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# Oxidant/anti-oxidant imbalances in patients with long bone fractures

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# OXIDANT/ANTI-OXIDANT IMBALANCES IN PATIENTS WITH LONG BONE FRACTURES

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A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine.

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## ABSTRACT

### *BACKGROUND*

The occurrence of fat embolisation following trauma is a well recognised clinical entity and may occur in up to 90% of patients with long bone fractures. However, the fat embolism syndrome (FES) which consists of hypoxia, acute lung injury, central nervous system depression, and axillary or subconjunctival petechiae is only noted in 0.5-3% of these patients. Various theories have been put forward to explain the syndrome but none have thus far been able to explain this discrepancy.

### *OBJECTIVES*

Our aim was to show that there is stimulation of the inflammatory system in patients with long bone fractures resulting in the production of lipid peroxides, and that an imbalance between the pro and anti-inflammatory mediators may be responsible for the hypoxia observed in most patients.

*METHODS*

Nineteen people with long bone fractures of the lower limbs were followed for 48 hours. Blood specimens were taken for arterial blood gas (ABG), lipid peroxides (LPO), vitamin C, glutathione, C-reactive protein (CRP), full blood count (FBC) at time of admission and 12, 24 and 48 hours after admission.

*RESULTS*

There was evidence of lung involvement with an increase in the alveolar/arterial difference (A-a$DO_2$) from a mean of 13.8 mmHg to 23.3mmHg. Thirteen out of 16 patients (who had accurate ABG's) showed an increased A-a$DO_2$. The white cell count (WCC) was raised initially with a mean of 13.8 x $10^9$/l. Within 48 hours, there had been a decrease to 8.9 x $10^9$/l ($p=0.003$). In keeping with an inflammatory response, there was a significant rise in the CRP in the 48 hours period from a mean of 9.7µg/l to 127.3µg/l ($p<0.0001$) and a decrease in the platelet count from a mean of 256 x $10^9$/l to 193 x $10^9$/l ($p=0.0003$) Furthermore, there was an increase in the lipid peroxides from 3.79nmol/ml to 5.81nmol/ml ($p=0.03$) over the first 24 hours and to 6.42nmol/ml ($p=0.20$) over the 48 hour period. The anti-oxidants

vitamin C and glutathione showed a decrease, vitamin C from 8.26μg/ml to 7.45μg/ml ($p=0.05$) and glutathione from 5.96μmol/ml to 3.84μmol/ml ($p=0.22$). Only one of the patients developed the fat embolism syndrome suggested by confusion, hypoxia, and upper body petechiae.

## CONCLUSION

Patients with fractured long bones appear to have stimulation of their inflammatory system. There is evidence of activation of the coagulation system in keeping with this process. Serum antioxidants maintain homeostasis and may be responsible for the prevention of the FES in most patients. However, even in asymptomatic individuals, there is evidence of pulmonary involvement. One explanation for the development of FES in patients with long bone fractures is an imbalance between the pro and anti-inflammatory mediators with the production of lipid peroxides leading to organ dysfunction such as ARDS.

**DECLARATION**

I declare that this thesis presented for the degree of Master of Medicine at the University of the Witwatersrand, Johannesburg, is my own work. This thesis has not been submitted for a degree or examination at any other university.

.....

Hayden Thomas Wesley White
(MBBCh)

16/11/98

Date

This thesis is dedicated to my wife Eleonore, for her love and support and to little Matthew, for reminding me why I undertook to do it in the first place.

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The following presentation arose from this paper:

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# CHAPTER 1

# THE FAT EMBOLISM SYNDROME

## 1.1 HISTORY

Lower (Lower, 1932) is credited with having first described the clinical manifestations of fat embolism following the injection of milk into experimental animals in 1664. However, it wasn't until 200 years later that the first histological description of the fat embolization process was described by Zenker (Zenker, 1862). In this paper he noted the presence of fat in the lung capillaries of a railwayman who was caught between the bumpers of two train wagons and who bled to death secondary to a liver laceration.

Classical fat embolism was described by Bergman (Bergman, 1873) as the clinical deterioration and death following a symptom free interval of a tinsmith who had fallen from a roof and sustained a comminuted fracture of the femur.

Fenger and Salisbury first described this syndrome in the American literature in 1879 (Fenger and Salisbury, 1879).

Theories as to the origin of fat embolization became increasingly controversial. In 1924, Gauss (Gauss, 1924) developed the mechanical theory. He postulated that fat from the marrow entered the blood stream and was carried to the lungs where it caused occlusion of vessels. Soon afterwards, in 1927, Lehman and Moore (Lehman and Moore, 1927) introduced the physicochemical theory in which they proposed that biochemical changes that occurred in the bloodstream caused fat to precipitate out of solution and to be carried to the lung.

