation, were significantly increased in pancreas tissues 24h after cerulein administration (E). GTE (25 mg/kg) significantly reduced the cerulein-induced elevation of MDA levels. No significant alterations in MDA levels were observed in sham groups.Data are expressed as mean±SEM from n=20 mice for each group.*p<0.001 vs sham °p<0.01 vs cerulein*

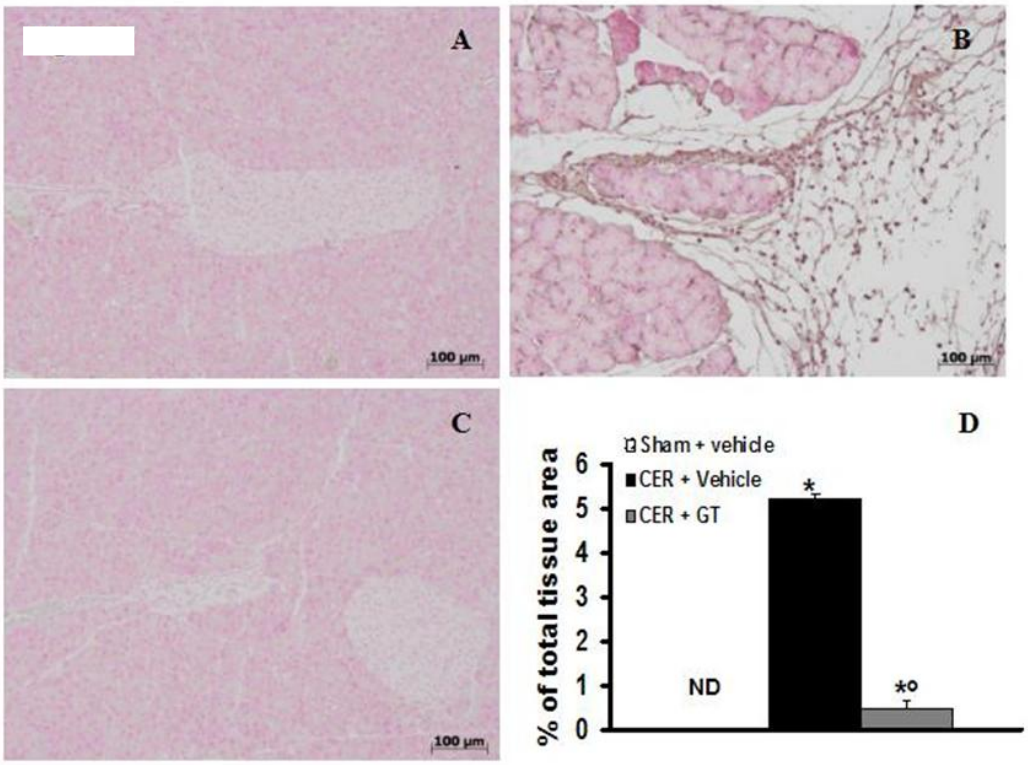



***Figure 1.7 Immunohistochemical localization of* Poly (ADP)-Ribose Synthetase (PARS) *in the pancreas.***

*No positive staining for PARS (A) was observed in pancreas sections from sham-GTE-treated mice, whereas positive staining was found in pancreas sections from cerulein-treated mice (B). The intensity of positive staining for PARS was substantially reduced after the administration of GTE (C). Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for PARS (D) from pancreas tissues was also assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. = not detected. Data are expressed as % of total tissue area. *p <0.01 versus sham. °p<0.01 versus cerulein.*

### *3.3.6 Effect of GTE on TGF-β and VEGF Expressions*

To assess whether GTE modulates leukocyte adhesion and infiltration through reduction in TGF-β and VEGF levels, we evaluated the expression of these molecules in the pancreas. In the pancreas tissue sections obtained at 24 hours after cerulein injection, positive staining for TGF- β and for VEGF was observed (*Figure 1.8B, F*; densitometry analysis, *Figure 1.8D* and *8H*) mainly localized in the acinar and ductal cells as well as in the vessel walls of the inflamed pancreas. The stainings for TGF- β and VEGF were visibly and significantly

decreased in the GTE-treated mice (P < 0.01; *Figure 1.8C, G*; densitometry analysis, *Figure 1.8D and 1.8H*). In addition, there was no staining for either TGF- β or VEGF in the pancreas obtained from the sham-saline (data not shown) and the sham-GTE mice (*Figure 1.8A, E;* densitometry analysis, *Figure 1.8D and 1.8H*).

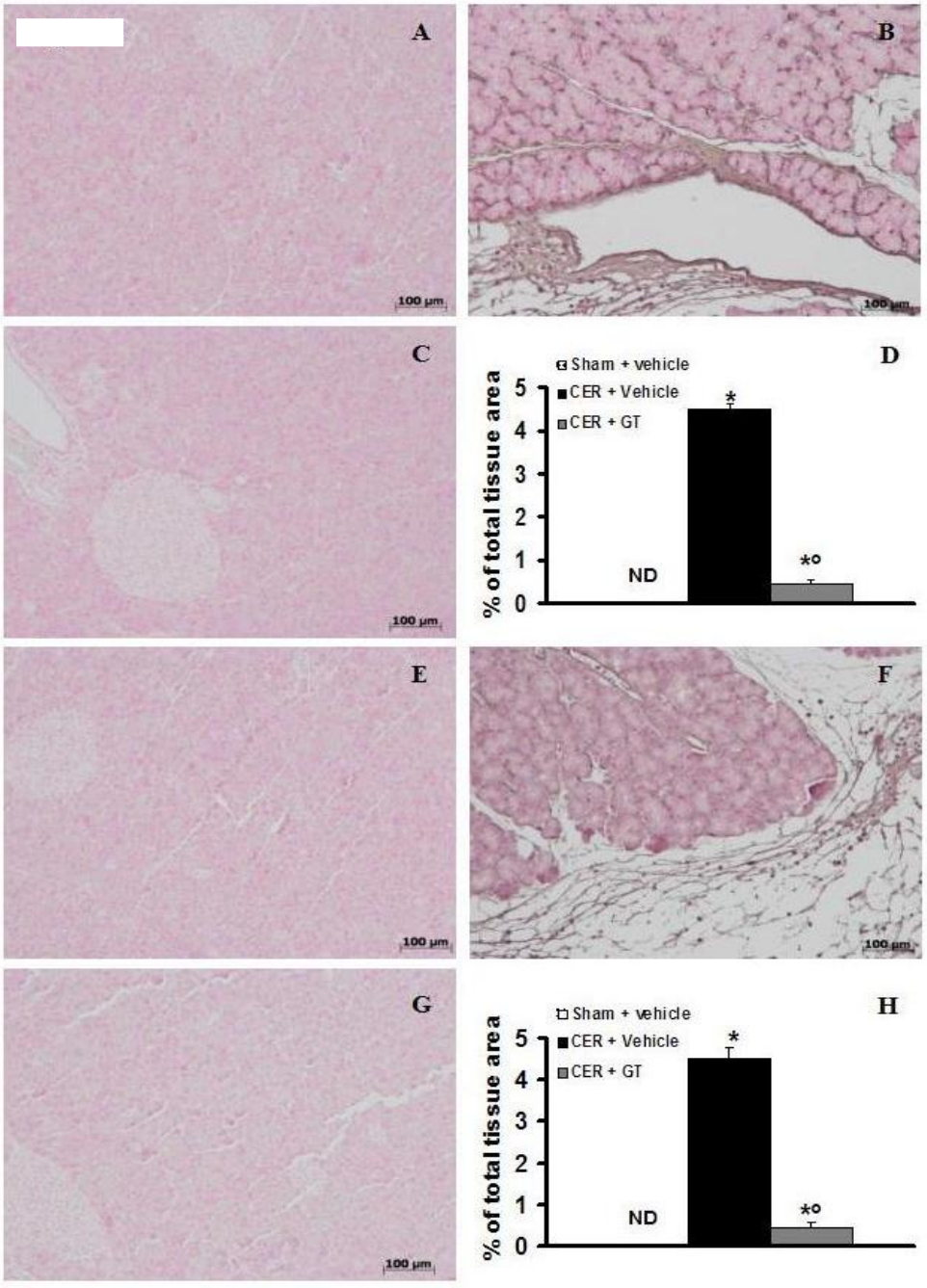



***Figure 1.8 Immunohistochemical localization of TGF-β and VEGF in the pancreas.***

*No positive staining for either TGF-β (A) or VEGF (E) was observed in pancreas sections from sham-GTE-treated mice. In contrast, intense positive staining for the two molecules was found in pancreas sections from cerulein-treated mice (B and F respectively). The intensity of positive staining for TGF-β*

*(C) and VEGF (G) was markedly reduced after the administration of GTE. Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for TGF-β (D) and VEGF (H) from pancreas tissues was also assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. = not detected. Data are expressed as % of total tissue area. *p < 0.01 versus sham. °p < 0.01 versus cerulein.*

### *3.3.7 Effect of GTE on Apoptosis*

The presence of apoptotic cells in pancreatic tissue is a measure of damage consequent to the inflammatory process.[95]To test whether tissue damage was associated with apoptosis induction, we carried out a TUNEL assay and measured Fas ligand, Bax, and Bcl-2 staining in the pancreas tissues. At 24 hours after cerulein administration, TUNEL assay confirmed the presence of apoptotic cells in sections obtained from the cerulein-treated mice (*Figure 1. 9B, C*). Positive immunohisto- chemical staining for Fas ligand and Bax (*Figure 1.10B and 1.11B*; densitometry analysis, *Figure 1.10D* and 1*.11D)* and negative staining or Bcl-2 (*Figure 1.11F*; densitometry analysis, *Figure 1.11H*) were also detectable in the pancreas tissues. In contrast, no positive staining for TUNEL assay (*Figure 1.9D*), Fas ligand, and Bax (*Figure 1.10C and 1.11C*; densitometry analysis, *Figure 1.10D and 1.11D*) and positive staining for Bcl-2 (*Figure 1.11G*; densitometry analysis, *Figure 1.11H*) were found in the tissue sections of the GTE-treated mice. Negative TUNEL assay (*Figure 1.9A*) and negative staining for Fas ligand, Bax, and Bcl-2 (*Figure 1.10A and 1.11A, E*; densitometry analysis, *Figure 1.10D and 1.11D, H*) were observed in the pancreas tissues of the sham-GTE groups. In addition, we analyzed by Western blot the expressions of Bax and Bcl-2 proteins. A substantial increase in Bax expression was found after cerulein administration, whereas GTE administration caused a significant decrease in the protein levels (*Figure 1.12A*; densitometry analysis, *Figure 1.12B*). A basal level of Bcl-2 was detected in pancreatic tissues from the sham groups, whereas in tissues collected from animals that had received cerulein, Bcl-2 expression was significantly reduced. Green tea polyphenol prevented cerulein-induced Bcl-2 degradation (*Figure 1.12C*; densitometry analysis, *Figure 1.12D*).

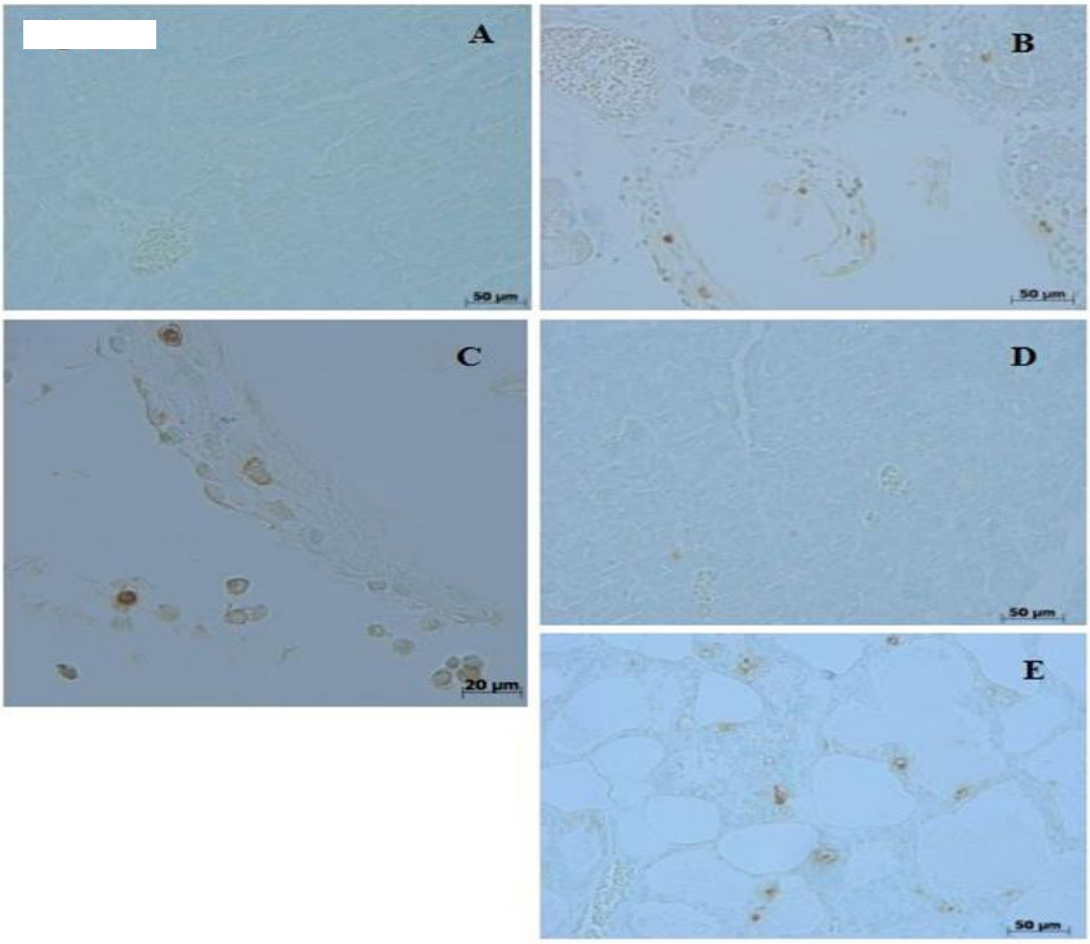



***Figure 1.9 TUNEL assay***.

*No apoptotic cells were found in sham-GTE mice (A), whereas sections obtained from cerulein-treated mice showed the presence of apoptotic cells (B, C). On the other hand, apoptosis was found reduced in sections from mice administered with GTE (D). TUNEL positive kit control is showed in (E). Figures are representative of at least three experiments performed on different experimental days.*

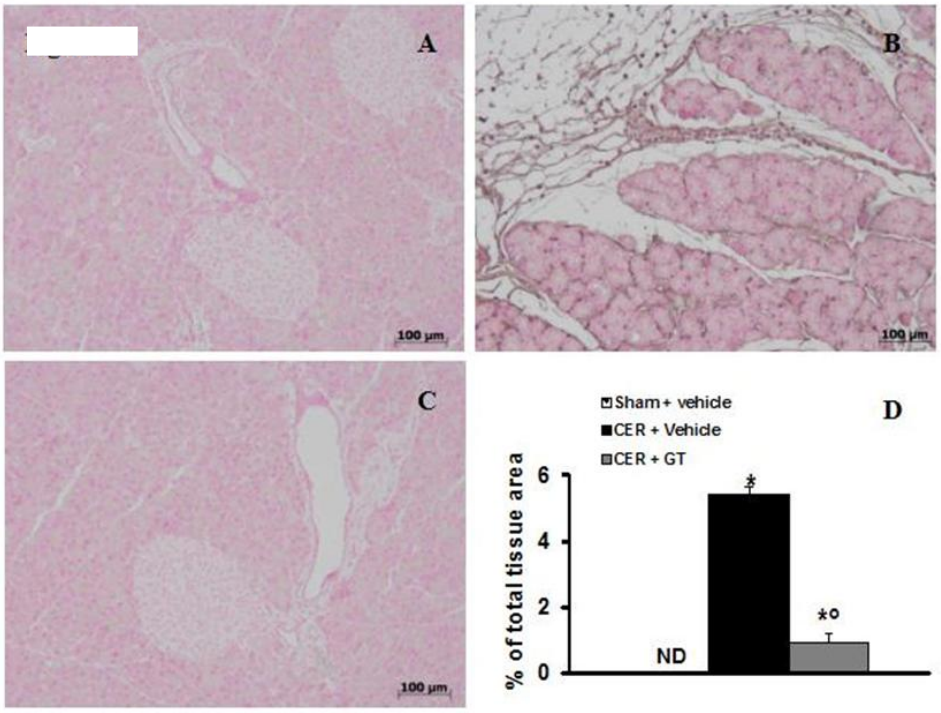



***Figure 1.10 Immunohistochemical localization of FasL in the pancreas***.

*No positive staining for FasL (A) was evidenced in sham-GTE-treated mice. Contrarily, sections obtained from cerulein-treated mice showed intense positive staining (B). The positivity for FasL was abolished in sections from GTE-treated mice (C). Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for FasL from pancreas tissues was also assessed (D). The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D. = not detected. Data are expressed as % of total tissue area. *p < 0.01 versus sham. °p < 0.01 versus cerulein.*

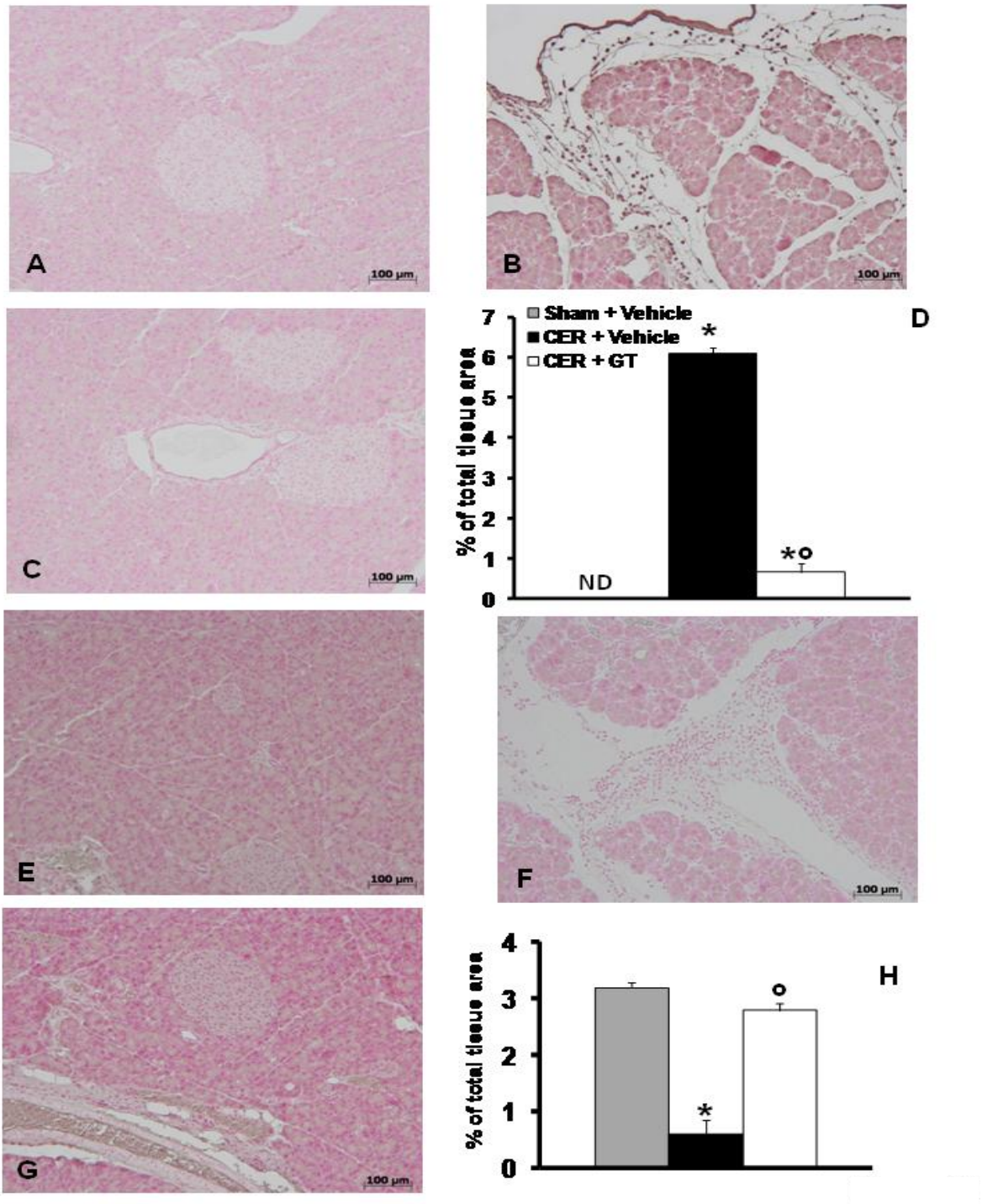



***Figure 1.11 Immunohistochemical localization of Bax and Bcl-2 in the pancreas.***

*No positive staining for Bax (A) and Bcl-2 (E) was evidenced in sham-GTE-treated mice. Sections obtained from cerulein-treated mice showed intense positive staining for Bax (B), and negative staining for Bcl-2 (F). Contrarily, the positivity for Bax was abolished in mice administered with GTE (C). An intense staining for Bcl-2 was also observed in GTE-treated mice (G). Figures are representative of at least three experiments performed on different experimental days. Densitometry analysis of immunohistochemistry photographs (n=5) for Bax (D) and Bcl-2 (H) from pancreas tissues was also assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer. N.D= not detected. Data are expressed as % of total tissue area.*p<0.01 vs sham, ºp<0.01vs cerulein*

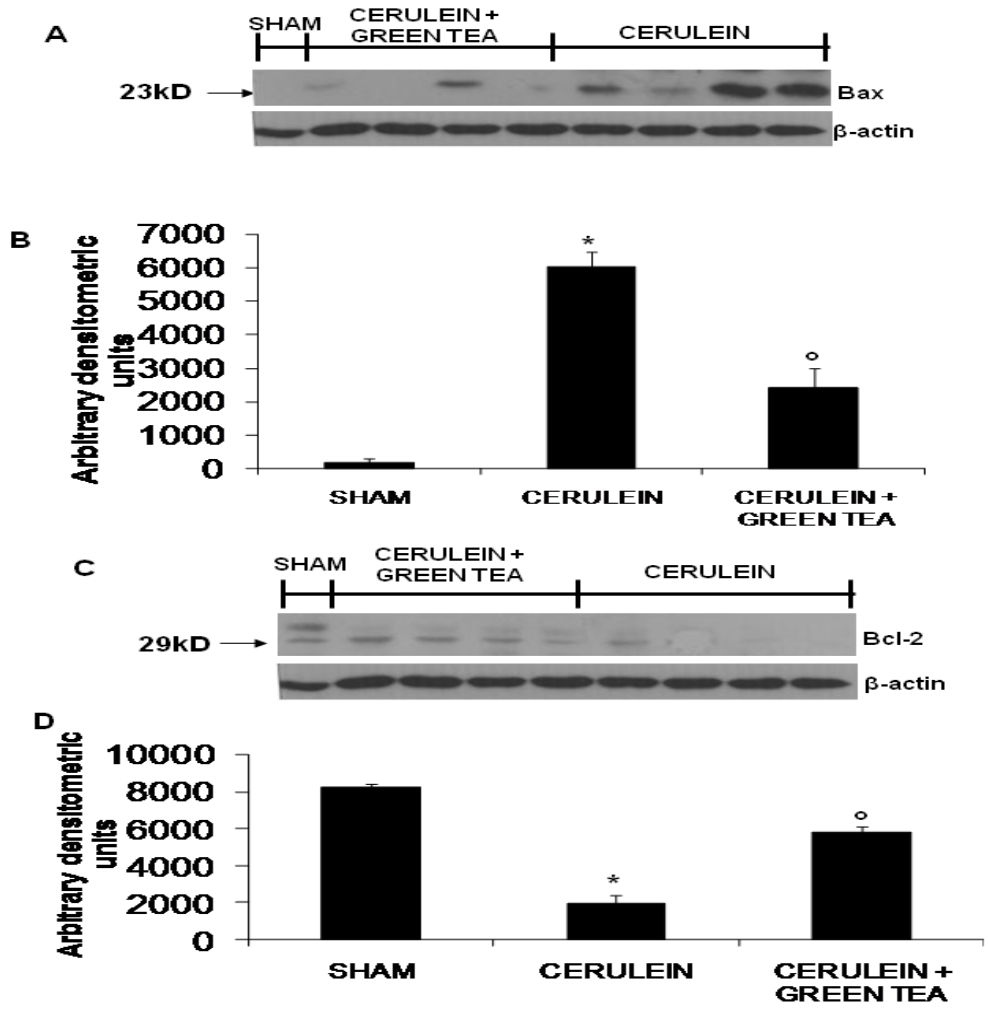



***Figure 1.12 Western blot analysis for Bax and Bcl-2.***

*A representative blot of lysates for Bax (A) and Bcl-2 (C) obtained from 5 animals per group is shown, and the correspondent densitometry analyses of all animals (B and D respectively) are reported. GTE caused a significant decrease in Bax levels and prevented cerulein-induced Bcl-2 degradation. The results in B and D are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs.cerulein.*

## 3.4 DISCUSSION

The pathophysiology of AP is a multifactorial process involving an intricate interplay of proinflammatory and anti-inflammatory pathways, with oxidative stress and cytokines being of major importance. Several attempts at targeting specific inflammatory mediators have been made, without as yet resulting in any effective therapeutic approach in man.[96] It has been recently shown that GTEs possess potent anti-inflammatory and antioxidative properties by decreasing NF-κB activation.[84 85 88 97]This study provides evidence that treatment of mice with GTE attenuates (1) the degree of cerulein-induced AP, (2) the activation of the transcription factor NF-κB, (3) the formation of proinflammatory cytokines, (4) lipid peroxidation and the formation of nitrogen-derived radicals, (5) PARS expression, and (6) the degree of apoptosis. All of these findings support the view that GTE exerts beneficial effects in experimental murine AP. In our model, the administration of cerulein caused a substantial increase in the serum levels of amylase and lipase as well as tissue damage, all of which were significantly reduced after the administration of GTE. After the initial pancreatic injury, migration of leukocytes occurs from the circulation into the acinar cells. The interaction of the activated leukocytes with the endothelial surface is mediated by adhesion molecules, such as P selectin and ICAM-1.[98] In mice administered with cerulein, we observed a substantial up-regulation of P selectin and ICAM-1, coupled with an increase of the specific granulocyte enzyme MPO. There was a significant reduction in the levels of P selectin, ICAM-1, and MPO activity in the GTE-treated mice. The influence of P selectin, ICAM-1, and neutrophils along with the interplay of VEGF is likely to have contributed to an end result of pancreatic oedema in our model, markedly reduced in mice treated with GTE. This demonstrates a potentially important, additional, protective anti-inflammatory mechanism for GTE. An emerging body of evidence suggests that NF-κB plays a major role in the early stages of AP.[81] In response to cerulein, there is rapid phosphorylation of IκB proteins, leading to their degradation and release of active NF-κB. This results in the transcription of a wide variety of genes involved in inflammation and progression of AP,

including those for TNF-α, IL-1β, leukocyte adhesion molecules, inducible nitric oxide synthase, TGF-β, and VEGF. The marked decrease in the quantity of lκB-α in our model suggests that the ameliorative effects of GTE are mediated through the NF-κB pathway. This was supported by the comparative appreciable decrease in the levels of the phosphor-NF-κB p65 (ser536) levels and the NF-κB p65 (nuclear) levels in the group treated with GTE. Thus, our study demonstrated that polyphenols down-regulate NF-κB activity in cerulein induced pancreatitis. Preventing NF-κB activation resulted, in turn, in reduced formation of proinflammatory cytokines such as TNF-α.

TNF- α, mainly produced by macrophages, is pivotal in many detrimental effects of AP such as up-regulation of other cytokines, oxidative stress, cell death, and endothelial activation.[99] There was a noticeable increase of TNF-α plasma levels and intrapancreatic TNF-α expression in mice injected with cerulein, and this up-regulation was found to be significantly reduced in the GTE-treated animals. Thus, it is possible that the beneficial effects of GTE in this study are also secondary to a reduction in the biosynthesis and/or the effects of proinflammatory cytokines.

Another pathway contributing to acinar cell damage early in the course of AP is mediated by reactive oxygen (ROS) and nitrogen species. At the molecular level, the effect is through molecular oxygen, nitric oxide, and their intermediary oxidation/ reduction products. Although integral to the intracellular signalling process, these molecules are damaging at high concentration. The effect of an uncontrolled production of ROS results in cell membrane lesions, DNA damage, increased lipid peroxidation, and activation of the NF-κB pathway.[100] Nitrotyrosine formation was initially proposed as a relatively specific marker for the detection of the endogenous formation “footprint” of peroxynitrite.[101] There is, however, evidence that certain other reactions can also induce tyrosine nitration, such as the reaction of nitrite with hypoclorous acid and the reaction of MPO with hydrogen peroxide.[102] Increased nitrotyrosine staining is therefore considered as an indication of increased nitrosative stress rather than a specific marker of the generation of peroxynitrite. Reactive oxygen species and peroxynitrite

produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids. In the present study, GTE caused a significant decrease in the levels of MDA (the product of lipid peroxidation), which were markedly elevated after the administration of cerulein. Moreover, ROS produce strand breaks in DNA, which trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme PARS, thereby resulting in the depletion of its substrate nicotinamide adenine dinucleotide (oxidized form; $NAD^+$) and a reduction in the rate of glycolysis. Because $NAD^+$ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, $NAD^+$ depletion leads to a rapid fall in intracellular adenosine triphosphate. This process has been termed the PARS Suicide Hypothesis.[103] We demonstrate here that GTE attenuates the increase in PARS activity in the pancreas from the cerulein-treated mice. The degree of pancreatic recovery after AP is closely correlated to the initial trauma.[104] Pancreatic regeneration from cerulein-induced pancreatitis is characterized by the proliferation of acinar cells and increased expression and deposition of collagen.[105] Transforming growth factor beta is a multifunctional polypeptide that contributes to tissue remodeling and repairs after an inflammatory episode.[106] It was demonstrated that a conditional loss of selective TGF-β signaling in the pancreas led to the amelioration of pancreatic fibrosis in mice.[105] Similar to the finding observed by Gress et al, [106] our cerulein-induced pancreatitis mouse model expressed raised levels of TGF-β on immunohistochemistry, which were substantially decreased after GTE treatment. This modulation of TGF-β may be clinically influential in reducing the potential for development of chronic pancreatitis. The endothelial cell-specific mitogen and chemotactic agent VEGF is involved in wound repair, angiogenesis of ischemic tissue, tumour growth, microvascular permeability, hemostasis, and endothelial cell survival. Markedly elevated intrapancreatic and serum VEGF levels have been demonstrated in experimental pancreatitis and in the clinical setting.[99 107] However, no relationships between serum VEGF levels and disease severity were observed. In the present study, we confirm that AP is associated with increased levels of VEGF, which also result in a significant pancreas oedema. Thus, the

reduced pancreatic oedema observed in our treatment group suggests that GTE may reduce vascular permeability and oedema formation through the modulation of VEGF expression. Acinar cell death is a hallmark of both experimental and human AP and occurs via two principal pathways, apoptosis and necrosis. It has been demonstrated that induction of apoptosis reduces the severity of experimental AP and is accompanied by an increased mitotic activity of acinar cells. On the other hand, its inhibition may worsen the disease.[95] The regulation of apoptosis at the molecular level in the course of AP is, however, still unclear. Recent evidence shows that a critical balance between the products of antiapoptotic and proapoptotic genes of the Bcl-2 and Bax family decides the fate of the cell.[108] Furthermore, it has been reported in cerulein-induced pancreatitis that Fas-FasL interaction may modulate the apoptotic cascade in the pancreas.[99] We have endeavored to identify the presence of apoptosis confirmed by the enhanced expression of Fas ligand and Bax protein and by the positive TUNEL assay. Bcl-2 proteins seemed to be down-regulated. On the contrary, mice that received GTE displayed high levels of Bcl-2 proteins with diminished concentrations of Fas ligand and Bax proteins compared with the cerulein-treated group. This demonstrates the regulatory effect of GTEs on the apoptotic pathway in the pancreas and that its inhibition may tip the balance toward a protective effect on cell death in cerulein-induced AP.

In conclusion, mounting evidence suggests that a multimodal approach at the molecular and cellular level is pertinent in the management of AP. The possibility of a medication targeting the initial acinar cell injury, however, may not be a feasible option in the clinical setting, as patient presentation would usually be after this phase. Our results show that GTE significantly ameliorated the effects of cerulein-induced experimental pancreatitis. Further evaluation of the mechanisms of action and possible use of green tea catechins as lead compounds for new drug discovery, may be considered in light of these encouraging results.

# 4 Recombinant human activated protein C (Xigris) attenuates murine cerulein-induced acute pancreatitis via regulation of NF-κB and apoptotic pathways.

## 4.1 Introduction

Protein C is a 60-kDa protein with an important regulatory action on coagulation.[109 110] Its thrombin mediated conversion to Activated Protein C[109 110] is impaired during sepsis.[111] The exogenous supplementation of activated protein C modifying the coagulation cascade in sepsis was examined by Taylor and colleagues reporting that activated protein C prevented the coagulopathic and lethal effects of *Escherichia coli* infusion in primates.[112]

Infusion of recombinant human Activated Protein C (Drotrecogin Alfa, Eli Lilly, Indianapolis, Indiana, USA) in patients with sepsis demonstrated a dose-dependent reduction in plasma levels of D-dimer, serum interleukin 6 (Il-6) and markers of coagulation and inflammation and was observed to be safe and well-tolerated.[113] Based on the findings of this study, the dose of 24 µg/kg/hr for 96 hours (by intravenous infusion) was selected for use in future studies.[113] The findings were further examined in a large, multi-centre, placebo-controlled, randomized clinical trial of xigris in severe sepsis (PROWESS) with a reduction in mortality as the primary end-point. The PROWESS study demonstrated a significant reduction in all-cause mortality in patients with severe sepsis in the intervention (xigris) arm. There was an increased incidence of haemorrhagic complications in the treatment arm although this did not attain significance. Amongst the listed exclusion criteria for PROWESS was “acute pancreatitis with no established source of infection”.[109] Acute pancreatitis (AP) was excluded because of the risks of haemorrhage associated with the inflamed pancreas.

Yet there is evidence both from experimental models[114] and from clinical studies[115] that micro vascular thrombosis in the pancreatic vascular beds is a mediator of pancreatic parenchymal necrosis and is also involved in the endothelium-inflammatory cell interplay. Further, the early stages of severe acute pancreatitis are characterised by a systemic

inflammatory response involving cellular and soluble cytokine mediators. Many of these processes are similar to those seen in sepsis and thus hypothetically would support the use of recombinant human Activated Protein C (rhAPC) *early* in the clinical course of severe acute pancreatitis, as a potential disease modifying agent.

In this context, Yamanel and colleagues evaluated the effect of recombinant human Activated Protein C in a rat model of taurocholate-induced haemorrhagic acute pancreatitis.[114] Their results showed that intervention with rhAPC was associated with a reduction in plasma tumour necrosis factor alpha and interleukin 6 levels and a reduction in pancreatic necrosis and overall injury scores without an increase in pancreatic haemorrhage. Alsfasser *et al* demonstrated that intervention with rhAPC was associated with a reduction in pancreatic and pulmonary myeloperoxidase levels and improved survival from 38% to 86% in rat taurocholate-induced acute pancreatitis.[76] However, this study did not demonstrate any difference in pancreatic necrosis or oedema scores in the intervention group compared to control nor did it shed light on the mechanism of action of rhAPC in experimental acute pancreatitis.

Recent evidence suggests that rhAPC has substantial anti-apoptotic properties and its ability to inhibit the NF-κB pathway, involved in the development of necrotising pancreatitis. The knowledge that inhibition of NF-κB activation reduces acinar cell injury[114] may be the mechanistic pathway through which rhAPC ameliorates the disease progression in the management of acute pancreatitis.

The aim of the present study is to undertake a detailed evaluation of the mechanisms of action of rhAPC in a well validated experimental model of acute pancreatitis with specific reference to the modulation of apoptotic and NF-κB pathways and down regulation of inflammation. This study is a necessary pre-requisite for any evaluation of rhAPC as a specific clinical treatment for acute pancreatitis.

## 4.2 Materials and methods

### *4.2.1 Animals*

Male Sprague-Dawley rats were purchased from Jackson Laboratories (Harlan Nossan, Italy). The study was approved by the University of Messina Review Board for the care of animals. The animals were housed in a controlled environment and provided with standard rodent chow and water *ad libitum*. Animal care was in compliance with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and the USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, and USA).

### *4.2.2 Drug administration.*

Recombinant Human Activated Protein C was administered intravenously at 500µg/kg. This dose was selected based on the increased excretion and metabolism found in the rat model (Betty Yan, Eli Lilly personal communication).

### *4.2.3 Induction of Acute Pancreatitis*

Pancreatitis was induced by intraperitoneal administration of 50µg/Kg of Cerulein hourly (x6) suspended in sterile saline solution.

### *4.2.4 Experimental Protocol.*

Two experimental protocols were evaluated: early intervention of rhAPC at the time of induction of acute pancreatitis (mechanistic study) and delayed intervention at 24h after induction of acute pancreatitis.

*Animals were arbitrarily distributed for the experiments with similar but separate controls for each of the protocols.*

#### 4.2.4.1 Cerulein Group

The animals were treated hourly (x6) with intraperitoneal administration of cerulein (50µg/Kg, suspended in saline solution)

#### 4.2.4.2 Sham + Vehicle Group

The animals in this group were treated identically to the *Cerulein Group* except that the 0.9% Normal saline was used instead of cerulein.

#### 4.2.4.3 Sham + rhAPC Group

Identical to the *Sham + Vehicle Group* except for the use of rhAPC

#### 4.2.4.4 rhAPC at induction of acute pancreatitis Group

rhAPC was administered within 1 hour of induction of pancreatitis (within 1h of the first ip administration of cerulein)

#### 4.2.4.5 rhAPC at 24 hours after induction of acute pancreatitis group

Here rhAPC was administered 24h after induction of pancreatitis.

The animals were sacrificed 24 hours after administration of rhAPC. Thus the two protocols had two different points of sacrifice, the 24 hours and 48 hours after induction of acute pancreatitis. Blood samples were obtained via intra-cardiac puncture. Pancreata was removed immediately, frozen in liquid nitrogen, and stored at -80$^{\circ}$C until assayed. Portions of this organ were fixed in formaldehyde for histological and immunohistochemical examination.

### *4.2.5 Haematological, Biochemical & Coagulation profile assays*

White blood cells (WBC), Haematocrit and Platelet count were analysed. Haematocrit was considered as a good indicator of severity and would correlate better than serum amylase and lipase levels.[91]

Serum amylase and lipase levels were measured at a veterinary laboratory. Results are expressed in international units per litre.

Recombinant Human Activated Protein C being a coagulation modifying medication prothrombin time, Partial thromboplastin time, International Normalized Ration (INR) and fibrinogen were measured.

The haematological, biochemical and coagulation profile were analysed at 24 and 48 hours after induction of AP.

### *4.2.6 Morphological Examination of the pancreas*

Paraffin-embedded pancreas samples were sectioned (5 µm) and stained with haematoxylin and eosin. Pancreata was examined by an experienced morphologist, who was blinded to the identity sample. Acinar-cell injury/necrosis was quantified by morphometry. For this study, 10 randomly chosen microscopic fields (×125) were examined for each tissue sample and the extent of acinar-cell injury/necrosis expressed as the percent of the total acinar tissue. The criteria for injury/necrosis were as follows: (i) The presence of acinar-cell ghosts or (ii) vacuolization and swelling of acinar cells and the destruction of the histoarchitecture of whole or parts of the acini, both of which would be associated with an inflammatory reaction.

### *4.2.7 Localisation of FAS ligand, BAX, BCL2, TNF-α, P-selectin, ICAM-1 and TGF-β by immunohistochemistry*

At 24 hours and 48hours after cerulein administration, the pancreata was fixed in 10 % (w/v) phosphate-buffered saline (PBS, 0.01 M, pH 7.4)-buffered formaldehyde and 8 µm sections prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3 % (v/v) hydrogen peroxide in 60 % (v/v) methanol for 30 min. The sections were permeabilized with 0.1 % (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2 % (v/v) normal goat serum in PBS for 20 min. Endogenous biotin and avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin, respectively (DBA, Milan, Italy). Sections were incubated overnight with anti-FAS ligand antibody (1:500 in PBS, v/v); anti-BAX antibody (1:500 in PBS, v/v), anti-BCL-2 antibody (1:500 in PBS, v/v), anti-TNF-α antibody (1:500 in

PBS, v/v), anti-P-selectin antibody (1:500 in PBS, v/v), anti-rat antibody directed at ICAM-1 (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy), anti-TGF-β antibody (1:500 in PBS, v/v) (DBA, Milan, Italy) or with anti-VEGF antibody (1:500 in PBS, v/v) (DBA, Milan, Italy). Specific labelling was detected with a biotin-conjugated goat anti-rabbit or goat anti-mouse IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). To verify the binding specificity for FAS ligand, BAX, BCL-2, TNF-α, P-selectin, ICAM-1 and TGF-β, some sections were also incubated with primary antibody only (no secondary) or with secondary antibody only (no primary).

### *4.2.8 Myeloperoxidase activity*

Myeloperoxidase activity, an index of PMN accumulation, was determined. Pancreata collected at the specified time point, was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x g at 4° C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM $H_2O_2$. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol of peroxide min $^{-1}$ at 37°C and is expressed in units per gram weight of wet tissue.

### *4.2.9 Malondialdehyde (MDA) activity*

Lipid peroxidation was assessed using malondialdehyde (MDA) levels of pancreatic tissue as an index. The MDA assessment was performed as previously described.[94] Pancreatic tissue collected 24 hours after induction of pancreatitis, was homogenized in 1.15% KCl solution. An aliquot (200 µL) of the homogenate was added to a reaction mixture containing 200 µL of 8.1% SDS, 1500 µL of 20% acetic acid (pH 3.5), 1500 µL of 0.8% thiobarbituric acid, and 600 µL of distilled water. Samples were then boiled for 1 h at 95°C and were centrifuged at 4000 RPM for 20 min at 4°C. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

### *4.2.10 Terminal Deoxynucleotidyltransferase-Mediated UTP nick-end Labeling (TUNEL) Assay.*

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (ApopTag, HRP kit DBA, Milan, Italy). Briefly, sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% $H_2O_2$ for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals visualized with diaminobenzidine.

### *4.2.11 Sub-cellular fractionation and western blot analysis for IkB-α, NF-κB p65 (nuclear), Bax and Bcl-2.*

Cytosolic and nuclear extracts were prepared as previously described [94] with slight modifications. Briefly, pancreas tissues from each rat were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, 1mM sodium orthovanadate, homogenised at the highest setting for 2 min and centrifuged at 1,000 g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in buffer B containing 1% Triton X-100, 150mM NaCl, 10mM TRIS-HCl pH 7.4, 1mM EGTA, 1 mM EDTA, 0.2mM PMSF, 20 µM, 0.2mM sodium orthovanadate. After centrifugation, 30 min at 15,000 g at 4°C, the supernatants containing the nuclear protein were stored at -80°C for further analysis. The levels of IκB-α, NF-κB p65 (nuclear), Bax and Bcl-2 were quantified in cytosolic fraction from pancreas tissue collected at 24 hours after cerulein administration. The filters were blocked with PM for 40min at room temperature and subsequently probed with specific monoclonal antibodies against IκB-α (Santa Cruz Biotechnology, 1:1000), or NF-κB p65 (nuclear) (Cell Signalling, 1:1000), or Bax (Santa Cruz Biotechnology, 1:1000) or Bcl-2 (Santa Cruz Biotechnology, 1:1000) in PMT at

4°C overnight. Membranes were incubated with peroxidase conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch) for 1 hour at room temperature. To ascertain whether blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin protein (1:10,000 Sigma-Aldrich). The relative expression of the protein bands of IκB-α (approx. 37 kDa), NF-κB p65 (nuclear, 65kDa), Bax (23 kDa) and Bcl-2 (29 kDa) was quantified by densitometric scanning of the radiographic films with GS-700 imaging densitometer (Bio-Rad) and analysed by a computer program (Molecular Analyst, IBM).

### *4.2.12 Materials*

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company (Milan, Italy). Primary monoclonal ICAM-1 antibodies for immunohistochemistry were purchased by Pharmingen. Reagents and secondary and non-specific IgG antibody for immunohistochemical analysis were from Vector Laboratories InC. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non pyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.)

### *4.2.13 Data analysis*

All values in the figures and text are expressed as mean ± SEM of the mean of “n” observations. In the *in vivo* studies, “n” represents the number of animals studied. In the experiments involving histological examination or immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days. The results were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. Nonparametric data were analyzed with the Fisher's exact test A $P < 0.05$ was considered significant.

## 4.3 Results

### *4.3.1 Haematological, Biochemical and coagulation profile*

Intraperitoneal injection of cerulein was associated with a significant rise in the serum levels of lipase and amylase and resulted in acute necrotizing pancreatitis (*Figure 2.1 a & b* respectively). The increase in lipase and amylase, were markedly reduced in ceruleintreated rats after rhAPC administration (*Figure 2.1a & b* respectively). No elevation in the serum levels of amylase and lipase were observed in sham groups. (*Figure 2.1 a&b*).

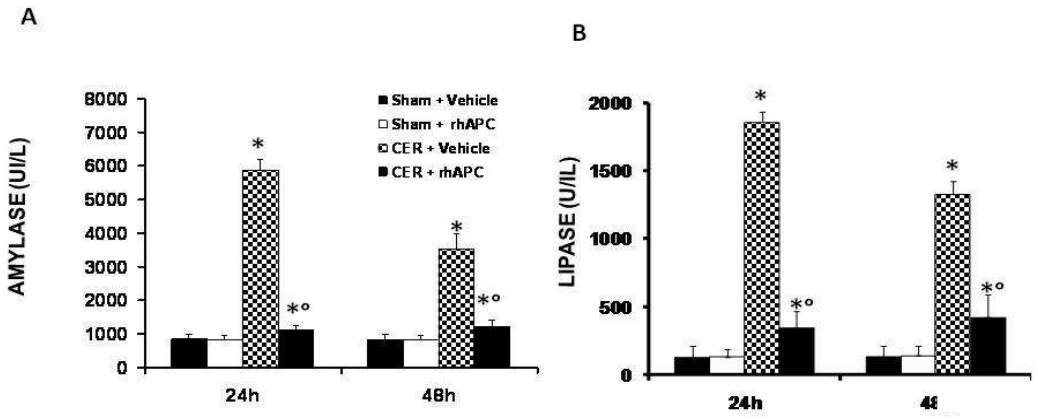



***Figure 2.1 Effects of rhAPC on amylase and lipase serum levels (U/l)***

*The administration of rhAPC significantly reduced the increase of amylase (A) and lipase (B) induced by cerulein. Each value is the mean ± SEM for n = 20. *p < 0.01 versus sham. °p < 0.01 versus cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

There was no significant change noted in the white cell count and Hematocrit levels within the different groups. The coagulation profile between the groups was essentially normal. Although a non-significant decrease in the fibrinogen and platelet counts was noted at 24

hours, an increase in the fibrinogen levels were noted at 48 hours compared to the platelets in the post induction phase group (*Figure 2.2*).