Since then, there have been over 2000 articles published on fat embolization. The pathogenesis however, remains elusive.

## 1.2 INCIDENCE

There is ample evidence that marrow fat embolization occurs in virtually all patients who sustain a long bone or pelvic fracture (Shier and Wilson, 1980). Nevertheless, only a small fraction actually develop the fat embolism syndrome (FES), which is characterised by pulmonary infiltrates, petechial haemorrhages, and mental disturbances, usually following long bone fractures.

Furthermore, fat embolization has been associated with a number of other conditions, unrelated to trauma (ten Duis, 199?). These include other skeletal injuries, soft tissue injuries, pancreatitis, extracorporeal circulation, high altitude and divers' decompression illness, sickle cell disease, burns, osteomyelitis, epilepsy, liposuction, and fatty liver.

Clinical evidence of the syndrome (ie. petechial rash, respiratory distress and/or confusion) is reported to occur in 0.5-3% of patients who sustain a single long bone fracture. (Bulger et al. 1997) In multiply injured patients, percentages as high as 30% have been reported with the likelihood proportional to the number of fractures and the size of the bone involved (Peltier, 1969). Although occasionally seen in patients with upper limb fractures, it is exceeding rare (ten Duis, 1997).

The syndrome is as much as 100 times less frequent in children than adults with comparable fractures (Limbird and Ruderman, 1978). There are two hypothesis for this:

- the fat content of the medullary cavity in children is lower and the proportion of haematopoietic tissue is much larger than in adults (ten Duis, 1997)

- the composition of bone marrow fat in children is different from adults; it contains less olein but a larger proportion of palmitin and stearin (Levy, 1990).

There is some evidence that the incidence of fat embolism is decreasing, perhaps as a consequence of an increased awareness on the part of medical staff and better supportive therapy (Guenter and Braun, 1981). It has been shown that early immobilisation and fixation of fractures can reduce this incidence. Furthermore, it is suggested that early resuscitation and haemodynamic support may contribute as there appears to be a relationship between fat embolism and hypovolaemic shock (ten Duis, 1997).

## 1.3 PATHOPHYSIOLOGY

Many theories have evolved regarding the aetiology of FES. One of the first came from Zenker (Zenker, 1862) who proposed that fatty foods present in the stomach at the time of the insult were taken up by the blood stream. This however was rejected by other observers who felt that the most likely source was the bone marrow itself. Still others felt

that this description was too simplistic and that biochemical forces played a significant role.

At present, two main theories dominate the literature. It should be noted however that neither can fully explain all the clinical and pathological observations present.

### *1.3.1 Mechanical Theory*

For many years it was thought that fat globules released from damaged marrow blocked the smallest branches of the pulmonary vasculature while smaller particles passed through the lung capillaries and enter the systemic circulation. Particles bigger then 9μm in diameter are capable of blocking lung capillaries and smaller particles are possibly able to pass through the lung capillaries and embolize the peripheries (Levy, 1990) Alternatively, fat emboli may reach the peripheries via an intracardiac shunt eg. a patent foramen ovale (Christie et al. 1995)

Pell (Pell et al. 1993) using transoesophageal echo observed that at the time a guide wire was inserted into the bone marrow canal for reaming, echogenic material passed into the right side of the heart. Reductions in arterial oxygen saturation correlated with the density of the echogenic

material. As the pulmonary artery and right heart pressures increased in response to multiple episodes of pulmonary embolization, this echogenic material paradoxically embolized through the foramen ovale into the left side of the heart if it was patent.

The literature provides support for the embolization theory. Bhaskaran (Bhaskaran, 1969) found bone marrow fragments in lung sections of patients who died from injuries. Other authors (Kerstell, 1971) showed that intravascular and perivascular fat in lung biopsies from injured patients appeared to have the same fatty acid composition as that from bone marrow. Furthermore, the anatomical structure of the marrow with its large, thin walled venous sinusoids supported by marrow fat deposits suggests that embolization is possible (Harry and Thomas, 1979).

The mechanical theory requires:

1. The presence of torn blood vessels to permit fat to enter the circulation
2. The liberation of free fat
3. A transient rise in marrow pressure to allow fat droplets to enter the vessel.

This has been supported by Morton and Kendall whose work indicated that the area of trauma is the site of origin of the pulmonary fat (Levy, 1990).