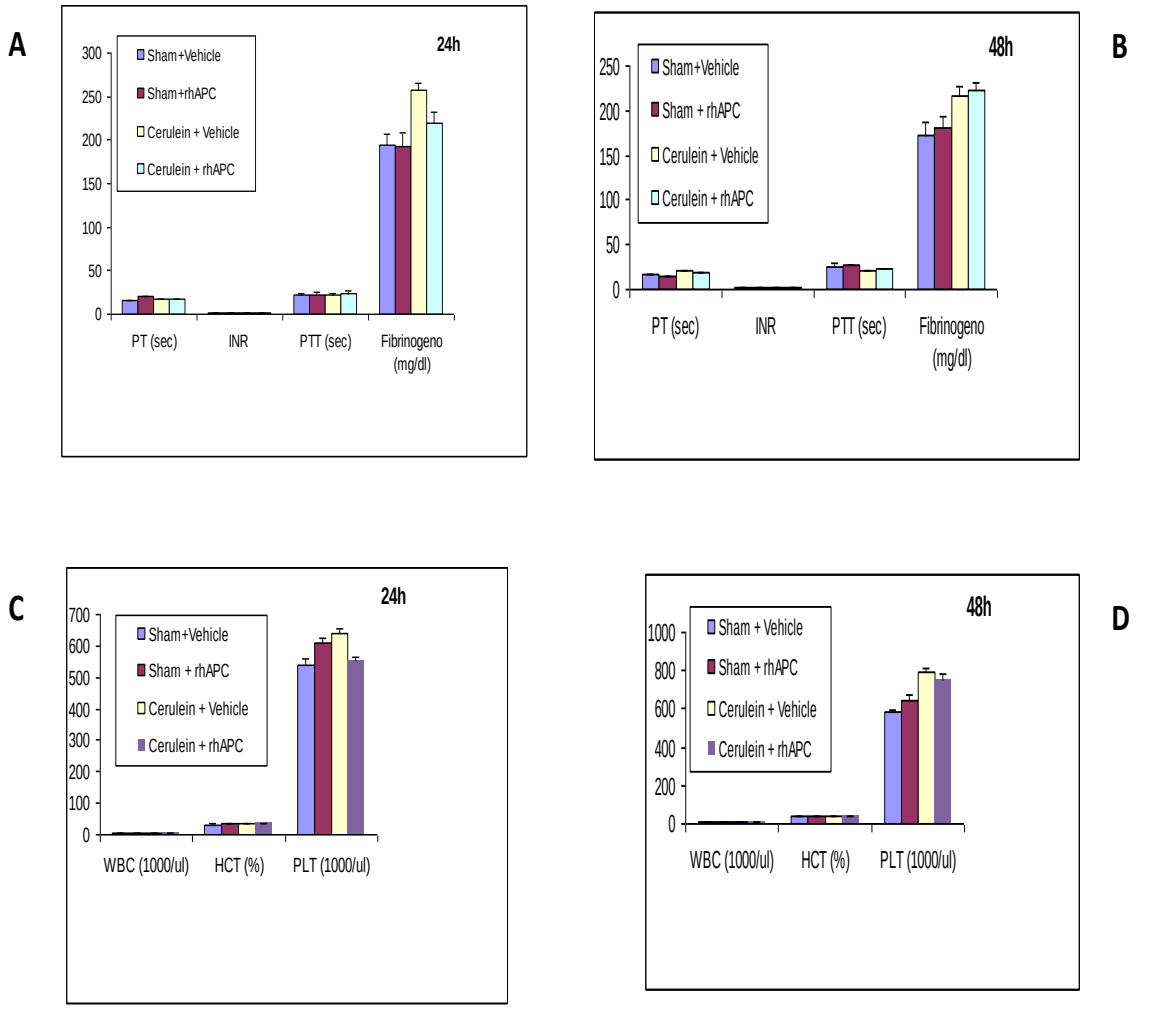



***Figure 2.2 Effects of rhAPC on hematocrit levels***

*There was no significant change noted in the white cell count and Hematocrit levels within the different groups. The coagulation profile between the groups was essentially normal. Although a non-significant decrease in the fibrinogen and platelet counts was noted at 24 hours, an increase in the fibrinogen levels were noted at 48 hours compared to the platelets in the post induction phase group (Figure 2). 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

### *4.3.2 Morphological Examination of the Pancreas*

Histological examination of the harvested pancreata at 24 hours and 48 hours after cerulein administration were characterized by inflammatory cell infiltrates and acinar cell necrosis and confirmed the presence of acute necrotising pancreatitis (*Figure 2.3 b, e*).

An inflammatory response characterized by the accumulation of water in pancreas, as an indicator of fluid content in the pancreas tissues, was also observed. In sham saline and sham- rhAPC treated rats, the histological features of the pancreas were typical of normal architecture (*Figure 2. 3 a, d, f*).

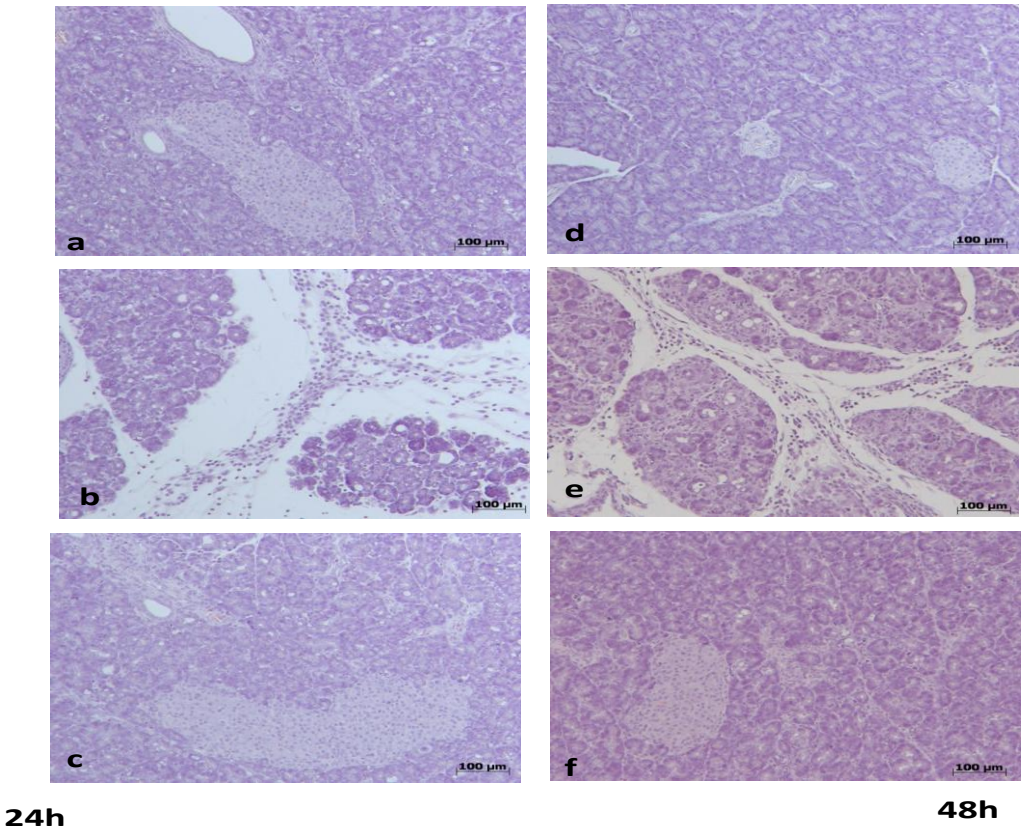



***Figure 2.3 Effects of rhAPC on morphologic changes of pancreatitis.***

*Representative haematoxylin/eosin-stained sections of pancreas from sham rhAPC-treated rats collected at 24 and 48 hours after cerulein induction (a, d), demonstrate that the histological features*

*of the pancreas were typical of a normal architecture. On the contrary, pancreas sections of cerulein-treated rats (b, e) demonstrate tissue damage characterized by interstitial oedema, acinar cell necrosis and inflammatory cell infiltrates. Pancreas section from rats that had received rhAPC after the injection of cerulein (c, f) showed significantly less histological alterations. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols. The figure is representative of at least three experiments performed on different experimental days.*

### 4.3.3 *Effect of rhAPC on IkB-α Degradation and expression of nuclear p65 subunit*

To investigate the cellular mechanisms by which treatment with rhAPC attenuates cerulein-induced acute pancreatitis, we evaluated IkB-degradation by western blot analysis and total nuclear NF-κB p65 subunit expression. A basal level of IkB-α was detected in pancreatic tissues from vehicle-treated animals; whereas in tissues collected from animals which had received cerulein, IkB-α expression was substantially reduced. rhAPC prevented cerulein-induced IkB-degradation (*Figure 2.4 a, a1*).

A significant increase in the expression of nuclear p65 NF-κB subunit was observed in pancreatic tissues collected at 24 and 48 hours after cerulein administration. Treatment with the rhAPC significantly reduced the expression of p65 subunit itself in the nucleus. Total p65 levels observed in animals treated with rhAPC were similar to those of the sham groups (*Figure 2.4 b, b1*).

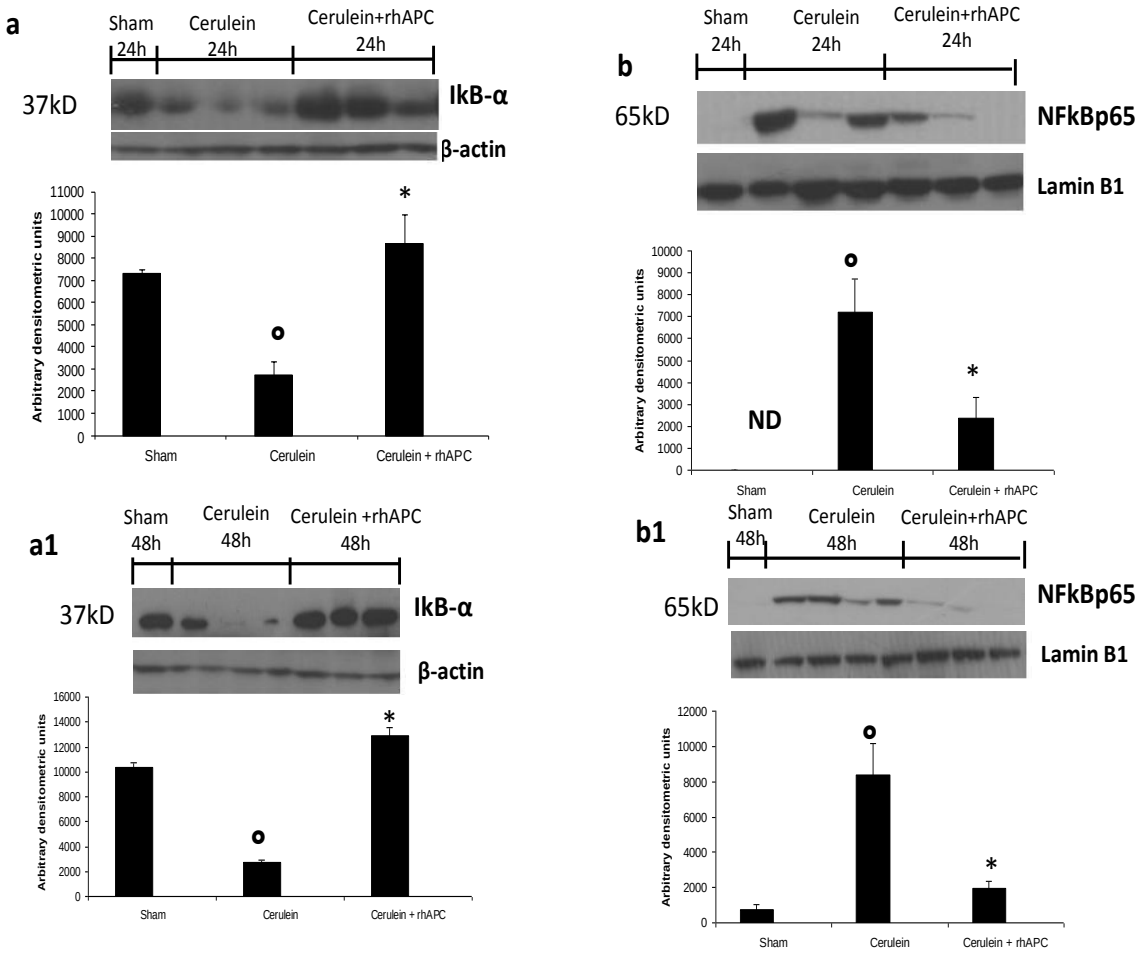



***Figure 2.4 Effects of rhAPC on IkB-α degradation and activation of nuclear NF-κB subunit p65.***

*A representative blot of lysates for IkB-α (a, a1), and nuclear p65 subunit (b, b1) and densitometry analyses of all animals for IkB-α (a1), and nuclear p65 (b1) subunit are reported. rhAPC prevented cerulein-induced IkB-degradation, as well as a marked decreased expression of the p65 subunit in the nucleus (a, a1 and b, b1). The results are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs. cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

### ***4.3.4*** *Effects of rhAPC on TNF-α expression*

We measured plasma levels and analyzed TNF-α and IL-1β expression in the pancreas by immunohistochemistry to test whether rhAPC modulates the inflammatory process through the regulation of TNF-α and IL-1β. In fact, immunostaining for TNF-α and IL-1β showed intense positivity at 24 hours and 48 hours after cerulein administration in the rats (*Figure 2.5 panel A and panel B b, e respectively*) and this positivity was found significantly reduced in rhAPC-treated rats (*Figure 2.5 panel A and panel B c, f respectively*). No positive staining

for TNF-α was observed in sham-rhAPC and sham-vehicle treated rats (*Figure 2.5 panels A and panel B a, d respectively*).

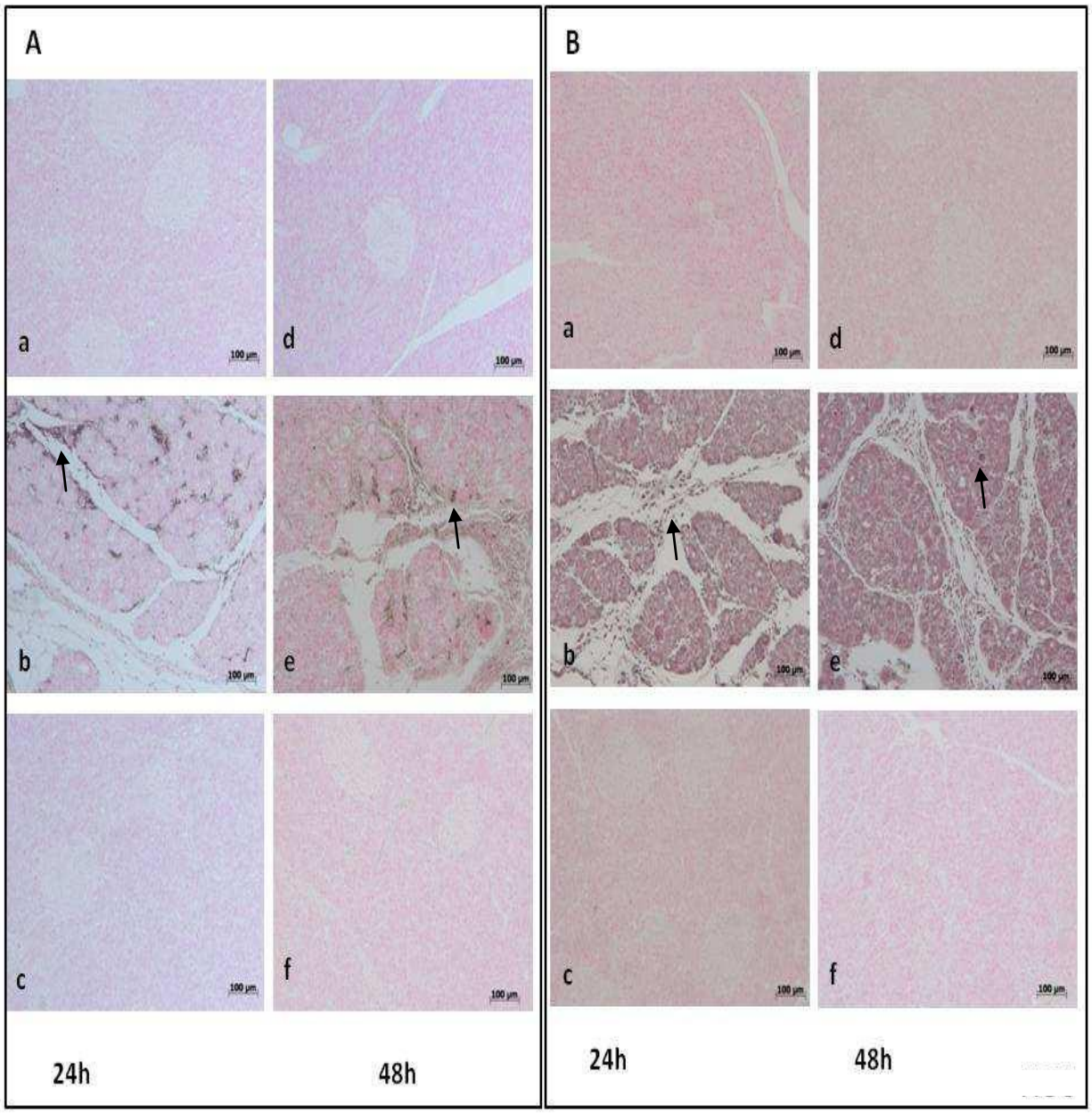



***Figure 2.5 Immunohistochemical localization of TNF-α and IL-1β in the pancreas***

*No positive staining for TNF-α and Il-1β were observed in pancreas sections collected at 24 and 48 hours after cerulein induction from sham-treated rats (panel A and B a, d respectively). In contrast, intense positive staining for the cytokines was found in pancreas sections from cerulein-treated rats (panel A and B, b & e, respectively (areas marked by arrow)). The intensity of positive staining for TNF-α and IL1β were markedly reduced after the administration of rhAPC (panel A and B c, f respectively). 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

### *4.3.5 Effects of rhAPC on ICAM-1, P-selectin expression and neutrophil infiltration*

The increase of immunohistochemical staining for the adhesion molecules ICAM-1 and P-selectin along the vessels wall of the inflamed pancreas was associated and correlated with the increase in myeloperoxidase activity. Tissue levels of myeloperoxidase directly correlate with the number of neutrophils in any given tissue.

Immunostaining for ICAM-1 and P-selectin, as well as myeloperoxidase activity (*Figure 2.8a*), were substantially enhanced at 24 hours and 48 hour after cerulein administration (*Figure 2.6; 2.7 b & e respectively*), and significantly reduced in pancreas from rhAPC-treated rats (*Figure 2.6; 2.7 c & f respectively*). Please note that no positive staining for ICAM-1 and P-selectin, were observed in pancreas samples obtained from sham-rhAPC-treated rats (*Figure 2.6; 2.7 a & d respectively*). An increase in ICAM and P-selectin expression were also evaluated by western blot analysis. A significant increase in the ICAM and P-selectin levels were observed in pancreatic tissues collected at 24 and 48 hours after cerulein administration (*Figure 2.6 g, g1 and h, h1 respectively*). Treatment with the rhAPC significantly reduced the ICAM and P-selectin expression (*Figure 2.7g, g1 and h, h1 respectively*). ICAM and P-selectin levels observed in animals treated with rhAPC were similar to those of the sham groups (*Figure 2.6 and 2.7 g, g1; h, h1 respectively*).

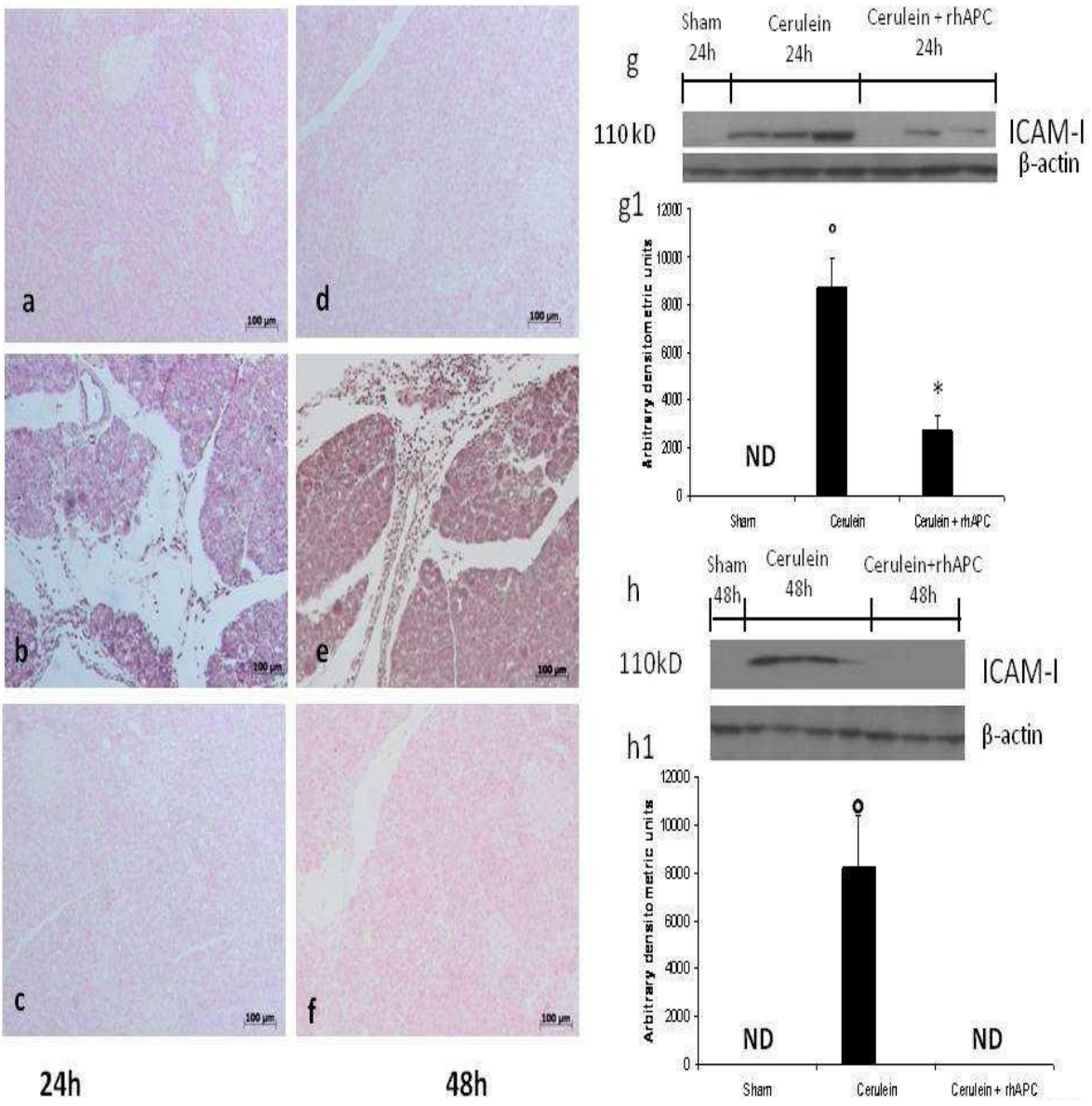



***Figure 2.6 Immunohistochemical localization of ICAM-1 in the pancreas.***

*No positive staining for ICAM-1 (a, d) was observed in pancreas sections collected at 24 and 48 hours after cerulein induction from sham-treated rats. In contrast, intense positive staining for the adhesion molecule was found in pancreas sections collected at 24 and 48 hours after cerulein induction from cerulein-treated rats (b, e respectively). The intensity of positive staining for ICAM-1 was markedly reduced after the administration of rhAPC (c, f). An increase in ICAM expression was evaluated by western blot analysis. A significant increase in the ICAM levels was observed in pancreatic tissues collected at 24 and 48 hours after cerulein administration (g, g1 and h, h1 respectively). Treatment with the rhAPC significantly reduced the ICAM expression (6g, g1 and h, h1 respectively). ICAM levels observed in animals treated with rhAPC were similar to those of the sham groups (5 and 6 g, g1; h, h1 respectively). The results are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs. cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

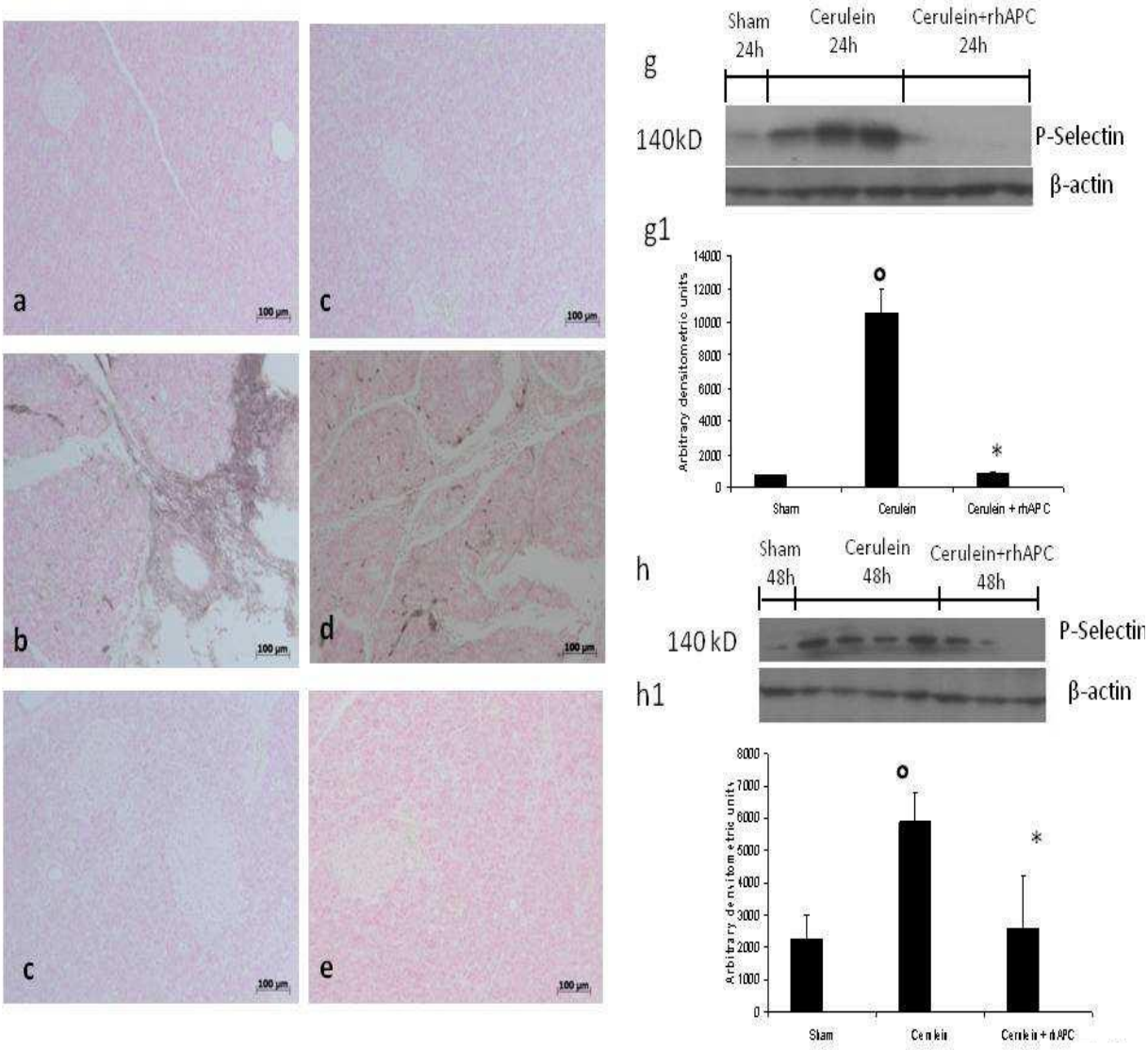



***Figure 2.7 Immunohistochemical localization of P-selectin***

*No positive staining for P-selectin (a, d) was observed in pancreas sections from sham rats. In contrast, intense positive staining for was found in pancreas sections from cerulein-treated rats (b, e respectively). The intensity of positive staining for P-selectin (c, f) was markedly reduced after the administration of rhAPC.*

*The P-selectin was also evaluated by western blot analysis. A significant increase in the P-selectin levels was observed in pancreatic tissues collected at 24 and 48 hours after cerulein administration (g, g1 and h, h1 respectively). Treatment with the rhAPC significantly reduced the P-selectin levels (g, g1 and h, h1 respectively). P-selectin levels observed in animals treated with rhAPC were similar to those of the sham groups (g, g1; h, h1 respectively). The results are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs. cerulein. 24 h & 48h represent time points of animals sacrificed in relation to the two protocols.*

### *4.3.6 Effect of rhAPC on lipid peroxidation*

The generation of reactive oxygen contributes significantly to the tissue necrosis and dysfunction associated with inflammation.[94] Increased tissue levels of malondialdehyde (MDA) were indicative of lipid peroxidation due to pancreatic injury (*Figure 2.8 b*). There was demonstrable decrease in the tissue MDA levels after administration of rhAPC. The test was negative in both sham-vehicle and sham-rhAPC-treated rats (*Figure 2.8 b*)

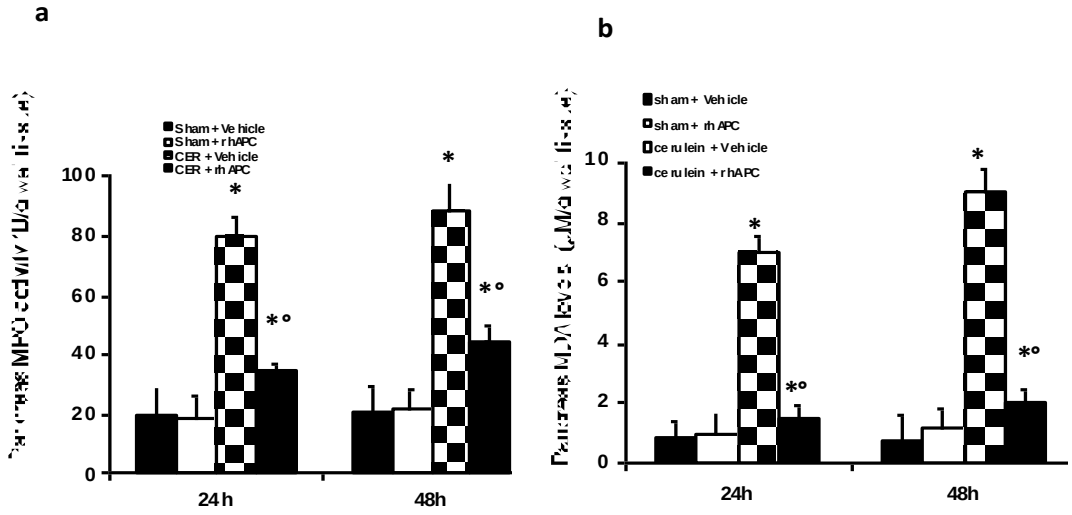



***Figure 2.8 Effect of rhAPC on neutrophil infiltration and lipid peroxidation***

*Myeloperoxidase (MPO) activity was significantly increased in the pancreas from cerulein-treated rats in comparison to that of sham-treated rats (a). The increase of MPO activity in the pancreas was significantly reduced by treatment with rhAPC. Data represent the mean ± standard error of the mean (SEM) of 20 rats for each group. *p < 0.01 versus sham treated rats.°p<0.01 versus cerulein.*

*In addition, malondialdehyde (MDA) levels, an index of lipid peroxidation, were significantly increased in pancreas tissues 24h after cerulein administration (b). rhAPC significantly reduced the cerulein-induced elevation of MDA levels (b). No significant alterations in MDA levels were observed in sham groups. Data are expressed as mean ± SEM from n= 20 rats for each group. *p <0.01 versus sham. °p<0.01 versus cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

### 4.3.7 *Effect of rhAPC on TGF-β.*

We evaluated the effect of rhAPC in the expression of TGF-β. In pancreatic tissue sections obtained at 24 & 48 hours after cerulein injection, positive staining for TGF-β was observed mainly localized in the acinar and ductal cells of the inflamed pancreas (*Figure 2.9 b and e*

respectively). The staining for TGF-β was visibly decreased in rhAPC-treated rats (*Figure 2.9 c and f respectively*)

No staining was noted for TGF-β in the pancreas obtained from sham-saline and sham-rhAPC treated rats (*Figure 2.9 a and d respectively*).

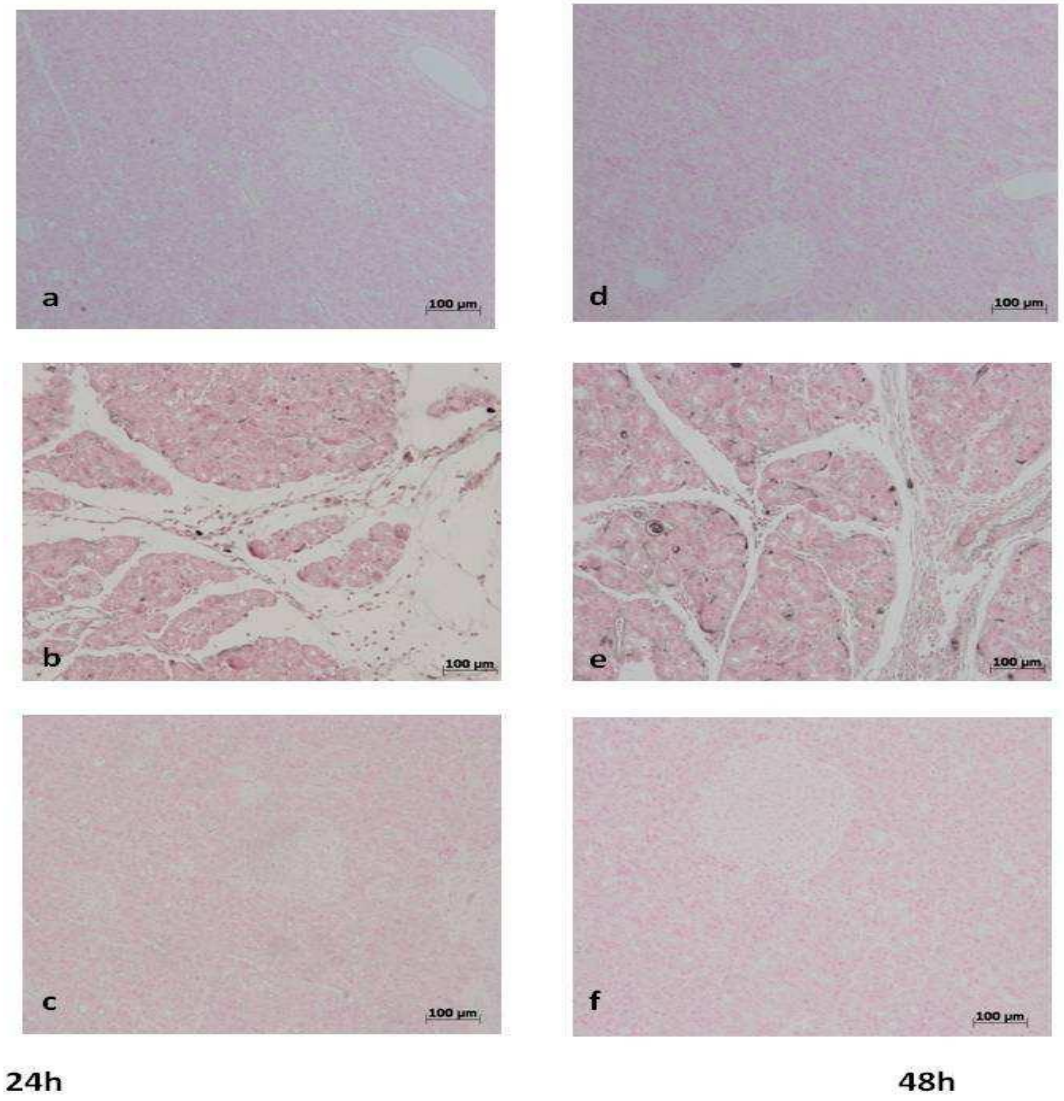



***Figure 2.9 Immunohistochemical localization of TGF-β in the pancreas.***

*No positive staining for either TGF-β (a, d) was observed in pancreas sections from sham-treated rats. In contrast, intense positive staining was found in pancreas sections from cerulein-treated rats (b, e respectively). The intensity of positive staining for TGF-β was markedly reduced after the administration of rhAPC (c, f). 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

### *4.3.8 Effect of rhAPC on apoptosis*

Damage due to inflammation may be assessed by the presence of apoptotic cells.[95] We carried out TUNEL assay and measured Fas-ligand, Bax and Bcl-2 staining in pancreas tissues to test whether tissue damage was associated with apoptosis. At 24 and 48 hours after cerulein administration, TUNEL assay confirmed the presence of apoptotic cells and intercellular apoptotic fragments in sections obtained from cerulein-treated rats (*Figure 2.13 b & e respectively*). In contrast, tissues obtained from rhAPC-treated rats contained a smaller number of TUNEL-positive cells (*Figure 2.13 c and f respectively*).In order, we evaluated a number of dark brown apoptotic cells for field and we counted a high number of apoptotic cells in sections obtained from cerulein at 24 and 48 hours after cerulein administration(*Figure 2.13 g*). On the contrary, the number of dark brown cells was significantly reduced in sections obtained from rhAPC-treated rats (*Figure 2.13 g*).

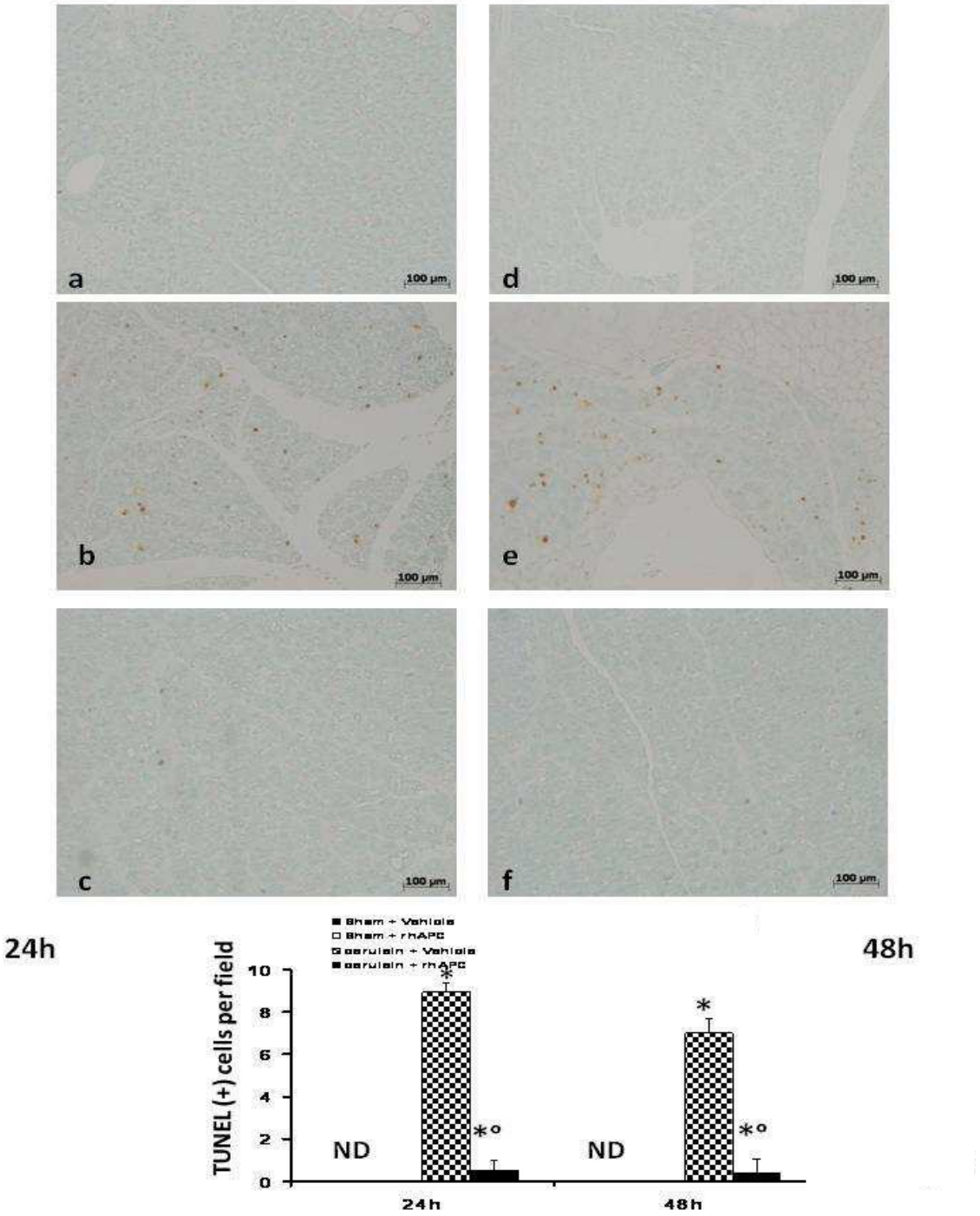



***Figure 2.10 TUNEL assay***.

*No apoptotic cells were found in sham-treated rats (a), whereas sections obtained from cerulein-treated rats showed the presence of apoptotic cells (b,e). On the other hand, apoptosis was found reduced in sections from rats administered with rhAPC (c,f). Figures are representative of at least three experiments performed on different experimental days. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

Positive immunohistochemical staining for Bax and Fas ligand was seen. (*Figure 2.10 and 2.12 b, e respectively*).Negative staining for Bcl-2 (*Figure 2.11 b, e*) was also detectable in the pancreas tissues. In contrast, no positive staining for Bax and Fas ligand (*Figure 2.10 and 2.12 c, f respectively*), and positive staining for Bcl-2 were found in tissue sections of rhAPC treated rats (*Figure 2.11 c, f*). Negative staining for Fas ligand, Bax and Bcl-2 were observed in pancreas tissues of sham-rhAPC groups (*Figure 2.10, 2.11 and 2.12 a, b respectively*).

In addition, we analyzed by western blot the expression of Bax and Bcl-2 proteins. A substantial increase in Bax expression was found after cerulein administration, whereas rhAPC administration caused a significant decrease in the protein levels (*Fig 2.10 g, g1 and h, h1*). A basal level of Bcl-2 was detected in pancreatic tissues from sham groups, while in tissues collected from animals which had received cerulein; Bcl-2 expression was significantly reduced. rhAPC prevented cerulein-induced Bcl-2 degradation (*Figure 2.11 g, g1 and h, h1*).

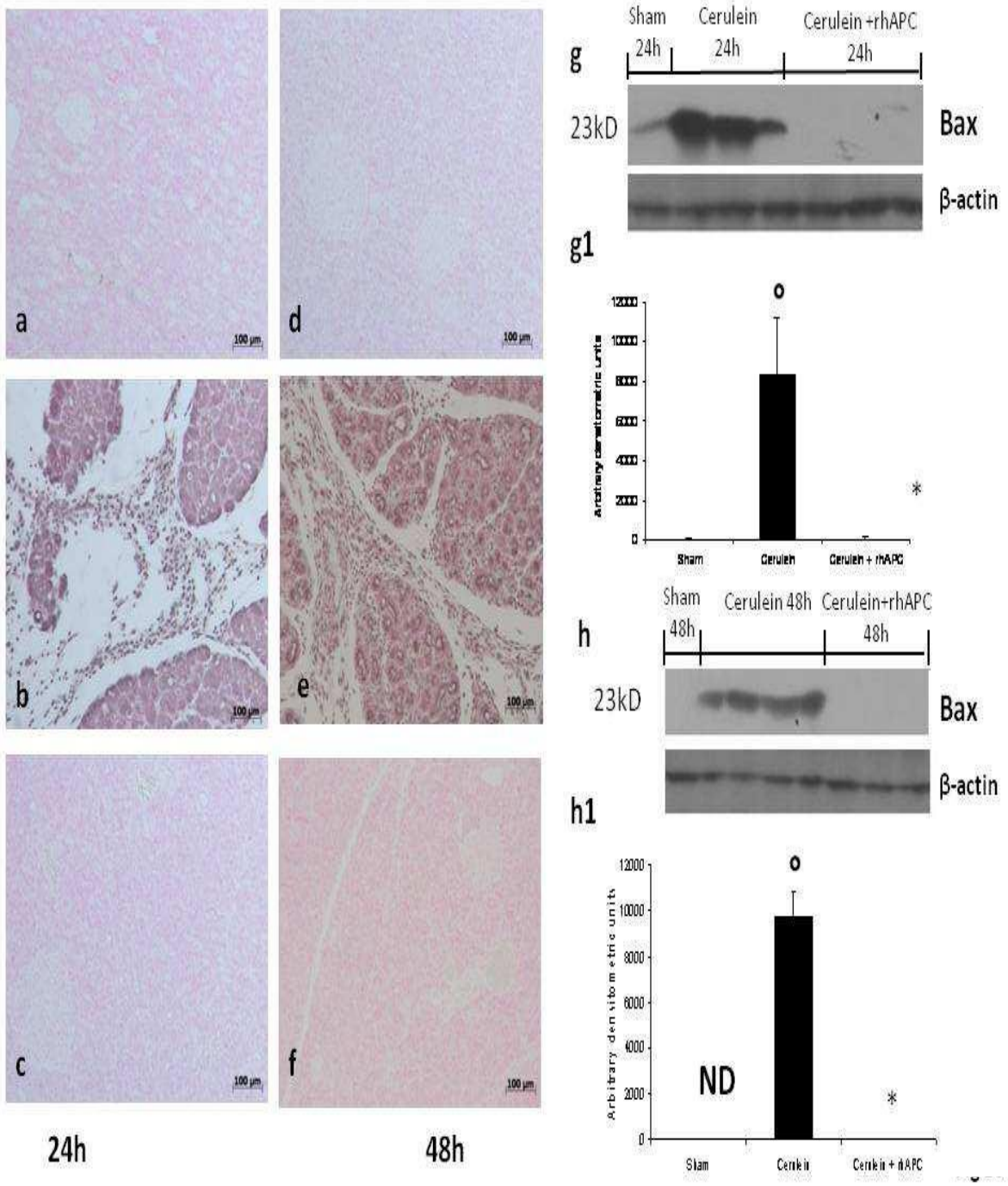



***Figure 2.11 Immunohistochemical localization of Bax in the pancreas.***

*No positive staining for Bax (a, d) was evidenced in sham-treated rats. Sections obtained from cerulein-treated rats showed intense positive staining for Bax (b, e). Contrarily, the positivity for Bax was abolished in rats administered with rhAPC (c, f). A representative blot of lysates for Bax (g, h) and the correspondent densitometry analyses of all animals are reported. rhAPC caused a significant decrease in Bax levels at 24 and 48 hours after cerulein induction (g, h). The results in g1 and h1 are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs. cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

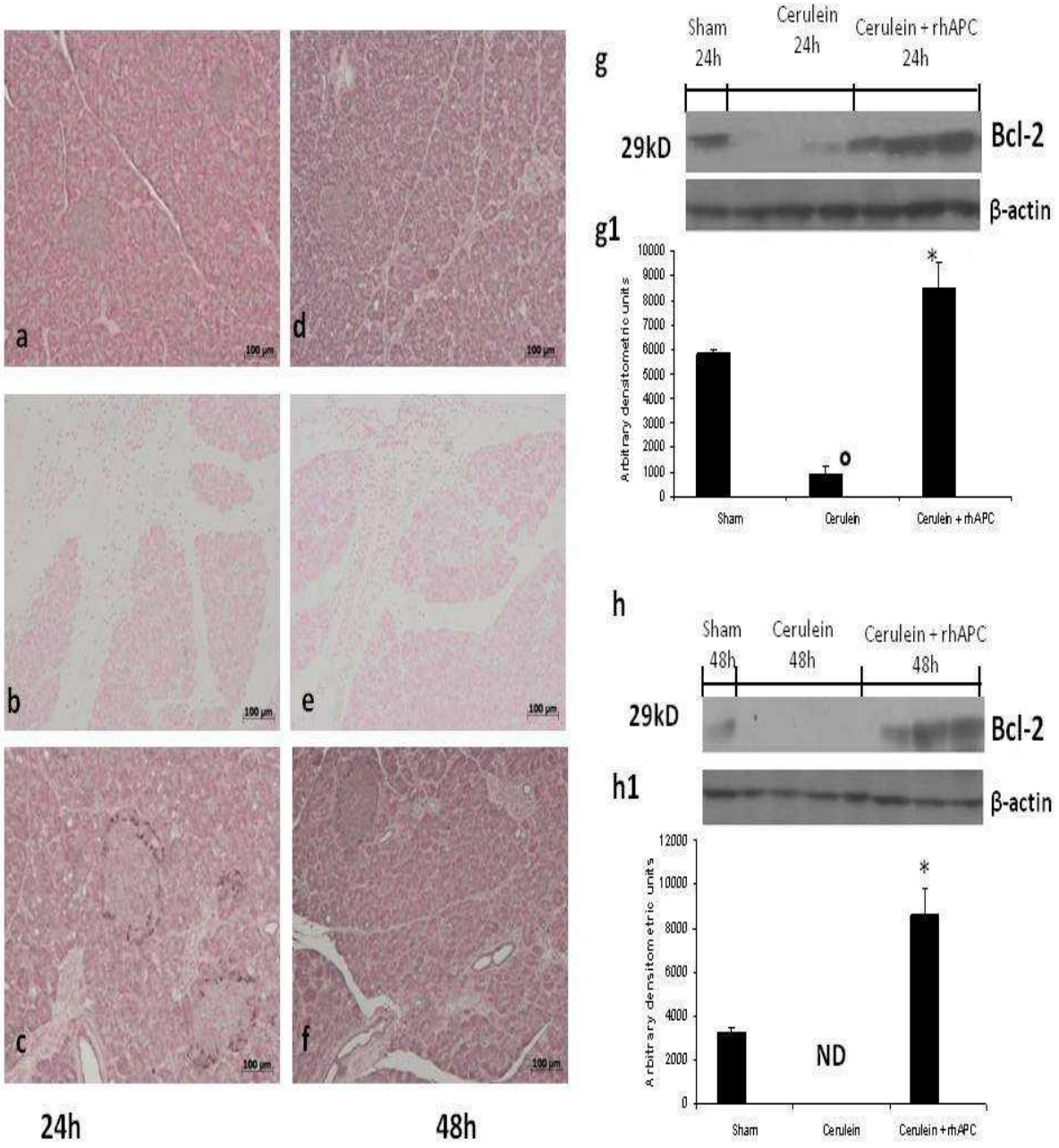



***Figure 2.12 Immunohistochemical localization of Bcl-2 in the pancreas.***

*No positive staining for Bcl-2 (a, d) was evidenced in sham-treated rats. Sections obtained from cerulein-treated rats showed negative staining for Bcl-2 (b, e). Contrarily an intense staining for Bcl-2 was also observed in rhAPC-treated rats (G). A representative blot of lysates for Bcl-2 (g, h) obtained from 5 animals per group is shown, and the correspondent densitometry analyses of all animals (g1 and h1 respectively) are reported. rhAPC prevented cerulein-induced Bcl-2 degradation. The results in B and D are expressed as mean ± SEM from 5 or 6 pancreas samples for each group. *p<0.01 vs. sham, ◦p<0.01 vs. cerulein. 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