There are some inconsistencies with the mechanical theory. Not everyone who sustains a long bone fracture develops the fat embolism syndrome. As previously noted, while approximately 90% of trauma patients develop fat embolism, less then 10% develop the FES. Proponents of the mechanical theory have attempted to explain this by suggesting that the quantity of fat released into the circulation may play á role. Peltier however, concluded that there was a sufficient amount of fat in one femur to cause enough mechanical obstruction of the pulmonary vasculature to seriously impair perfusion and gas exchange (Peltier, 1969). Furthermore, dysfunction usually develops only 12-24 hours after the fracture has occurred and often as late as 48 hours. There are also many reports in which features of fat embolism are present in patients with small fractures (ten Duis, 1997).

The source of the fat is another controversial area as there is evidence that it may arise from extramedullary fat (Gresham, 1985). Quire and associates demonstrated that the cholesterol content of fat emboli is

about 30%, while the cholesterol content of fat in marrow and adipose tissue is only about 1% (LeQuire et al. 1959). This suggests that much of the fat in the fat emboli comes from the bloodstream itself (Shier and Wilson, 1980).

The mechanical theory alone is therefore insufficient to explain the FES. Consequently, the biochemical theory was developed to explain these inconsistencies.

### *1.3.2 Biochemical Theory*

The biochemical theory is based on the assumption that fatty acids whether freely circulating or formed within the pulmonary system cause endothelial damage and are directly toxic to pneumocytes (Levy, 1990). Capillary leakage, perivascular bleeding, platelet adhesion and clot formation are considered to be the main factors responsible for tissue damage and organ dysfunction (Shier and Wilson, 1980). Free fatty acids are released into the blood following the lysis of triglycerides released from the fracture site. This process takes place with the help of the enzyme lipoprotein lipase which is increased in the serum following long bone fractures (Harry and Thomas, 1979; Szabo et al. 1977).

Catecholamines released as a result of the stress response to injury result in lipolysis by means of activation of the adenyl cyclase system which catalyses inactive lipase to the active form. The active lipase hydrolyzes depot triglycerides to free fatty acids and glycerol (Havel, 1968). These travel to the lung and are logged in the capillaries where they are directly toxic to the endothelial cells.

Ordinarily, the fatty acids in the plasma are bound to albumin and are non toxic. However, if the amount of fatty acids produced is high (or albumin is low), unbound fatty acids can cause a severe chemical pneumonitis, resulting in decreased surfactant activity and increasing interstitial pulmonary oedema (Shier and Wilson, 1980).

Fats from the peripheral circulation may also cause obstruction of pulmonary capillaries. A chemical event at the site of injury may cause the release of mediators that affect the solubility of lipids, causing coalescence and subsequent embolization. Normal chylomicrons (which are less than 1μm in diameter) may coalesce and form fat globules 10-40μm in diameter, which are capable of occluding the lung

capillaries (Levy, 1990). Hulman (Hulman, 1988a) suggests that it may be the increased levels of C-reactive protein (CRP) in the peripheral circulation that are responsible for this process.

Bergentz in 1961 (Bergentz and Nilsson, 1961) was the first to suggest that intravascular aggregation of red blood cells following trauma and fat embolism were linked. More recently, it has been suggested that intravascular coagulation plays a role in this syndrome. Fibrin occurs in pulmonary vessels more often in lethal cases of fat embolism than in other post traumatic deaths (Shier and Wilson, 1980). Intravascular coagulation is thought to be initiated by thromboplastin released from the fat and the presence or absence of fibrin is dependent on the degree of fibrinolytic response to the clotting. FFA have also been shown to increase platelet aggregation and promote hypercoagulation which can result in platelet/fibrin clots in the microcirculation of the lung (Hoak et al. 1967). Platelet activation proceeds to platelet adhesion and the release of serotonin, ADP, histamine, and phospholipids. These amplify fibrin formation and worsen $PaO_2$ (Shier and Wilson, 1980). In patients whose platelets decrease, there is a significant drop in $PaO_2$ (McCarthy et al. 1973).

Because the same changes morphologically can be produced in the lungs by intravenous injection of thromboplastin and because fat which is heated to destroy thromboplastin does not produce these changes, it is reasonable to suggest a link between intravascular coagulation and fat embolism (Shier and Wilson, 1980).

Certain aspects of the biochemical theory have recently been challenged. In a study by Schnaid (Schnaid et al. 1987) in which patients with major trauma, with and without long bone fractures were compared, FFA and catecholamines levels were identical. This casts doubt upon the role of FFA in FES. A shortcoming of this study however, was that none of their patients had FES and it is therefore possible that FFA's are increased only in this group.