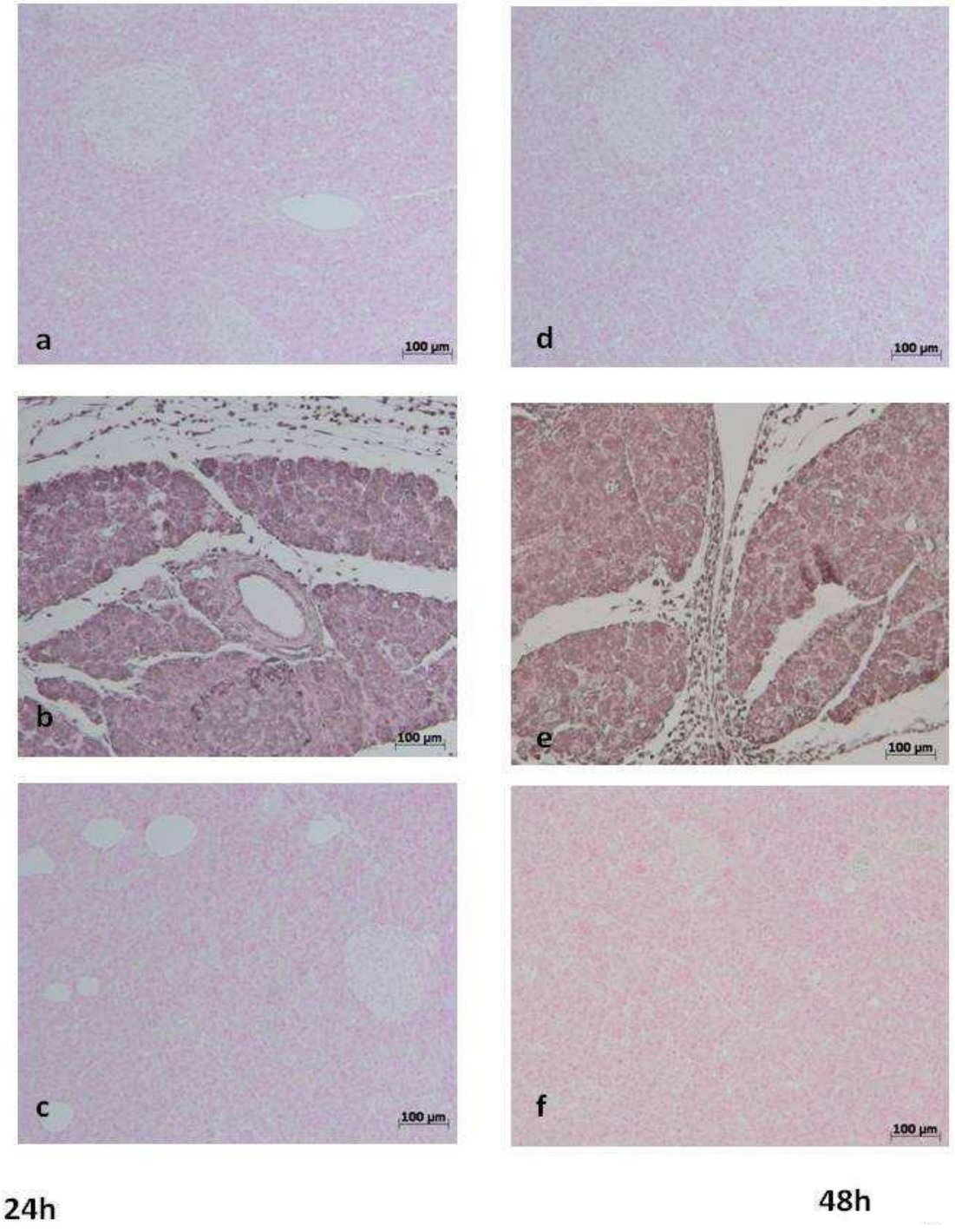



***Figure 2.13 Immunohistochemical localization of Fas Ligand in the pancreas.***

*No positive staining for Fas Ligand (a, d) was evidenced in pancreas sections collected at 24 and 48 hours after cerulein induction from sham-treated rats. Contrarily, sections obtained from cerulein-treated rats showed intense positive staining (b, e). The positivity for Fas Ligand was abolished in sections from rhAPC-treated rats (c, f). 24 h and 48h represent time points of animals sacrificed in relation to the two protocols.*

## 4.4 Discussion:

Recombinant Human Activated Protein C has been widely used in the management of sepsis.[116]

Exploiting the similarity of microvascular thrombosis between acute pancreatitis and sepsis our study has demonstrated the potential use of Recombinant Human Activated Protein C in acute pancreatitis.

Administration of cerulein in this study resulted in a demonstrable increase in the inflammatory markers, increase in the amylase and lipase levels and histological features of acute pancreatitis. There was marked reduction in these parameters on administration of rhAPC in both the early and late intervention protocols.

The disease related risk of intra-abdominal haemorrhage has raised specific concerns about the safety of the drug with anticoagulant properties being used in patients with acute pancreatitis. Neither was there any pancreatic manipulation leading to pancreatic haemorrhage during experimentation, nor was there any demonstrable evidence in our study of rhAPC affecting the coagulation cascade

Intervention with recombinant human Activated Protein C significantly attenuated pancreatic injury in this model with reduction in plasma enzymes, histological features of pancreatic injury, cytokine and endothelial adhesion molecule expression and perhaps unexpectedly, the features of apoptosis. These effects of recombinant human Activated Protein C were seen both in groups where the drug was administered early and when the drug was given 24h after induction of pancreatic injury.

A marked increase in the levels of IkB-α and significant drop in the NF-κB p65 (nuclear) levels were noted in the pancreatitis group treated with rhAPC. This would suggest that the ameliorative effect of rhAPC on acute pancreatitis may be mediated through the NF-κB

pathway. Suppression of NF-κB activation, in turn, may have influenced the release of pro-inflammatory cytokines such as TNF-α.

The breadth of markers of pancreatic injury examined in this study provides insight into the possible multivalent actions of recombinant human Activated Protein C in this model. Effects on production of pro-inflammatory cytokines such as TNF-α may influence both local and remote injury and together with the down-regulation of endothelial adhesion molecules ICAM-1 and P-selectin may lead to a reduction in neutrophil infiltration and neutrophil-mediated oxidative injury.

The anti-apoptotic effect of intervention with recombinant human Activated Protein C is a potential paradox in the setting of pancreatic parenchymal injury. Cell death by apoptosis is regarded as generating a lesser inflammatory signal than death by necrosis and thus although the global actions of intervention with recombinant human Activated Protein C are anti-inflammatory, an anti-apoptotic effect may have adverse consequences on the apoptosis-necrosis cell death balance and raises questions requiring further study. A longer time course clinical model may be invaluable to investigate this as the relatively truncate 48h time course of the present protocol may be an insufficient duration to unmask any potential late “pro-necrotic” effects of intervention with recombinant human Activated Protein C.

A key finding of this study is that intervention with recombinant human Activated Protein C is not associated with pancreatic or remote-organ haemorrhage. Caution must be exercised in extrapolating from this finding as in the clinical setting, haemorrhage is typically a late feature associated with untreated or inadequately drained sepsis. The present findings provide some assurance that early intervention with recombinant human Activated Protein C in the setting of necrotizing pancreatitis is not associated with haemorrhage but further work (again possibly with a longer time course (72 hours) model) is required to ascertain that there are no haemorrhage-related late effects (for example related to accentuated necrosis).

This study augments the findings of the experimental studies on recombinant human Activated Protein C in acute pancreatitis. Specifically, intervention is associated with a profound reduction in inflammation that is seen either with early administration or with administration 24h after induction of acute pancreatitis. This reduction of inflammation is mediated by modulation of NF-κB/IkB-α cleavage and the western blot data suggest that at least a component of the action of recombinant human Activated Protein C in experimental acute pancreatitis is mediated by the NF-κB pathway. In turn, this may modulate pancreatic cytokine production and neutrophil diapedesis. A complex pattern of action of the intervention agent is seen in the anti-apoptotic effects of recombinant human Activated Protein C as demonstrated by TUNEL. Diversion of programmed cell death from an apoptotic to a necrotic pathway may have deleterious consequences and requires further study.

In summary, this study provides the most detailed examination to date of the effect of intervention with recombinant human activated protein C in experimental acute pancreatitis. There is a profound, global anti-inflammatory effect which is mediated, at least in part, by action at the NF-κB and apoptotic pathways. There is no evidence that intervention is associated with increased pancreatic or remote organ haemorrhage. Taken together with the evidence of reduction in mortality of experimental acute pancreatitis provided by the Alsfasser study[76], this study provides a foundation towards evaluation of recombinant human activated protein C in clinical severe acute pancreatitis.

# 5 Recombinant human activated protein C modulates the sub-cellular expression of Caspases in L-Arginine induced Acute Pancreatitis

## 5.1 Introduction

The disease severity of acute pancreatitis (AP) forms a continuous spectrum, from the relatively mild form characterised by abdominal pain without remote organ injury to a severe illness with catastrophic multiple organ failure. Mild acute pancreatitis is essentially a self-limiting illness requiring supportive care, whereas the severe form entails early, dynamic and sustained management to minimise mortality.

Disease progression of severe AP is dominated by a sequel of peri-pancreatic sepsis[115] and multi- organ failure. Although peri-pancreatic sepsis follows a similar course to other forms of intra-abdominal sepsis, there is an increased risk of catastrophic intra-abdominal haemorrhage[115]. The pathophysiology of bleeding at this stage may be related to the effects of pancreatic juice in the lesser sac as a consequence of pancreatic ductal blow-out[117]

Exploiting the similarity in the pathophysiology of severe AP and sepsis raises the possibility of utilising recombinant human activated protein C(rhAPC) [59] presently being used for severe sepsis. This 55kD glycoprotein analogue of vitamin K-dependent endogenous protein C is synthesised from genetically engineered human cells[59]. rhAPC is known for its effect on the anticoagulant pathway and its cytoprotective effect. Acting through the anticoagulant pathway it plays an important role in the maintenance of haemostasis, inhibiting thrombin generation and stimulating fibrinolysis.[59] Various studies have explored the antiapoptotic action of rhAPC as means of exerting its cytoprotective effect directly on cells [45 118 119]. These antiapoptotic effects are known to involve non-compartmentalised specific enzymes cysteine proteases (commonly known as caspases) which are essential for the normal survival of healthy cells but held under tight control under normal conditions. The

activated cellular caspases exercise their effect through two separate pathways; the intrinsic and extrinsic pathways. [6, 46, 42] The pathways are composed of numerous enzymes, but three of those are positioned in crucial points in the entire process of apoptosis. Caspase 9 is regarded as the "canonical Caspase" of the intrinsic mitochondrial pathway whereas Caspase -8 is the key initiator of death receptor mediated apoptosis through the extrinsic pathway. The main down stream effector Caspase responsible for cleaving the majority of cellular substrates in apoptotic cells is Caspase 3.[47]

Identification of changes in cells due to apoptosis incorporates various techniques. Although the mainstay of apoptosis detection, electrophoresis is limited by its applicability in detecting morphological changes in individual cells[119]. The morphological staining methods available such as the Ethidium Bromide and acridine orange, DAPI, Hoechst staining, annexin V staining and DNA ladder assays require multiple steps which may result in damage of the cell membrane and change in cell population distribution of live, apoptotic and /or necrotic cells[120]. The TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling) procedure although being frequently used requires experience in assessment as it is subject to misinterpretation due to presence of necrotic cells which also generate fragmented DNA[119].

Utilising immunohistochemical staining methods and computer assisted image analysis of rat pancreatic tissue, this part of my study identified and quantified for the first time, the effect of Recombinant human activated protein C on the expression of Caspase 8, 9 and 3 in an L-Arginine induced acute pancreatitis model.

## 5.2 Methods

### *5.2.1 Study Design*

The study utilised adult male Sprague-Dawley rats with a median weight of 280g (range 230g-340g), conducted under a home office licence *(P.I.L 40/7973 – issued 11/03/2005)* with ethics committee approval. The study conformed to the University of Manchester ethical

guidance on animal welfare during experimentation. The additional controls for the study on the same type of animals were also carried out at the University of Messina, Italy, under their strict ethical guidance on animal welfare.

### *5.2.2 Animal Care*

Prior to the commencement of the experiment the animals were housed in metabolic cages with controlled temperature and 12-hour light–dark cycles for at least 1 week. During the course of the experiment animals were monitored in compliance with current animal care regulations and analgesia and supplemental fluids were administered.

### *5.2.3 Induction of Acute Pancreatitis*

There is a concentration dependent increase in the degree of pancreatic injury in the L-arginine induced experimental rat pancreatitis model. At high doses mortality secondary to chemical peritonitis was found as a feature. In the interests of refinement of study design and reduction of animal waste, selection of a concentration of 300mg/100g body weight of L-arginine represented a pragmatic compromise between the extent of pancreatic injury and animal mortality. The dose selection part of the project was carried out by predecessor. Data from animals that died during the dose selection are not included in this thesis

Pancreatitis was induced by an intraperitoneal bolus of 300mg/100g body weight of L-arginine diluted in 0.15 M saline which was administered to non-anaesthetised rats to induce pancreatitis.

### *5.2.4 Drug Administration*

Recombinant human activate protein C (source -Eli Lilly, Indianapolis USA) used for the study was 50 µg/100g body weight.

### *5.2.5 Group allocation.*

The rats were randomly allocated to four main groups with 14(controls), 12(acute pancreatitis), 6 (Pre-treatment), 5(post-treatment) animals in the specified groups:

#### 5.2.5.1 Vehicle Control

Intraperitoneal injection of sterile normal saline in a volume equivalent to treatment groups.

#### 5.2.5.2 Drug Control

Intravenous administration of rhAPC in an equivalent volume to treatment groups to animals which had no AP induction.

#### 5.2.5.3 Normal Control Group

These animals had no pharmacological intervention and they were under observation during the period of the experiment.

#### 5.2.5.4 Acute Pancreatitis group

The animals were treated with intraperitoneal administration of L-Arginine, with no subsequent treatment.

#### 5.2.5.5 Pre- Treatment group

Recombinant human activated protein C was administered 30 minutes before inducing Acute Pancreatitis. This group represents the greatest likelihood of demonstrating organ injury amelioration following therapeutic intervention with rhAPC. Even though in the clinical setting it is not possible to carry out pre-emptive treatment results from this group are necessary to demonstrate that rhAPC can modulate experimental acute pancreatitis.

#### 5.2.5.6 Post-Treatment group

Recombinant human activated protein C was administered 30 minutes after induction of acute pancreatitis. This group investigates the potential for APC to ameliorate organ injury in an ideal therapeutic setting before extensive disease evolution. In clinical practice, current trends recognise that the optimum timing for intervention in acute pancreatitis is prior to full-scale evolution of organ injury. Enzymatic markers of pancreatic injury are evident at 10-15 minutes in the serum in this model.[121]

### *5.2.6 Tissue harvesting and processing*

Following strict guidelines, animal were sacrificed at 48 hours, from induction of AP, the pancreatic tissue was harvested and immediately immersed in 10% buffered formalin (prepared from 4% paraformaldehyde in 0.1%PBS, pH 7.6) for 18-24 hours, washed in buffer and routinely processed to paraffin wax blocks. 5µm thick consecutive sections were cut from each paraffin block ensuring that adequate amount of pancreatic tissue was visible at the cross-section.

### *5.2.7 Haematoxylin & eosin staining*

Haematoxylin and eosin staining were performed on the slides as per standard protocols and used for general histological assessment of the tissue.

### *5.2.8 Assessment of blood samples*

Samples were analysed for full blood profile as well as for amylase and lipase levels. The investigation was carried out at a regional veterinary laboratory using an Auto lab system (Central diagnostic services, The Queen's Veterinary School Hospital, Cambridge).

### *5.2.9 Cytokine assays:*

#### 5.2.9.1 Enzyme Linked Immunosorbent Assay for TNF-α

A 96- well polystyrene micro plate solid phase commercial ELISA Kit (R& D Systems Minneapolis, USA) containing E.coli-expressed recombinant rat TNF α and antibodies raised

against the recombinant factor were used. This procedure employed the quantitative sandwich enzyme immunoassay technique. The rat serum required a two- fold dilution with Calibrator diluent consisting of a buffered protein base. A standard absorbance curve was generated by serial dilution of the rat TNF-α standard. Fifty microlitre of diluted sample was added to the rest of the wells at room temperature. Fifty microlitre of standard containing recombinant rat cytokine in a buffered protein base with preservatives was pipetted in to each of the wells. After gentle mixing, the plates were incubated for two hours at room temperature. To remove any unbound substances mechanical washing of the plates with buffer solution (buffered surfactant with preservative) was employed. Using a multichannel pipette, 100 µl of Conjugate (polyclonal antibody against TNF α) was added in to each well. The plates were incubated for two hours at room temperature and washed with buffer solution. Hundred microlitre of substrate solution was added to the wells and incubated for 30 minutes in a dark room. On adding 100 µl of stop solution containing diluted hydrochloric acid solution in to each well, the blue colour in some of the wells changed to yellow. After ensuring thorough mixing, optical density of each well was assessed within 30 minutes using a MRX Micro plate Reader (Dynex Technologies Inc.) with Revelation software, set to 450nm. The sample values were then read off the standard curve.

#### 5.2.9.2 Enzyme Linked Immunosorbent Assay for IL-1β

A 4.5 hour solid phase ELISA designed to measure rat IL-1 was used (R& D Systems Minneapolis, USA). The 96- well polystyrene micro-plate commercial ELISA Kit was coated with polyclonal antibody specific for rat IL-1β. The rat serum required a three- fold dilution with Calibrator diluent consisting of a buffered protein base. A standard absorbance curve was generated by serial dilution of the rat IL-1β standard. Fifty microlitre of diluted sample was added to the rest of the wells at room temperature. Fifty microlitre of rat IL-1β standard containing recombinant rat cytokine in a buffered protein base with preservatives was pipetted in to each of the wells. After gentle mixing, the plates were incubated for two hours at room temperature. To remove any unbound substances mechanical washing of the

plates with buffer solution (buffered surfactant with preservative) was employed. Using a multichannel pipette, 100 µl of rat IL-1β Conjugate (polyclonal antibody against IL-1β) was added and the plates were incubated for two hours at room temperature. The plates were consequently washed with buffer solution. Hundred microlitre of substrate solution was added in to the wells and incubated for 30 minutes in a dark room. On adding 100 µl of stop solution containing diluted hydrochloric acid solution in to each well, the blue colour in some of the wells changed to yellow. After ensuring thorough mixing the optical density of each well was assessed within 30 minutes using a MRX Microplate Reader (Dynex Technologies Inc.) with the Revelation software, set to 450nm. The sample values were then read off the standard curve.

#### 5.2.9.3 Enzyme Linked Immunosorbent Assay for IL-6

A 96- well polystyrene micro plate solid phase commercial ELISA Kit (R& D Systems Minneapolis, USA) containing E.coli-expressed recombinant rat IL-6 and antibodies raised against the recombinant factor were used. This procedure employed the quantitative sandwich enzyme immunoassay technique. No dilution of rat serum was carried as per manufacturer's advice. A standard absorbance curve was generated by serial dilution of the rat IL-6 standard. Fifty microlitre of the rat serum sample was added to the rest of the wells at room temperature. Fifty microlitre of standard containing recombinant rat cytokine in a buffered protein base with preservatives was pipette in to each of the wells. After gentle mixing, the plates were incubated for two hours at room temperature. To remove any unbound substances mechanical washing of the plates with buffer solution (buffered surfactant with preservative) was employed. Using a multichannel pipette, 100 µl of Conjugate (polyclonal antibody against IL-6) was added in to each well. The plates were incubated for two hours at room temperature. The plates were then washed with buffer solution. Hundred microlitre of substrate solution was added in to the wells and incubated for 30 minutes in a dark room. On adding 100 µl of stop solution containing diluted hydrochloric

acid solution in to each well, the blue colour in some of the wells changed to yellow. After ensuring thorough mixing the optical density of each well was assessed within 30 minutes using a MRX Micro plate Reader (Dynex Technologies Inc.) with the Revelation software, set to 450nm. The sample values were then read off the standard curve.

### *5.2.10 Immunohistochemical analysis for Caspase 3*

The 5µm tissue sections were deparaffinised in four changes of Xylene (10 minutes each) and rehydrated in five changes of industrial methylated spirit (IMS: 4x99% and 1X95%: 5 minute changes) followed by distilled water ($dH_2O$) and Tris-buffered saline (TBS). Antigen retrieval (AR) technique for each antibody was first followed according to manufacturer's recommendation, but each Caspase antibody was tested on tissue 1) without pre-treatment, 2) with microwaving in citrate buffer (pH6.0) 3) pressure cooking in citrate buffer (pH6.0) and 4) enzymatic pretreatment with 0.1trypsin. Testing all those antigen retrieval methods and then choosing was performed to ensure that the best signal to background staining ratio was obtained. This precaution was necessary as the planed computer assisted image analysis required a very low background staining to avoid overestimation of the results.

Breifly, the microwave AP was achieved placing tissue sections in citrate buffer (pH 6) in a ceramic pressure cooker in a microwave (850W) oven set at high power for 15 minutes. The power was then lowered to 450 W for 10 minutes with a subsequent cooling period of 40 minutes and washed in buffer. To block endogenous peroxidases, tissue was incubated with peroxidase blocking agent (DAKO ChemMate$_{TM}$.Glostrup, Denmark) for 10 minutes at room temperature, washed with distilled water and Phospate Buffered Saline with 0.05% Tween (PBS/Tween20) (Sigma Aldrich, USA).

Non-specific staining was blocked with the protein block (Dako) for 10 minutes at room temperature and then the tissue sections were incubated with Caspase 3 antibody (Biocare) diluted at 1:50 with DAKO antibody diluent. After overnight incubation at 6°C slides were washed with $dH_2O$ and PBS-Tween 20. The sections were treated with biotinylated anti-

rabbit IgG secondary antibody (Vector Labs, Peterborough, UK) diluted 1:300 with antibody diluents (Dako) for one hour, washed with PBS Tween 20 and incubated horse radish peroxidase avidin D (Vector Laboratories Inc, CA, USA); 1:300 PBS. The reaction was developed using SG chromogen (Vector Laboratories) lightly counterstained with Eosin and coverslipped using synthetic mountant.

### *5.2.11 Immunohistochemical analysis for Caspase 9*

A similar technique to immunostaining for active Caspase 3 was used with polyclonal antibody to active Caspase 9 (Acris Antibodies GmbH, Himmelreich) at a dilution of 1:100. No antigen retrieval was used as advised by the manufacturers.

### *5.2.12 Immunohistochemical analysis for Caspase 8*

A similar protocol to immunostaining for active Caspase 3 was followed for Caspase 8, using the same antigen retrieval technique. Rabbit polyclonal antibody to Caspase 8 (ab4052, abcam®) was used at the recommended dilution of 1:100.

### *5.2.13 Quantification of apoptosis*

#### 5.2.13.1 Image analysis

Immunostaining patterns were examined by light microscopy (Leitz DM RB microscope). From each of these slides a representative region was identified at low power. The representative region was the area where the histologically confirmed damage to the tissue caused by L-Arginine was most pronounced. In all sections in which peritoneum was discernible, damage was most pronounced subperitoneally, diminishing towards the central part of the organ.The entire removed pancreatic tissue from each animal destined for immunohistochemical study was embedded in a paraffin block. Consecutive sections from each block were used for immunohistochemical staining for caspases.

Digital images were obtained from at least 10 high power field (HPF) per tissue section (at 40x magnification) using a Delta Pix camera. The digital images were analysed using Leica

QWin Standard V2.4 programme (Leica Microsystem Imaging, Cambridge, UK) using an in-house macro written in QUIPS. The microscope, camera and computer were calibrated according to a standardised procedure that was followed for each primary antibody. This enabled automatic computer detection and uniform measurement of immunopositive features. The programme was set to detect colour intensities in a fixed and constant range. The colour intensity allocated for oedema of tissue was subtracted from the total fixed representative region. This resulted in a measurement of pancreatic tissue area in a fixed frame.

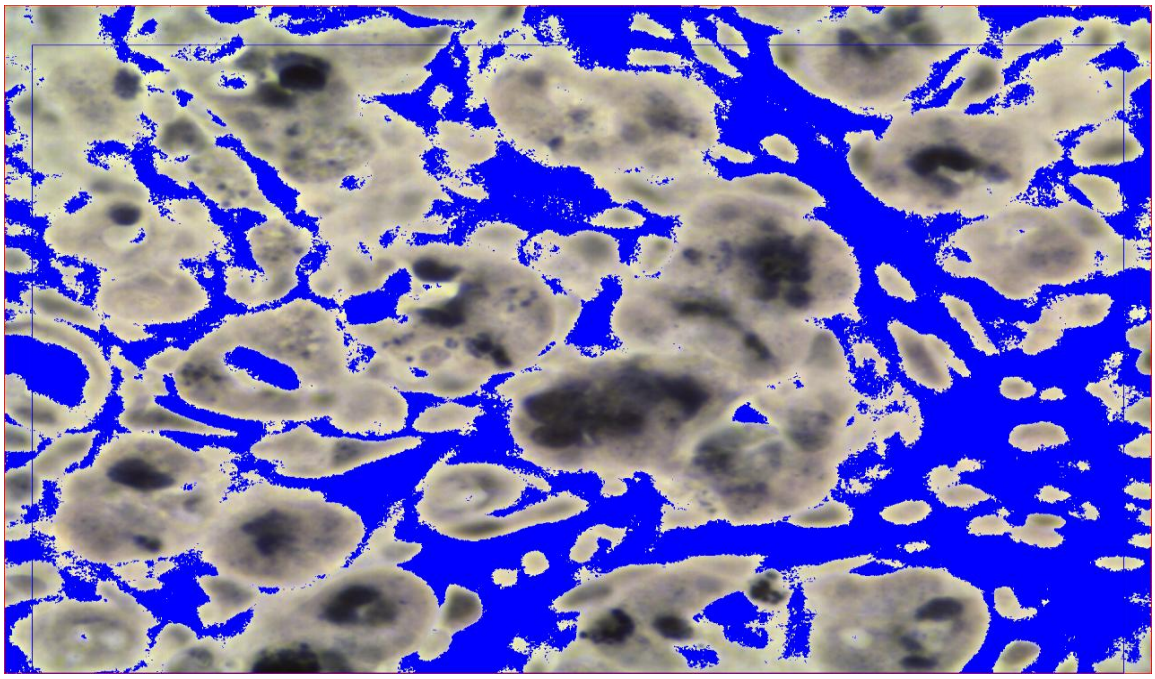


***Figure 3.1 The area covered by oedema is identified and false- coloured by the computer programme as blue***

The resultant non-oedematous tissue dense region was assessed for antibody staining based on the allocated uniform colour intensity.

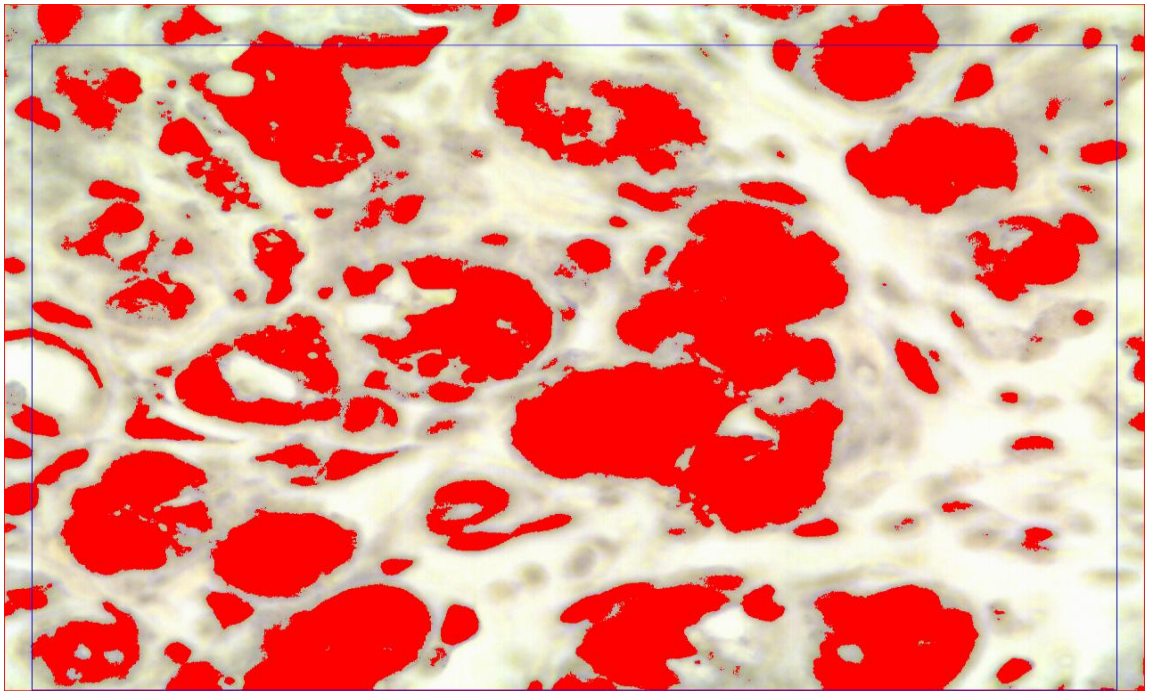


***Figure 3.2 Area covered by red signifies antibody staining***

This immunostained area was then quantified for each of the ten HPFs.

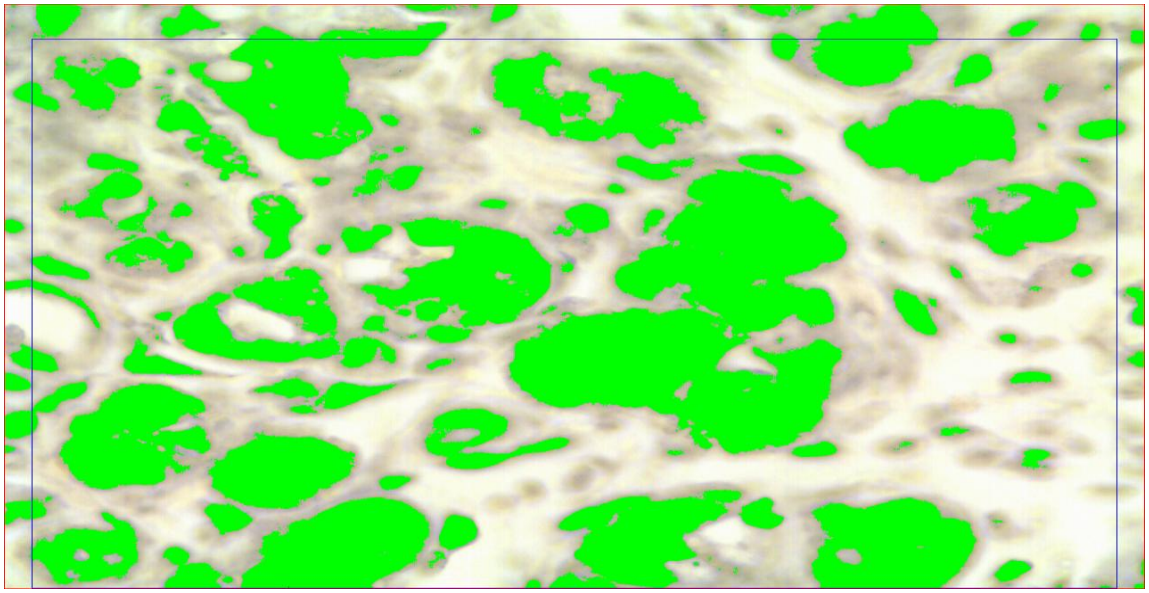


***Figure 3.3 Area covered by green signifies quantification of apoptotic area***

The cumulative result of immunostained area for each animal was then averaged per $mm^2$ and then the average of each group for each Caspase was used for comparative analysis.

| Field # | Area | Meas.Frame |
|---|---|---|
| 1 | 5899.05 | 19802.00 |
| 2 | 7030.38 | 19802.00 |
| Total | 12929.429 | 39603.996 |
| Mean | 6464.714 | 19801.998 |
| Std Dev | 565.668 | 0.000 |
| Std Error | 399.988 | 0.000 |
| Max | 7030.383 | 19801.998 |
| Min | 5899.046 | 19801.998 |
| 2-s Range | 2262.674 | 0.000 |

***Table 3: An example of data obtained per high power field***

| Pan 3.2 | | | | Slide No |
|---|---|---|---|---|
| Field # | Area | Total area/frame | | |
| 1 | 1382.89 | 19802 | 18419.11 | |
| 2 | 2435.26 | 19802 | 0.132214 | |
| 3 | 1491.71 | 19802 | 18310.29 | |
| 4 | 1556.3 | 19802 | 0.084996 | |
| 5 | 969.08 | 19802 | 18832.92 | |
| 6 | 1965.72 | 19802 | 0.104377 | |
| 7 | 1707.15 | 19802 | 18094.85 | |
| 8 | 1977.89 | 19802 | 0.109307 | |
| 9 | 620.35 | 19802 | 19181.65 | |
| 10 | 983.6 | 19802 | 0.051278 | |
| 11 | 573.03 | 19802 | 19228.97 | |
| 12 | 1580.35 | 19802 | 0.082186 | |
| 13 | 549.25 | 19802 | 19252.75 | |
| 14 | 1225.62 | 19802 | 0.063659 | |
| 15 | 149.6 | 19802 | 19652.4 | |
| 16 | 1919.25 | 19802 | 0.09766 | |
| 17 | 363.84 | 19802 | 19438.16 | |
| 18 | 4515.98 | 19802 | 0.232325 | |
| 19 | 1483.84 | 19802 | 18318.16 | |
| 20 | 3230.5 | 19802 | 0.176355 | |
| Total | 30681.22 | 396040 | | Total |
| Mean | 1534.061 | 19802 | | Mean |
| Std Dev | 997.627 | 0 | | Std Dev |
| Std Error | 223.076 | 0 | | Std Error |
| Max | 4515.984 | 19802 | | Max |
| Min | 149.599 | 19802 | | Min |
| 2-s Range | 3990.508 | 0 | | 2-s Range |

***Table 4: An example of data obtained & calculations from 10 high power fields***

#### 5.2.13.2 Semiquantitative assessment

Based on a visual analogue scoring system a semi quantitative scoring system was devised to establish intensity of staining.

Five randomly selected different high power fields from the periphery to the centre of the tissue were selected per slide using the same microscope setting for each slide to avoid bias. They were then graded, scored and tabulated based on the intensity of staining.

### *Statistical analysis*

Data are presented as medians and non-parametric tests are used for comparisons unless otherwise specified. Statistical analyses were carried out in Excel (Microsoft® Office Excel® 2007(12.0.6331.5000)) and Stats Direct statistical software Version 2.4.1 (Stats Direct, Altrincham, UK)

## 5.3 Results:

### *5.3.1 Biochemical Analyses*

Amylase and lipase levels were raised in the AP group compared to the control group. Although the values were not significantly different from the acute pancreatitis group a decrease in these biochemical parameters were noted in both the treated groups. This part of the project was carried out by my predecessor.

### *5.3.2 Pancreatic Histology*

Diffuse injury characterised by oedema, leukocyte infiltration, acinar cell disruption with areas of haemorrhage and necrosis were noted in the *Acute Pancreatitis Group*.

A reduction in the degree of pancreatic injury was noted with the *Pre- Treatment Group.* The rhAPC administered after induction of AP was associated with similar patterns of injury to the acute pancreatitis group. This part of the project was carried out by my predecessor.

### *5.3.3 Cytokine data*

#### 5.3.3.1 TNF-α

There was a non significant rise in levels of TNF-α in the acute pancreatitis group compared to control. Levels in both treatment groups did not significantly differ from other groups (*P=0.19, Kruskal-Wallis*).

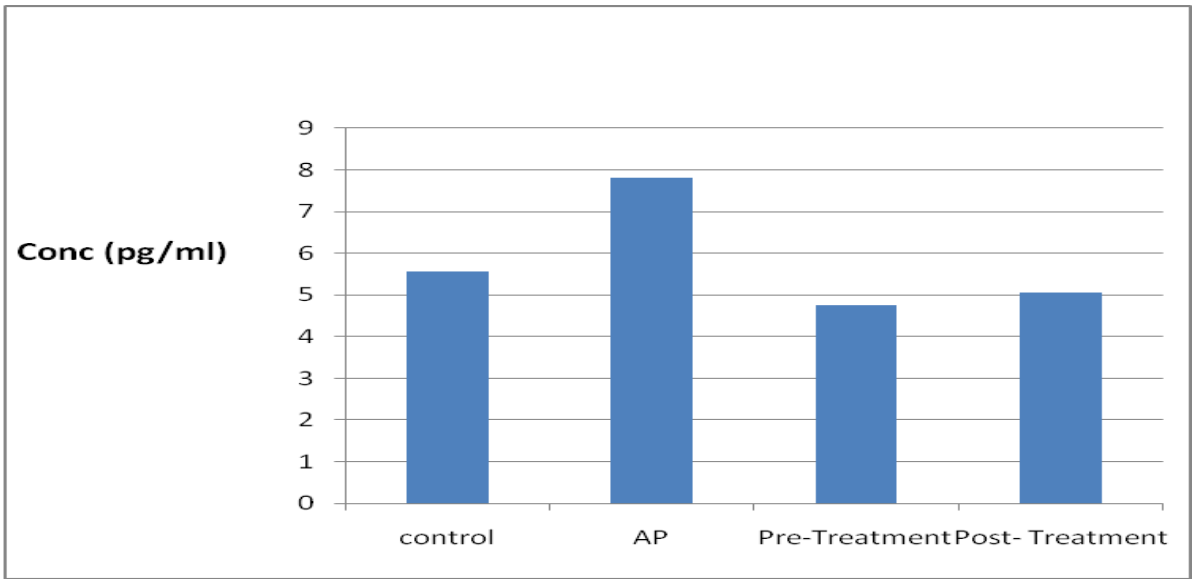



***Figure 3.4 Comparative levels of TNF-α***

### 5.3.3.2 IL-1β

There was a non significant rise in levels of IL-1β in the acute pancreatitis group.

Compared to control, levels in both treatment groups did not significantly differ from other groups (*P=0.28, Kruskal-Wallis*)

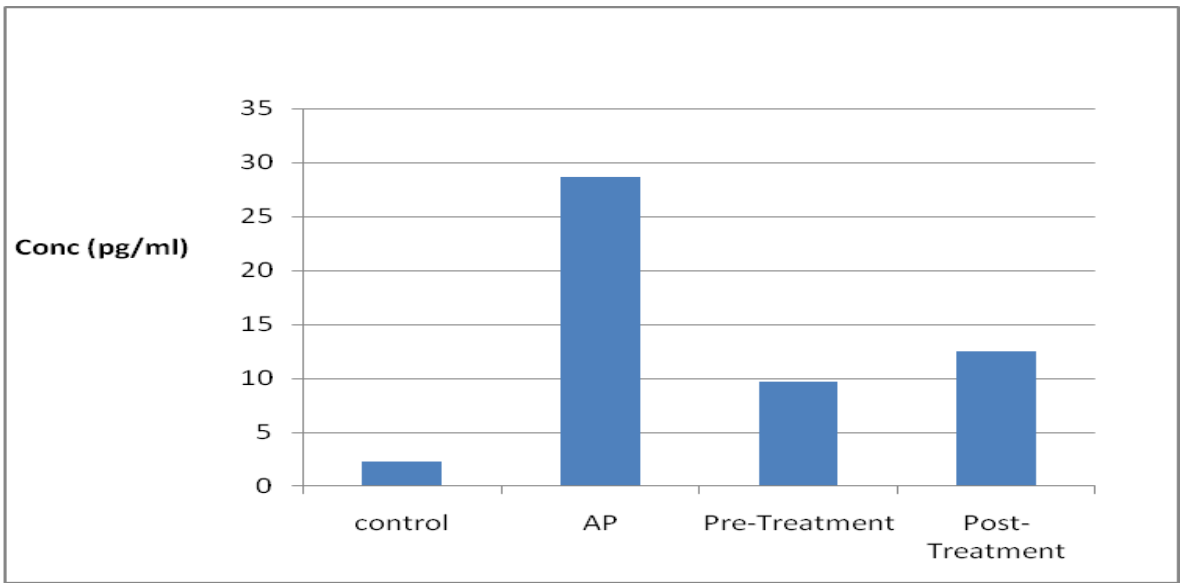



***Figure 3.5 Comparative levels of IL-1β***

### 5.3.3.3 IL-6

There was a non significant rise in levels of IL-6 in the acute pancreatitis group compared to control. Levels in both treatment groups did not significantly differ from other groups (*P=0.14, Kruskal-Wallis*)

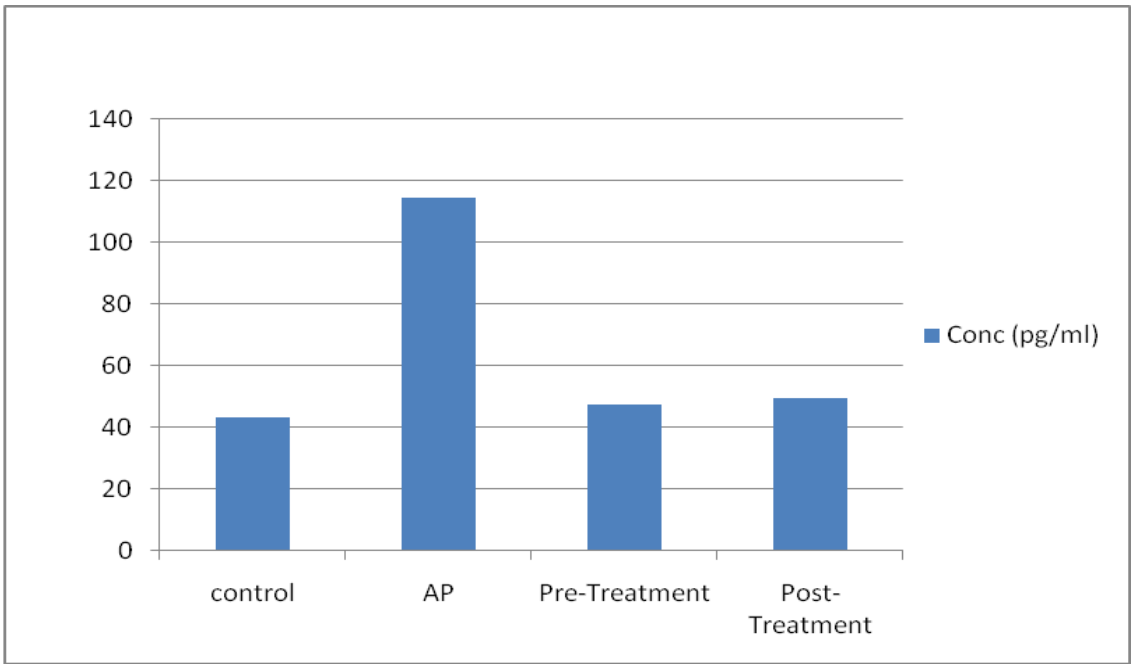
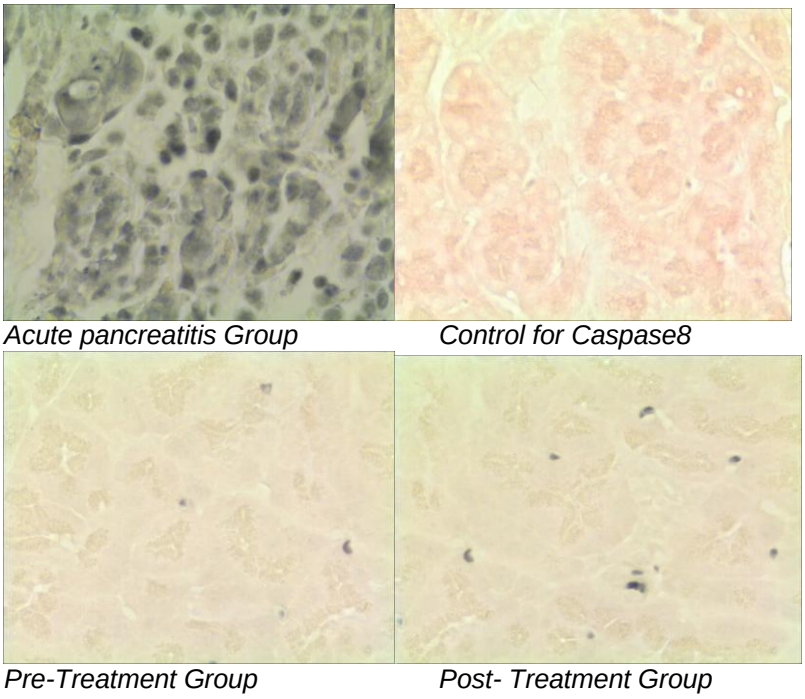



***Figure 3.6 Comparative levels of IL-6***

### *5.3.4 Caspase Expression data*

#### 5.3.4.1 Caspase 8



*Acute pancreatitis Group* *Control for Caspase8*

*Pre-Treatment Group* *Post- Treatment Group*

***Figure 3.7 Immunohistochemical analysis of Caspase 8***

There was a demonstrable increased level of expression of Caspase 8 in the pancreatitis group compared to the controls. There were no significant differences between the groups. *(Kruskal Wallis test P = 0.2558).* The *Kruskal-Wallis: pair wise comparison (Conover-Inman)* between the groups were not significant either

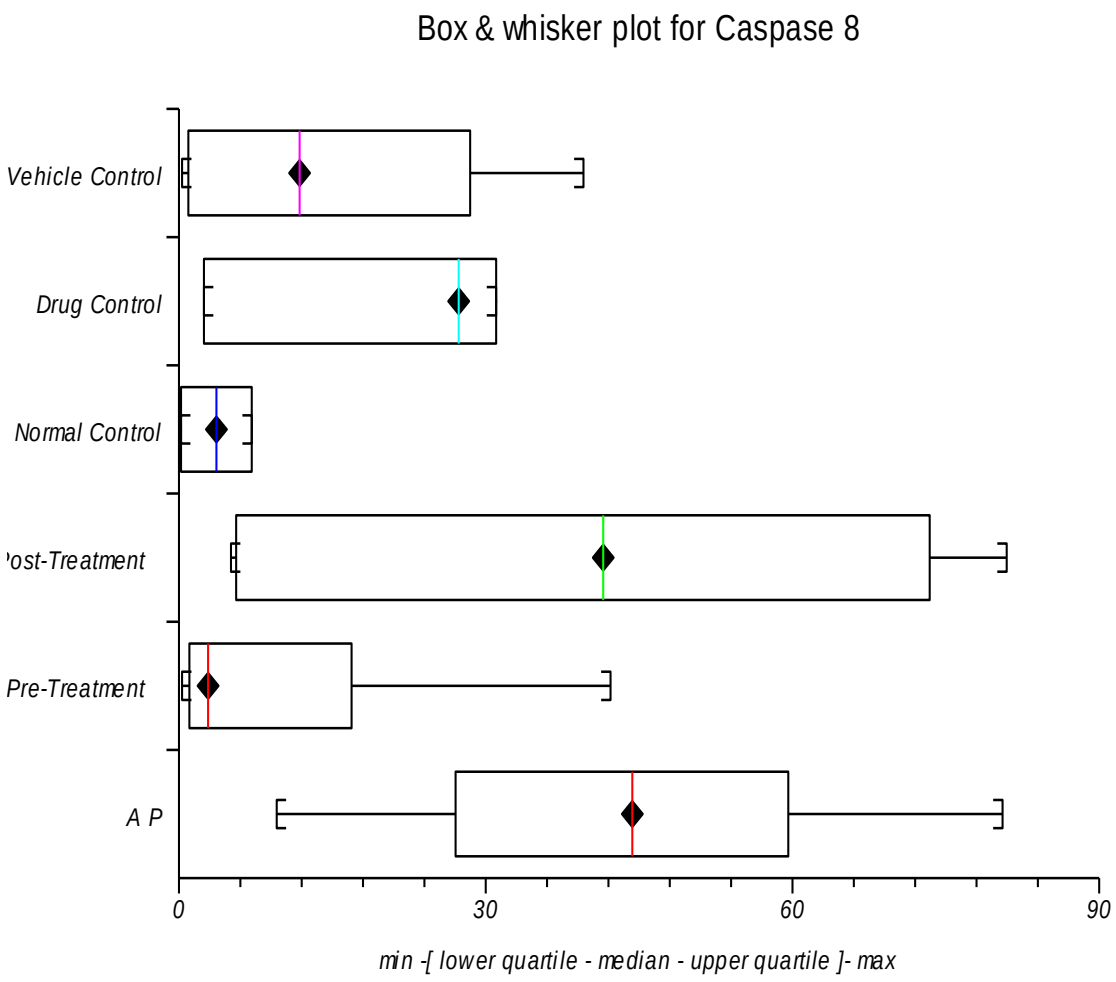
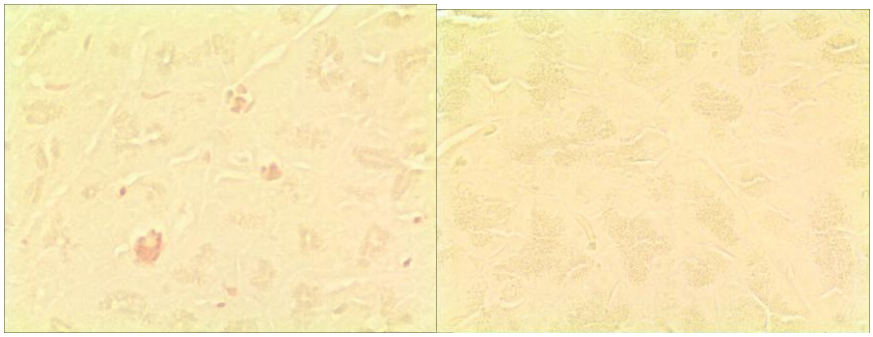
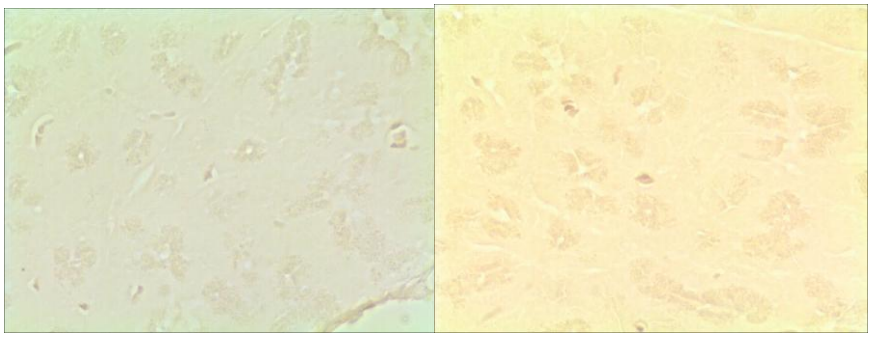



***Figure 3.8 Box & whisker plot for Caspase 8***

### 5.3.4.2 Caspase 9



*Acute pancreatitis Group* *Control Group for Caspase 9*



*Pre- Treatment Group* *Post- Treatment Group*

***Figure 3.9 Immunohistochemical analysis of Caspase 9***

Only a mild increase in expression of Caspase 9 was noted in the pancreatitis group with a decrease in the treatment groups. There were no significant differences between the groups. (*Kruskal Wallis test P = 0.2558).* The Kruskal-Wallis: pair wise comparison *(Conover-Inman)* between the groups were not significant either.