## 1.4 NON-TRAUMATIC FAT EMBOLIZATION

As previously stated fat embolization has been well described following non traumatic events. Most theories of fat embolization in the absence of trauma are based on Lehman and Moore's (Lehman and

Moore, 1927) concept that emboli are composed of aggregated chylomicrons formed when an unknown substance breaks the stability of the emulsion of these chylomicrons in the blood stream. A recent hypothesis suggests that the acute phase reactant, CRP, is involved in the agglutination of very low density lipoproteins (VLDL) and chylomicrons in the blood to form fat macro globules which then embolize. This occurs via a calcium dependant process which has been well demonstrated in experimental models (Hulman, 1988a; Hulman, 1988b).

## 1.5 PATHOLOGY

Pathologically, vessels in the lung smaller than 20μm in diameter become mechanically occluded by fat emboli. The obstruction is worsened by platelets and fibrin which adhere to the emboli and form a plug. The lung lipase hydrolyses the neutral fat to toxic free fatty acids that damage the endothelium, inactivate lung surfactant and increase capillary permeability. The consequence of this is the development of the acute respiratory distress syndrome (ARDS). (Gresham, 1985)

## 1.6 CLINICAL

The classical fat embolism syndrome comprises the symptom complex of petechial rash, pulmonary distress and mental disturbances usually occurring within 24-48 hours following a long bone fracture. This latent period is less then 12 hours in 3% of patients, 12-24 hours in 10%, 24-48 hours in 45%, 48-72 hours in 33% and greater than 72 hours in 9% (Shier and Wilson, 1980).

It is important to distinguish between fat embolism and the fat embolism syndrome. Fat embolism refers to the presence of fat globules in the lung parenchyma and peripheral circulation after a long bone fracture. Patients are usually asymptomatic but may manifest certain biochemical abnormalities including decreased $PaO_2$, raised CRP and decreasing platelet count. Classical FES is a serious manifestation of fat embolism that involves progressive respiratory insufficiency and cerebral and skin manifestations.

Most patients with long bone or pelvic fractures, despite being asymptomatic will show quite marked reductions in $PaO_2$ levels following trauma. These may occur within a few minutes of the

incident and persist for a few days without other features of FES being present. These patients do not develop cyanosis. The hypoxia is often intermittent and may recur several times during the post trauma period (Tachakra, 1976). There may also be other evidence of subclinical fat embolism including, increased platelet activation, thrombocytopenia, and intravascular coagulation. However, although these manifestations are similar to those of the classic fat embolism syndrome, they are much less severe (Shier and Wilson, 1980).

Because of the variable severity of the disease, certain authors have distinguished three different grades of fat embolism (Riska and Myllynen, 1982). In the first grade, the primary manifestations are skin petechiae with one or two additional mild signs. The second grade, is recognisable by the presence of skin petechiae and other characteristic features of FES, however the symptoms are mild and no specific treatment is indicated. The third grade, the clinical fat embolism syndrome, is characterised by the presence of the typical diagnostic criteria, and is a severe condition in which the patient is in need of respiratory assistance.

The clinical pattern of this syndrome has a bimodal distribution; some patients clearly demonstrate a fulminate course with the onset of symptoms within 12 hours of injury. These patients tend to have a higher mortality with prominent neurologic involvement, leading to coma, and also massive embolization, which results in right ventricular failure and cardiovascular collapse. Most patients tend to have a more slowly progressive course manifesting symptoms of variable severity 24-72 hours after injury (Bulger et al. 1997).

### *1.6.1 Pulmonary signs*

Pulmonary involvement occurs in 75% of patients with FES (van Besouw and Hinds, 1989). The initial symptom is hypoxia induced tachypnoea and hyperventilation. Hypoxia is caused by a ventilation/perfusion (V/Q) mismatch induced by inflammation and damage to the capillary endothelium with platelet adhesion, capillary leakage and subsequently micro-atelectasis. $PaO_2$ and $PaCO_2$ are both decreased and respiratory failure develops as a consequence of primary acute respiratory distress syndrome. Some studies have shown that the degree of pulmonary shunt (arterial-alveolar oxygen difference) is a good early indicator of FES and may indicate high risk patients (Levy, 1990). The chest radiograph is initially normal but later shows multiple

bilateral patchy areas of interstitial or alveolar infiltrate. These patchy infiltrates often progress to opacify each lung diffusely.

Patients who develop ARDS may require mechanical ventilation with high levels of oxygen, pressure targeted ventilation and positive end expiratory pressure. This may lead to other complications such as barotrauma, pneumothorax and gram negative infections (Levy, 1990).