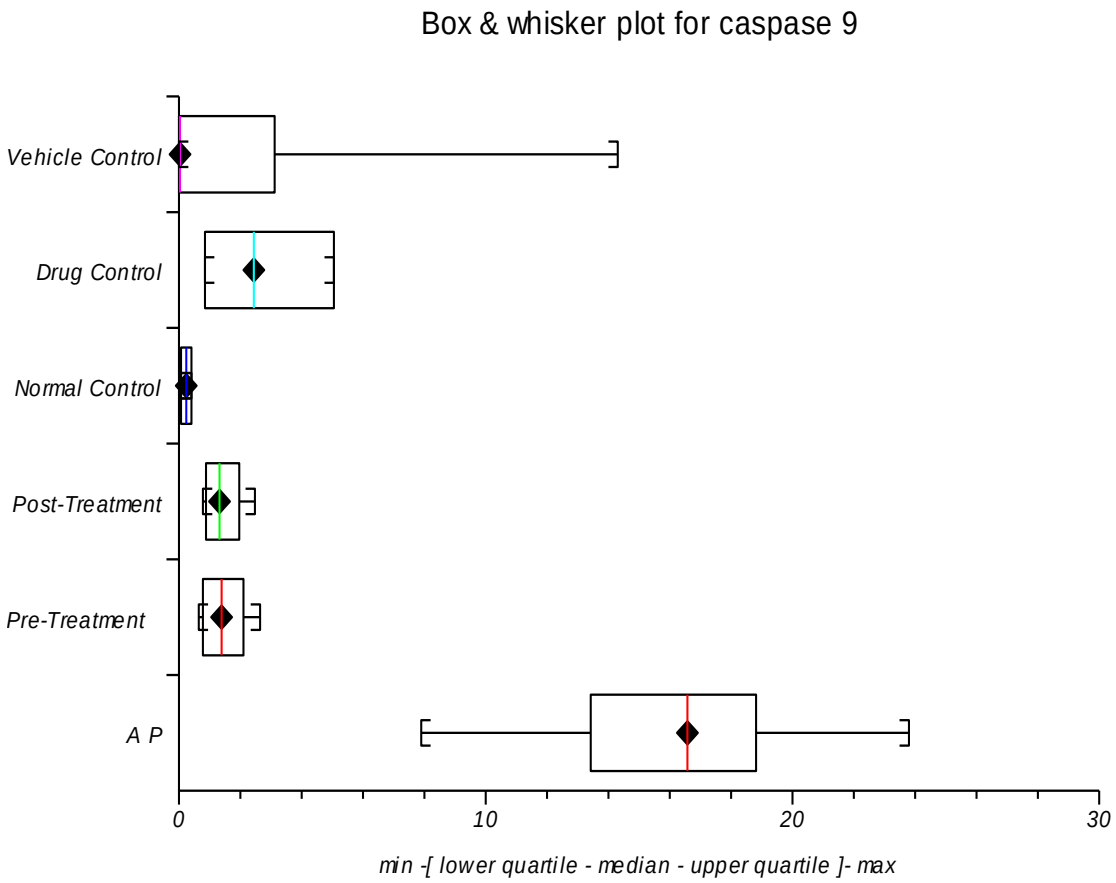
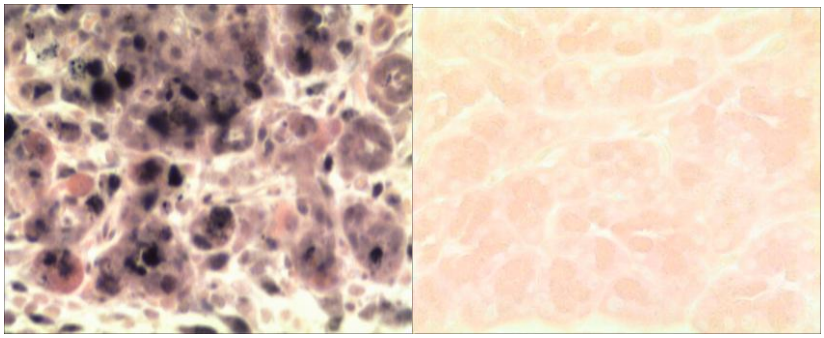
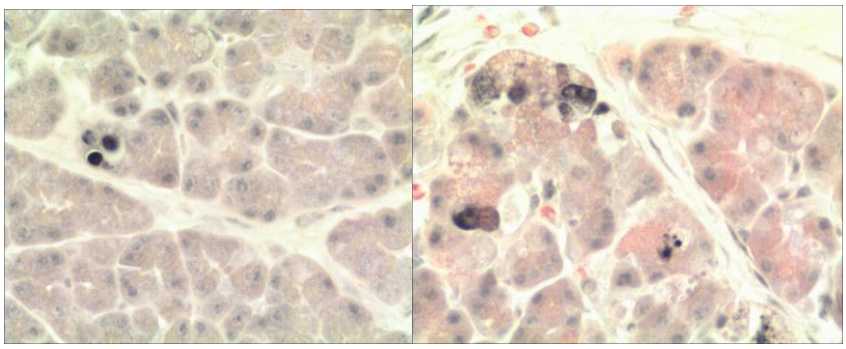



***Figure 3.10 Box & whisker plot for caspase 9***

### 5.3.4.3 Activated Caspase 3



*Acute pancreatitis Group* *Control Group for Caspase 3*



*Pre- Treatment Group* *Post- Treatment Group*

***Figure 3.11 Immunohistochemical analysis of Caspase 3***

There was a non significant elevation in the expression of Caspase 3 in the acute pancreatitis group. Levels in both the treatment groups did not significantly differ from the other groups. (*Kruskal Wallis test P = 0.2558.)*The *Kruskal-Wallis: pair wise comparison (Conover-Inman)* between the groups were not significant either.

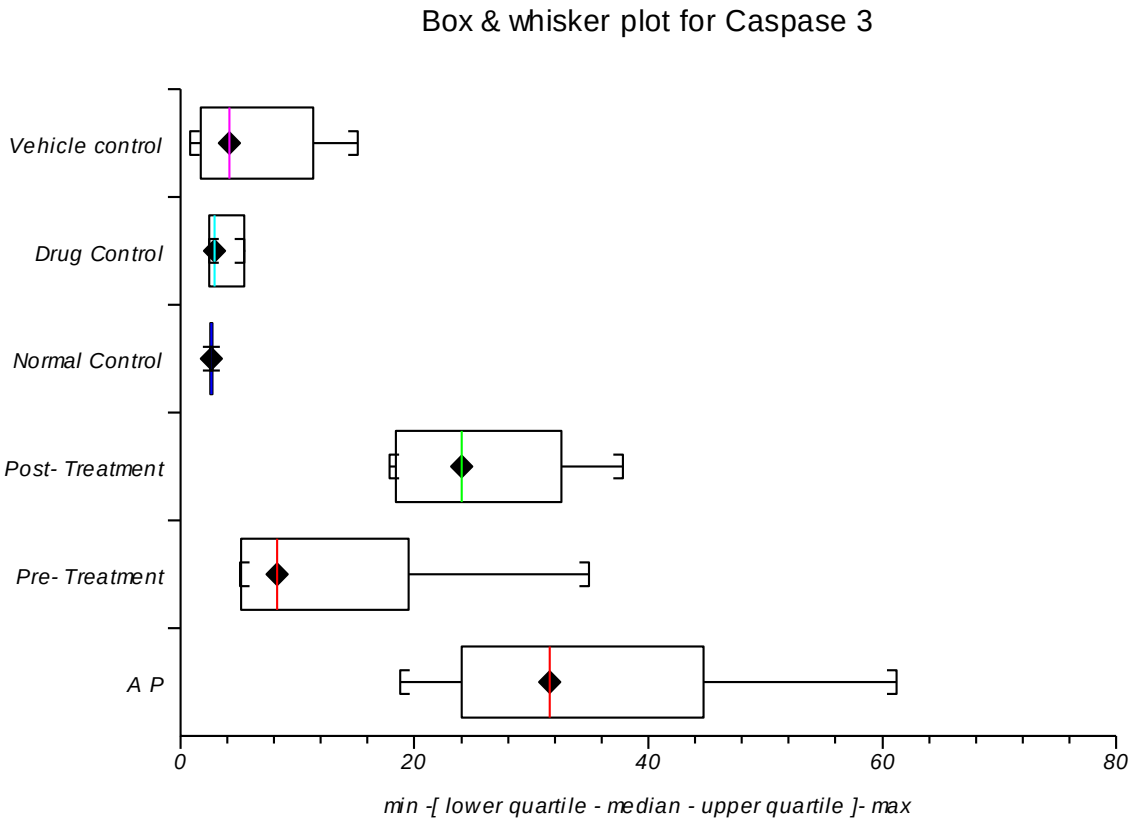



***Figure 3.12 Box & whisker plot for Caspase 3***

### 5.3.4.4 Comparative expression of Caspase 8, 9, 3

A non significant increased expression of Caspase 8 was noted in all the groups compared to the other caspases analysed (*P=0.1631, Kruskal-Wallis*). Caspase 9 was the least expressed of the three caspases. A cumulative expression of caspase 3 would be expected being an effector caspase but that expression is not observed in this study. *(P=0.15, Kruskal-Wallis).*

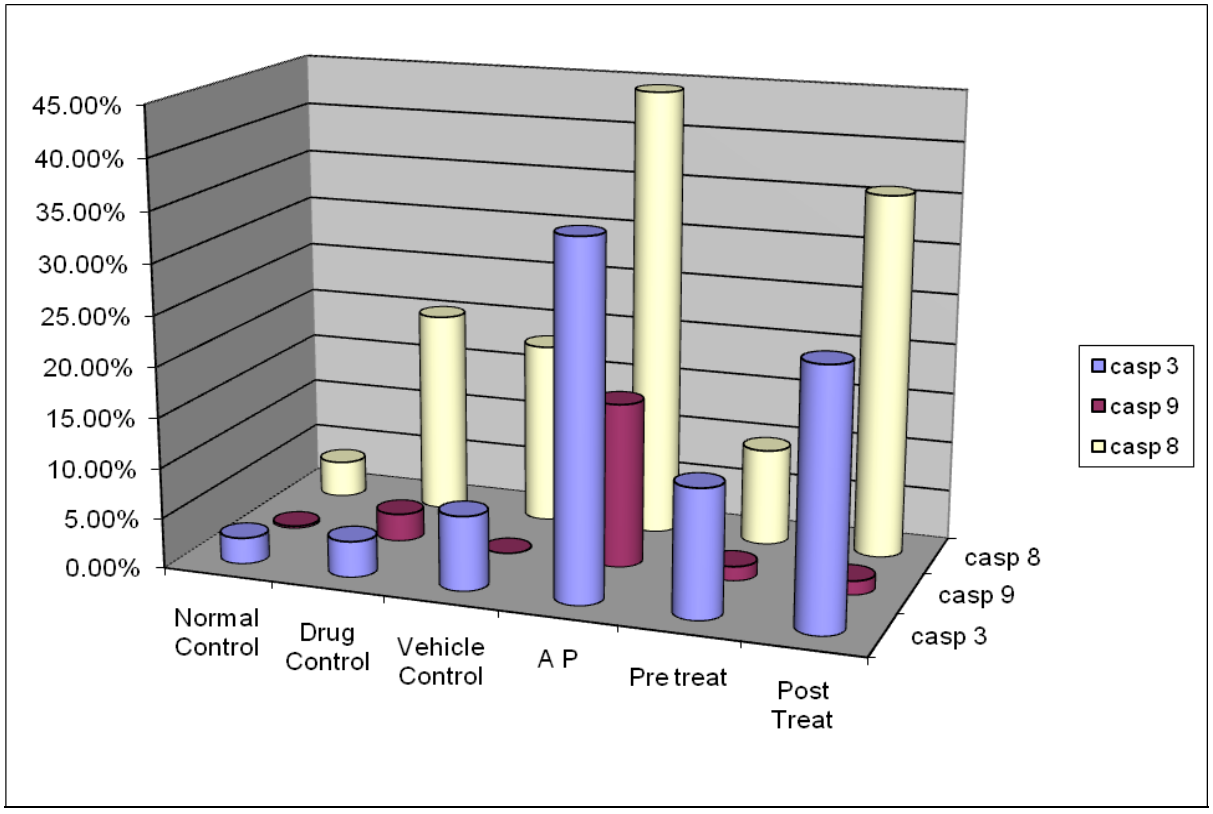



***Figure 3.13 Comparative expression of Caspase 8, 9, 3 using Image analysis***

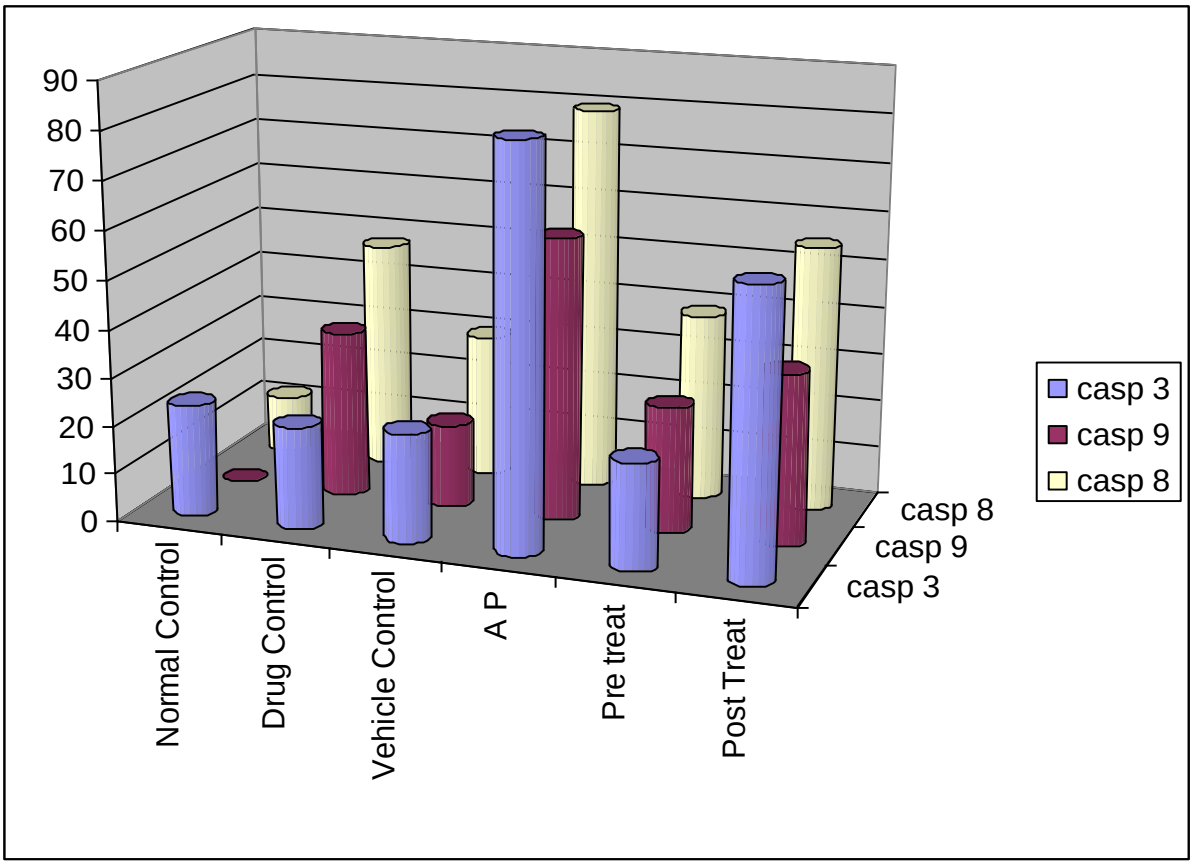



***Figure 3.14 Comparative expression of Caspase 8, 9, 3 using semi quantitative assessment***

## 5.4 Discussion:

The coagulation cascade is involved in the early phases of acute pancreatitis [122 123] and preventing thrombosis or bleeding by maintaining a normal coagulative state in the pancreatic microcirculation is a desirable objective.

Our study demonstrated hyperlipasemia and characteristic histological features of AP in the acute pancreatitis group. There was demonstrable increase in the levels of TNF α, IL-1β and IL-6 after induction of acute pancreatitis. The stimulation of these acute phase proteins indicates the activation of the inflammatory process taking place in acute pancreatitis.

Little is known of the effect of rhAPC on the apoptotic pathway in acute pancreatitis. Although experimental studies have examined these effects using different parameters[76 77 114 124] none of these studies have examined the effect of rhAPC on the apoptotic pathway in acute pancreatitis. The focus of this project was to demonstrate the sub cellular modulation in the expression of caspases on pancreatic tissue treated with rhAPC.

The demonstrable increase in levels of TNF α observed in our study may have played an integral part in the activation of the initiator caspases[125]. This activation of the extrinsic pathway through the expression of initiator caspase 8 was evident in the acute pancreatitis group. A definite but non significant decrease in expression of caspase 8 was noted in both the treatment groups.

The progression of apoptosis via the extrinsic pathway was evaluated by quantifying the expression of caspase 3. There was quantifiably increased positive expression of the executioner caspase 3 in the acute pancreatitis group with a non-significant reduction noted in the treatment groups.

As the apoptosis cascade takes place, it would be expected that there is a quantitative amplification of expression of caspase 3 compared to caspase 8[126], but our study demonstrates that this does not happen supporting the possibility that rhAPC may act prior to or at Caspase 3.

Although considerable crosstalk exists between the extrinsic and intrinsic pathways, no demonstrable increase was noted in the expression of caspase 9.

This non-significant reduction in expression of caspase 9 may support the fact that apoptosis in this model was not triggered by an internal mechanism rather by an external mechanism. Thus the difficulty in claiming that Caspase 9 is inhibited by rhAPC.

Our study restricted by the number of animals involved, resulted in a demonstrable but non-significant effect in the expression of apoptosis.

In summary, this preliminary study demonstrates the effect of Recombinant human activated protein C prior to and post induction of experimental acute pancreatitis resulting in amelioration and reduction in the death of cells via the apoptotic pathway. Manipulating death signalling mechanisms may represent therapeutic targets in the treatment of acute pancreatitis.

Further experimental evaluation of this role of recombinant human activated protein C in acute pancreatitis is justified as this study provides insight in to the sub cellular basis for the potential efficacy of activated protein C in acute pancreatitis.

# 6 Functional Protein C levels during the early phase of clinical acute pancreatitis

## 6.1 Introduction:

Protein C is a 60 kilo Dalton, vitamin K-dependent, anticoagulant protein playing an integral role in haemostatic regulation.[53] The Protein C anticoagulant pathway is involved in the control of thrombosis, limitation of inflammatory response and may also decrease endothelial cell apoptosis in response to inflammatory cytokines and ischaemia .[54] Thrombin, thrombomodulin, the endothelial cell protein C receptor (EPCR), protein C, and protein S are the essential components of the protein C pathway. The activation of protein C is enhanced dramatically when protein C is bound to the EPCR [54 55] Recombinant human activated protein C is not only an anticoagulant but also has an important link between coagulation, inflammation, apoptosis and vascular permeability.[57] During severe sepsis, an acquired defect in the protein C pathway is noted due to protein C consumption, protein S inactivation and reduction in activity of various complexes resulting in a deficiency of protein C.[54] The question of whether exogenous supplementation of activated protein C could modify the coagulation cascade in sepsis was examined in a key primate study with Taylor and colleagues reporting that rhAPC prevented the coagulopathic and lethal effects of Escherichia coli infusion in primates.[112] The anticoagulant and anti-inflammatory activities of rhAPC have been recognised and the human recombinant form (Xigris, Eli Lilly, Indianapolis USA) is now licensed for use in clinical sepsis.[109] Current clinical evidence suggests that there are clear parallels between the systemic inflammatory response of sepsis and that seen in acute pancreatitis (AP) an acute inflammatory disease of the pancreas which in its severe form is associated with pancreatic necrosis and a systemic inflammatory response.[127] There is experimental evidence that the pancreatic necrosis of the severe disease variants is associated with pancreatic parenchymal micro vascular thrombosis.[114] Studies by Radenkovic and colleagues have shown significant changes in coagulation profiles in patients undergoing surgical necrosectomy for acute pancreatitis.[52] There were noticeable

changes in the levels of protein C, anti-thrombin III and d-dimer indicating an exhaustion of fibrinolysis and coagulation inhibitors in patients with poor outcome during the course of the disease.[52] Lindstrom and colleagues examined the interaction between protein C and activated protein C in 31 patients with multiple organ failure as a result of severe acute pancreatitis.[58] Their study demonstrated the wide intra-patient variations in protein C levels at various stages of the disease and that activated protein C levels were lower in patients with severe disease with multiple organ failure. These findings raise the prospect of the use of recombinant human activated protein C early in the disease course of acute pancreatitis. In experimental acute pancreatitis, our group and others have demonstrated that rhAPC attenuates disease severity.[76-78] However little is known about endogenous protein C levels early in the disease course of human acute pancreatitis. This information is an important and necessary precursor to any potential therapeutic intervention study using recombinant human activated protein C. The aim of the present study is to assess whether there is depletion of functional protein C levels early in acute pancreatitis.

## 6.2 Patients and Methods

### *6.2.1 Study setting & Population*

The study was undertaken in a university teaching hospital serving an inner-city urban population during the period 28th May 2008 to 30th April 2009. A consecutive series of 60 patients admitted with a diagnosis of acute pancreatitis (AP) were recruited to the study. One patient was excluded from subsequent analyses as the blood samples were misplaced. Two further patients received anticoagulant therapy and were also excluded leaving a final study population of 57. The clinical diagnosis of AP was based on a combination of acute abdominal pain and a three-fold elevation of serum amylase above the upper reference limit. Patients who were pregnant, lactating, below eighteen years of age and those on anticoagulant medication were excluded. Patients with ongoing severe acute pancreatitis who were admitted as tertiary referrals during the period of this study to our critical care unit

for management by our specialist hepato-pancreatobiliary service were not included. Patient-level data were collected on demographics, aetiology and clinical outcome. The median [128] age was 41 (18- 81) years and 29 were women. Seventeen patients (29%) underwent computed tomography within the first week of admission with confirmation of the diagnosis of acute pancreatitis in all. The aetiology of the episodes of acute pancreatitis was gallstones in 25 (44%), alcohol in 21 (37%) and others in 11(19%). Seven patients received antibiotics. The protocol of the Hepato-pancreato-biliary unit at the Manchester Royal Infirmary is to use prophylactic broad-spectrum antibiotics in patients with predicted severe acute pancreatitis with an imipenem-type drug (meropenem). Patients with mild acute pancreatitis are not treated with antibiotics unless there is a specific clinical indication. The duration of antibiotic therapy is for seven days. After the course of antibiotics, it is stopped or continued based on the patient's clinical symptoms and signs. All patients were prescribed thromboprophylaxis with low molecular weight heparin

Protein C activity was assessed at admission, 24hours and 48 hours. Other coagulation and haematological parameters (prothrombin time [PT], activated partial thromboplastin time [aPTT] and platelet count) were measured at these time points. The laboratory normal range for PT and aPTT were 10.6 - 13.6 seconds and 21- 34 seconds respectively. The normal range for platelet count was 150- 400 x 109/ litre. Variables required for calculation of APACHE II and Logistic Organ Dysfunction Score (LODS) were collected on admission, at 24 and 48 hours. The severity of the disease was based on the 1992 Atlanta Classification. Data were entered prospectively into an electronic database and batch analysed at completion of recruitment.

### *6.2.2 Functional Protein C assay*

A 2.7 ml sample of venous blood was collected into a sterile vacutainer containing 3.2% (0.1M) trisodium citrate anticoagulant solution at a ratio of 9:1. The samples were centrifuged at 3,500 revolutions per minute for 10 minutes to separate plasma and platelets.

The plasma was stored at - 40°C. The frozen plasma was thawed at 37°C for 15 minutes and analysed in batches. The synthetic chromogenic substrate method STAR- StachromR Protein C kit (Diagnostica Stago, Asnieres, France) was used for the quantitative determination of functional Protein C levels in plasma. A fully automated study technique was used. The principle adopted in quantification was that Protein C is activated by a specific activator (derived from the venom of Agkistrodon C. Contortrix). The quantity of enzyme formed is measured by its amidasic activity on a synthetic chromogenic substrate causing the release of paranitroanaline which is measured on a plate reader at 405 nm. The intensity of the colour produced is directly proportional to the level of protein C in the test plasma (laboratory reference range 69-154 units/decilitre). External quality control (QC) was provided by the United Kingdom National External Quality Assessment Service (UKNEQAS).

### *6.2.3 Ethics committee approval*

Approval was obtained for the study from the Regional Ethical committee (Ref No. 08/H1014/30). A protocol target of 60 patients was requested (and approved by committee) for this preliminary exploratory phase study.

### *6.2.4 Statistical analyses*

Data are presented as medians [128] and non-parametric tests are used for comparisons unless otherwise specified. The Stats Direct statistical software Version 2.4.1 (Stats Direct, Altrincham, UK) was used.

## 6.3 Results

### *6.3.1 Profile of acute pancreatitis.*

The median time from onset of worst pain to admission was 6 (1- 24) hours. The median length of stay was 5 (0-35) days. Two patients (4%) had High Dependency or Intensive care unit (level II or level III critical care support respectively).[129] There were no in-hospital deaths in this cohort. Seventeen (30%) patients had contrast-enhanced computed tomography (CT) within a median of 4 days (0-8 days) after admission. Eleven of the 25 patients with gallstone pancreatitis underwent laparoscopic cholecystectomy during the index admission. Five patients had endoscopic retrograde cholangiopancreatography ± endoscopic sphincterotomy within eleven days of presentation with a median bilirubin level of 42(21-98 µmol/L). Two patients had pseudo cyst drainage. No patients in this group required necrosectomy.

| | **Total (n = 57)** | **APACHE II <8 (n = 43)** | **APACHE II ≥ 8 (n = 14)** | ***P* value** |
|---|---|---|---|---|
| **Age** | 41 (18-81) | 35 (18-69) | 66 (39-81) | |
| **Gender (female: male)** | 29:28 | 23:20 | 6:8 | 0.49 ($\chi^2$) |
| **Median time from symptom onset to admission (hours)** | 6 (1 - 24) | 10 (1 – 22) | 5 ( 3 – 24) | P = 0.08 |
| **Functional Protein C levels** | 99.5 (41-180) | 104(43-180) | 93 (41-169) | P = NS |
| **Platelet count** | 296 (29-502) | 299(55-759) | 263 (29-502) | P = NS |
| **PT** | 12.9 (10.8-31.2) | 12.9 (10.8-15.1) | 13.4 (11.2-30.8) | P = NS |
| **aPTT** | 26.2 (18.2-42.6) | 26.25 ( 18.8-42.6) | 25.2 (18.2-40.6) | P = NS |

***Table 5: Haematological, coagulation and protein C profiles in patients with APACHE ≥ 8 acute pancreatitis compared to those with APACHE II < 8***

### *6.3.2 Organ Dysfunction Scores.*

The median APACHE II score on admission was 4 (0-12) and the median LODS score on admission was 0 (0-4). The scores dropped to 2(0-9) and 0 (0-2) respectively 24 hours after admission. Fourteen (25%) patients had an APACHE II ≥ 8 on admission with a median APACHE II and LODS score in this sub-group of 9 (8-12) and 1 (0-4) respectively.

### *6.3.3 Coagulation Profile.*

The median Prothrombin time (PT) on admission was 12.9 seconds (10.8 - 30.8) and at 24 hours, 13.5 (11.0 -25.8). The median aPTT on admission was 26.2 seconds (18.2- 42.6) and at 24 hours was 28.1 (24.0 - 42.1). The median Platelet count was 296 (29-502) on admission and 241(20-502) at 24 hours. Coagulation profiles and platelet counts in patients in the subgroup with an APACHE II score of ≥ 8 are seen in the table 5.

### *6.3.4 Functional Protein C levels*

The median functional Protein C level on admission was 97 (41 - 178) units/decilitre. At 24 hours, functional protein C levels were 96 (46-170). In 18 patients remaining as in-patients at 48 hours, functional protein C levels were 93.5(51-184). These values were not significantly different (*Kruskal- Wallis multiple group comparisons P =0.741*). The median functional Protein C Level on admission for patients with an APACHE II ≥8 compared to those with an APACHE II <8 can be seen in the table 1. There was no significant difference in functional protein C levels between those with APACHE ≥ 8 and patients with lower APACHE II scores (*P = 0.163, 95.1% CI = -29 to 6; Mann-Whitney U test*). The relation between organ dysfunction and functional protein C levels is seen in table 2. When data were categorised by admission LOD scores (0, 1 or >1), there was no difference in functional protein C levels (*P=0.549*). When admission values in these three sub-groups were compared to values at 24 hours in the same groups there was again no significant difference (*P=0.4187*) although functional protein C values in patients with LODS >1 at 24h were at the lower range of laboratory reference population.

| | Admission LODS = 0 | Admission LODS = 1 | Admission LODS >1 | *P* Value |
|---|---|---|---|---|
| **Number of patients** | 30 | 17 | 10 | |
| **Admission protein C** | 97.5 (70 – 180) | 104.5 (77 – 159) | 92.5 (41 – 169) | *P*=N.S |
| **Protein C at 24 h** | 92 (46 – 145) | 104 (71 -170) | 80.5 (58 – 114) | *P*=N.S |

***Table 6: Relationship between organ dysfunction and functional protein C levels***

### *6.3.5 Correlation between APACHE II scores and Functional Protein C Levels*

Correlation plots between APACHE II and functional protein C levels at admission and at 24 hours are seen in figures 4 .1 and 4.2.

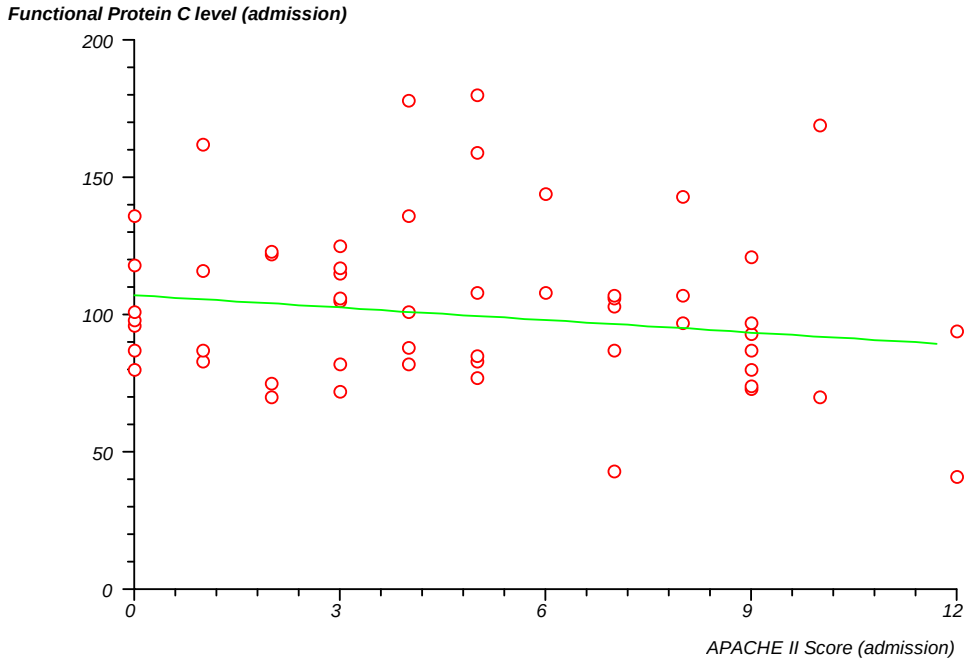



***Figure 4.1 Nonparametric linear regression plot of functional protein C levels on admission compared to admission APACHE II scores.( Kendall's rank correlation coefficient = -0.117)***

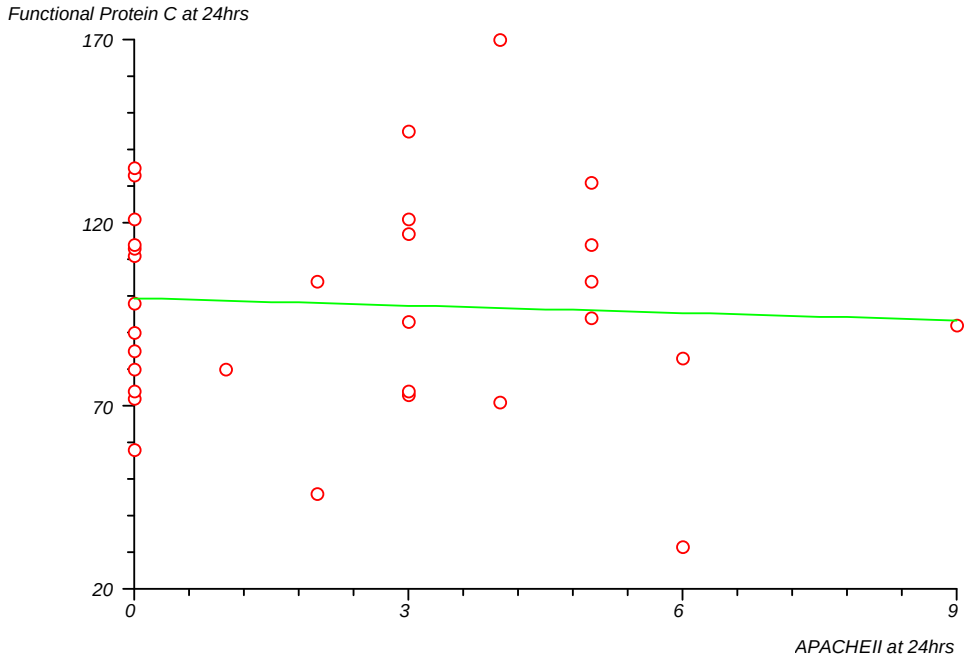



***Figure 4.2 Nonparametric linear regression plot of functional protein C levels at 24 hours compared to APACHE II scores at 24 hour (Kendall's rank correlation coefficient = -0.039)***

## 6.4 Discussion

Protein C is now understood to be an important mediator of the interaction between coagulatory and inflammatory pathways in human sepsis. Endogenous levels are depleted in acute sepsis and treatment with recombinant human activated protein C has a beneficial effect on outcome. As an acute systemic inflammatory response with changes in the coagulation and inflammatory pathways is also characteristic of human acute pancreatitis, it is a logical extrapolation to ask whether protein C levels are similarly depleted in this disease. Confirmation of this finding in acute pancreatitis would then sit well with parallel evidence from us and others demonstrating that the clinical course of experimental acute pancreatitis is ameliorated by intervention with recombinant human activated protein C and suggest that this drug may be evaluated as a treatment for early severe acute pancreatitis. To evaluate protein C levels early in the disease course of human acute pancreatitis we recruited a cohort of patients admitted to our unit with acute pancreatitis. The median duration between onset of symptoms and admission was short (6 [1- 24]) hours and thus our findings are representative of protein C changes early in the disease. Tertiary referral patients were excluded as many of these individuals presented with late established sepsis. Our technique of using the chromogenic substrate method is useful in detecting both Type I (quantitative deficiency with low functional protein C [activity] and immunological activity [antigen levels]) and Type II (qualitative deficiency with low activity but normal antigenic levels) deficiency in the functional activity of protein C. None of the patients had functional Protein C levels more than 2 standard deviations below the mean of laboratory's reference range indicating that none of the study population had an inherent heterozygous protein C deficiency.[53] We believe that this study examines for the first time, the variations that occur in functional Protein C levels on admission, 24 hours and 48 hours of patients admitted with acute pancreatitis. The key findings were that functional protein C levels were within the normal range on admission and at 24 hours in this cohort. As the majority of patients had mild acute pancreatitis, a sub-group analysis was undertaken of the patients with an

admission APACHE of ≥ 8. There was no significant difference between values in this group and the group with admission APACHE II scores of <8 but the numbers of patients with APACHE ≥ 8 acute pancreatitis was small. If the current trend for categorisation of acute pancreatitis by organ dysfunction is considered, there were no statistically significant differences in functional protein C levels between patients with a LODS of 0 and those with a LODS > 1 either on admission or at 24 hours. When the entire cohort data are examined by linear regression, a weak negative association is observed between admission APACHE II and functional protein C levels with a similar finding being present when APACHE II at 24 hours is compared to functional protein C at 24 h. Our findings are at first sight in contrast to the results of a very recent study from Belgrade by Radenkovic and colleagues[52]. In a cohort of 91 patients with acute pancreatitis, they demonstrated that protein C levels were significantly lower in patients with organ failure, both on admission and at 24 hours. One important difference is that they did not measure functional protein C levels. Another important difference between the groups is the high proportion (42%) of their organ failure patients with infected necrosis. There were 8 deaths in this group in contrast to none in our study. As these patients were admitted relatively early in their disease course it may be that the majority of patients in Belgrade with acute pancreatitis were treated by Radenkovic's unit. In contrast, in a major Western European conurbation such as ours, patients with severe disease receive their initial care in a considerable number of hospitals across the region, all with high-level critical care. Tertiary transfer to our specialist unit is reserved for patients with on-going sepsis who were excluded from our study as we wanted to find a population representative of those admitted on a routine basis to general acute hospitals.

Conclusions that can be drawn from these data are that it is unlikely that functional protein C activity is impaired in human mild acute pancreatitis. This is entirely consistent with the clinical setting of a mild self-limiting illness and suggests that current management strategies aimed at resuscitative and supportive care with vigorous attention to detection and treatment of gallstone attacks is correct. In contrast, there remains at least a theoretical niche for

pharmacological intervention in severe acute pancreatitis. Whilst patients with severe disease may come to fulfil sepsis-criteria late in their disease course and receive treatment with rhAPC (accepting that there are ongoing studies evaluating the efficacy of this drug), the prospect of early intervention to blunt the inexorable progression of the systemic inflammatory response cascade is an attractive scenario but one for which no evidence is generated from our present data. A greater sample number, especially of patients with severe disease and exploration of other components in the protein C pathway is required to continue the theme of inquiry initiated by the present study.

# 7 Setting up of the Phase II Clinical study

Based on laboratory and clinical evidence of the effect of recombinant human activated protein C on acute pancreatitis along with supportive clinical data from PROWESS study, a phase II study was set up. The protocol was compiled with the help of my supervisor on the evidence obtained from our experimental data and supportive evidence from other similar studies. Patients admitted to hospital with acute pancreatitis and an APACHE II score ≥ 15 have a 30% risk of death.[15] In addition, these episodes are associated with prolonged critical care stay, often in excess of 30 days.[130]

## 7.1 Aim

This study aims to undertake a preliminary evaluation of 24-hour infusion of rhAPC administered early in the disease course of severe acute pancreatitis (APACHE II ≥ 15).

## 7.2 Hypothesis

The hypothesis tested is that early administration of recombinant human activated protein C will preserve pancreatic parenchymal microvascular patency and thus ameliorate pancreatic injury. In our experimental model of L-arginine-induced experimental pancreatitis[131], administration of recombinant human activated protein C was associated with amelioration of pancreatic injury. Bearing in mind that in previous large multi-centre trial of rhAPC, early severe acute pancreatitis was an exclusion criterion in view of the risk of pancreatic haemorrhage. A key finding of our experimental study was that there was no evidence of rhAPC -induced pancreatic haemorrhage. Thus the present study represents a logical progression of our current programme of work and takes the evaluation of rhAPC to clinical severe acute pancreatitis.

## 7.3 Primary objective

The primary objective of this study is to undertake a preliminary evaluation of the role of recombinant human activated protein C administered early in the disease course of human severe acute pancreatitis. The principal end-points are assessment of safety of treatment and assessment of effect of intervention on organ failure, length of ITU stay, mortality and markers of coagulation and inflammatory response.

## 7.4 Secondary objectives

Interim analysis may indicate the feasibility or otherwise of a preliminary health economic analysis. Given that clinical experience with the use of rhAPC in severe acute pancreatitis is extremely limited, the proposed study will provided valuable baseline information that will help in two key areas: defining the nature of the acute pancreatitis population who may benefit from treatment with rhAPC and assessing the risk-benefit balance of intervention with rhAPC. A matched case control analysis on a clearly defined population will provide further information in the differences observed between treatment and control populations. Control patients will be selected from our current database of Severe Acute Pancreatitis with matching based on APACHE II and LOD scores.

## 7.5 Study population and dose

The proposed study takes the format of an inception cohort study: 30 consecutive patients with severe acute pancreatitis (APACHE II ≥ 15, within 24 hours of admission to hospital) will be treated with a 24-hour infusion of rhAPC (24 μg/kg/hr for 24 hours by intravenous infusion).

## 7.6 Endpoints

Endpoints to be assessed include biochemical and radiological markers of inflammation and rheological indices of haemorrhage. Outcome will also be compared to results in an age, gender and severity matched historical cohort of patients with acute pancreatitis.

## 7.7 Scientific critique and funding

The protocol was sent for peer review to a panel of experts in the field (Professor Siriwardena sent the protocol to Associate Professor Dileep Lobo, University of Nottingham, Mr Ross Carter of Glasgow Royal Infirmary).

The protocol was also reviewed by the research group of Eli Lilly, Indianapolis, USA). Based on the suggestions and positive feedback, funding was sought through an *Initiator Investigated trial (IIT)*. As this was independent clinical research using an Eli Lilly drug and the drug being registered for severe sepsis in the United Kingdom where the research is conducted, Eli Lilly and Company (Indianapolis, Indiana, USA) agreed to provide free study drug and possible financial support after assessment of the scientific quality of the proposed research. All the research supported by this program would be conducted by the applicants and their affiliated institutions.

## 7.8 Regional ethical committee approval

Regional ethical committees are setup to safeguard the safety, dignity and rights of the patients participating in research, being entirely independent of the research sponsors and investigators. I completed the ethics committee application form with guidance from my supervisor.

The **Leeds (West) Research Ethics Committee** (Reference Number: **07/H1307/201**) after deliberation and amendments awarded a favourable ethical approval for the study.

## 7.9 Medicines and Healthcare products Regulatory Agency (MHRA)

In our application to the Medical Health and Regulatory Authority (MHRA), we proposed a preliminary clinical assessment with a strong focus on safety ensuring that there is rigorous monitoring for adverse effects, in particular, for treatment-related haemorrhage. The EudraCT number **2007- 003635 - 23** was obtained which is the unique identifier required for

all trials conducted with an investigational medicinal product in any European Union Member State. An MHRA approval was obtained for the study.

## 7.10 Site Specific Assessment (SSA)

Site specific assessment was required to assess the suitability of the site and Principal investigator to conduct the trial. This was obtained from the North West ethics committee.

## 7.11 Sponsor approval by NHS Research & Development wing.

The Central Manchester and Manchester Children's Hospital NHS Trust accepted the role of sponsor for the project "A preliminary evaluation of the safety profile of 24-hour infusion of human recombinant activated protein C (Xigris) early in severe acute pancreatitis. XIG-AP 1" (Pin 10607) delegating the responsibilities of the sponsor to the Chief investigator, Professor Ajith K Siriwardena.

# 8 Integrated Discussion

Effective management of acute pancreatitis has for centuries eluded mankind. The disease has a wide spectrum of presentation; the milder form is usually a self limiting condition whereas the severe form presents as a highly morbid and frequently lethal attack [132]. The ability to predict disease progression on admission would aid in the comprehensive and multidisciplinary management of patients.

The perfect predictor has been an elusive factor hindering the management of the disease. Over the years, various biochemical markers have been identified but have not been conclusive. On systematically reviewing literature and identifying appropriate biochemical markers in predicting progression of acute pancreatitis, the ideal predictor would be a combination of biochemical, clinical and contemporary organ dysfunction scoring systems[17]. Early prediction of disease progression however, is important in the better management of the disease. As previously mentioned, acinar cell injury and death are the earliest events that occur in acute pancreatitis. Identification of potential pharmacological interventions offered through valuable insight in to experimental and clinical acute pancreatitis may lead on to the development of various natural and synthetic potential disease modifiers.

Green Tea Extracts (GTE) consumed in many parts of the world as a natural remedy has been examined as a potential therapeutic medication. Experimental results have demonstrated the effect of GTE on the oxidative pathway significantly ameliorating the effects of pancreatic injury. The various green tea catechins especially Epigallocatechin-3-gallate (EGCG) can perhaps be useful lead compounds for new drug discovery against various molecular targets[133].

With no specific targeted therapy for severe acute pancreatitis at present, various medications have been tried and tested. The possibility of a medication targeting the initial acinar cell injury may not be a feasible option as patient presentation and management would usually be after this phase. As the disease progresses, severe acute pancreatitis is

characterised by pancreatic necrosis [134]. The hypothesis of preserving pancreatic parenchymal microvascular patency and thus ameliorating pancreatic injury through the early administration of rhAPC has facilitated in identifying a treatment for acute pancreatitis. rhAPC converted from its inactive precursor, protein C, by thrombin acts through fibrinolysis and inhibition of thrombosis[135]. Studies on rhAPC in experimental acute pancreatitis examined in detail the modulation of rhAPC on inflammatory markers, morphology, microvascular thrombosis and apoptosis. Two murine acute pancreatitis models were used, the L-Arginine model and the Cerulein model. Both models stimulate acute pancreatitis, are concentration dependent and cause pancreatic necrosis. The L-arginine-induced rat model is a good model for necrotizing pancreatitis but has a narrow safety margin. For the more intricate mechanistic studies, a cerulein-mouse model was selected: experimental mortality is less. It is acknowledged that there are difficulties inherent in comparing different models.

The results have encouraged the Academic HPB team at the Manchester Royal Infirmary, Manchester UK, to take this idea further in the way of a clinical trial of rhAPC early in the course of severe acute pancreatitis. Prior to taking the huge step from bench to bed side, the variations in protein C levels were examined as a precursor to the Phase 2 trial of administering rhAPC early in the clinical course of severe acute pancreatitis.

Good research raises more questions than it answers and this thesis is no exception to that rule. The future of the main projects that constitute this thesis have been highlighted

## 8.1 Epigallocatechin-3-gallate (Green tea polyphenol extract) ameliorates pancreatic injury in cerulein induced murine acute pancreatitis.

Mounting evidence suggests that a multimodal approach at the molecular and cellular level is pertinent in the management of AP. The possibility of a medication targeting the initial

acinar cell injury, however, may not be a feasible option in the clinical setting, as patient presentation would usually be after this phase. Our results show that GTE significantly ameliorated the effects of cerulein-induced experimental pancreatitis. Further evaluation of mechanisms of action and phase 2 clinical applications may be considered in light of these encouraging results.

## 8.2 Recombinant human activated protein C (Xigris) attenuates murine cerulein-induced acute pancreatitis via regulation of NF-κB and apoptotic pathways.

Recombinant Human Activated Protein C in our model helped in ameliorating pancreatic tissue damage via regulation of NF-κB and apoptotic pathways without concomitant reversal of coagulopathy. The supportive literature from around the world on the use of rhAPC lends credibility in the translation of this finding to clinical practice paving way in identifying a treatment for acute pancreatitis.

## 8.3 Recombinant human activated protein C modulates the sub-cellular expression of Caspases in L-Arginine induced Acute Pancreatitis

In summary, this exploratory study demonstrates the effect of recombinant human activated protein C prior to and post induction of experimental acute pancreatitis resulting in amelioration and reduction in the death of cells via the apoptotic pathway. Further experimental evaluation of this role rhAPC in acute pancreatitis is justified as it would provide insight in to the sub cellular basis for the potential efficacy of rhAPC in acute pancreatitis

## 8.4 Functional Protein C levels during the early phase of clinical acute pancreatitis.

In human AP, functional protein C levels are conserved in mild disease. However, there is evidence that levels are depleted early in severe disease suggesting a parallel between the pathobiology of severe sepsis and severe AP. A greater sample number, especially of patients with severe disease is underway to continue the theme of inquiry initiated by the present study.

## 8.5 A preliminary evaluation of the safety profile of twenty four hour infusion of Human recombinant activated protein C (Xigris) early in severe acute pancreatitis.

Having laid the foundation for this Phase II clinical trial my successor at present is recruiting participants to the trial.

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