### *1.6.2 Cerebral signs*

Cerebral involvement is seen in 86% of patients (van Besouw and Hinds, 1989). Patients are usually asymptomatic for the first 24 hours. Signs may precede the development of pulmonary symptoms. Initially these are subtle but later restlessness, drowsiness, confusion and eventually coma ensue. These signs occur independent of the degree of hypoxia. As patients have multiple injuries, other causes of confusion such as subdural haematoma must be excluded.

The mechanisms are controversial, however paradoxical embolization through a patent foreman ovale may play a role (Christie, 1995). When it does occur it usually follows extensive lung involvement with high pulmonary artery pressures.

Most patients make a full recovery, but a small percentage are left with residual signs such as epilepsy or blindness.

### *1.6.3 Petechial rash*

This is found 50-60% cases and appears 24-48 hours after the incident. The mechanism involves embolization of fat globules within the dermal capillary network (Alho, 1980). It may last for only 6 hours but although it may last for longer, it is self limiting and disappears within 7 days. The commonest sites are the axillae, the anterior chest and neck, around the navel, and the conjunctiva and mucous membranes. It has been postulated that the location may be due to haemodynamic properties of fat globules and the position of the supine patient (Tachakra, 1976). Pathologically they are similar to petechiae seen in brain and lungs.

### *1.6.4 Other Clinical Manifestations*

Several minor symptoms accompany the clinical picture fairly consistently. The most common are an early fever, which may be evident on initial presentation, retinal changes (exudates, oedematous patches, cotton wool spots, haemorrhages and intravascular fat globules), jaundice, renal changes and tachycardia. These often precede major symptoms (Levy, 1990) and although of themselves are

of no major clinical significance, they may serve as early warning signs of impending serious event.

ECG changes including right heart strain and non-specific T wave changes may occur later.

## 1.7 LABORATORY FINDINGS

Laboratory findings include hypoxia, thrombocytopenia, anaemia hypocalcaemia and decreased fibrinogen (McCarthy et al. 1973). The most sensitive of these is hypoxia. Many other tests, including fat globules in the urine, sputum or blood, elevated lipase and blood lipids have been proposed but have not been shown to be of diagnostic or predictive value (Nolte et al. 1974; Hutchins and Macnicol, 1985; Levy, 1990; Gurd, 1970).

## 1.8 DIAGNOSIS

FES is mainly a diagnosis by exclusion. It is often masked in multiple trauma by other injuries. Furthermore, many of the signs and symptoms are non-specific ie. tachycardia, fever, confusion, anaemia,

thrombocytopenia. In order to standardise the diagnosis, Gurd in 1970 proposed the following criteria.

### *1.8.1 Gurd's Criteria for diagnosis of FES* (Gurd, 1970)

***Major***

- petechial rash
- respiratory symptoms plus bilateral signs with positive radiographic changes
- central nervous system depression

***Minor***

- tachycardia
- pyrexia
- emboli present in retina
- fat present in urine
- sudden drop in haematocrit or platelet count
- increased erythrocyte sedimentation rate (ESR)
- fat globules in sputum
- sudden thrombocytopenia

He considered one major criteria and four minor criteria to be diagnostic.

He later refined the criteria to include fat globules in the peripheral circulation (Gurd, 1974).

Some studies however, have questioned the usefulness of Gurd's criteria and (Lindeque et al. 1987) have shown them to be neither sensitive nor specific. Lindeque (Lindeque et al. 1987) and others

(Bulger, 1997; Pollak and Myers, 1978) proposed that the diagnosis be based on measurement of the partial pressure of oxygen in peripheral blood.

Others have suggested the use of a fracture index as a semiquantitative means of diagnosing FES (Schonfeld et al. 1983). Each of seven signs is assigned a particular score and a cumulative score greater than 5 is necessary for a positive diagnosis (See Table 1.1).

*Table 1*

| **Symptom** | **Score *** |
|---|---|
| Petechiae | 5 |
| Diffuse alveolar infiltrates | 4 |
| Hypoxia $PaO_2$ <60mmHg | 3 |
| Confusion | 1 |
| Fever | 1 |
| Heart rate >120 bpm | 1 |
| Respiratory rate >30 bpm | 1 |

* Total score > 5 is diagnostic of FES

### *1.8.2 Other Diagnostic Techniques*

The lack of sensitive clinical and laboratory criteria for the diagnosis of FES has led investigators to investigate other potentially diagnostic techniques. The usefulness of bronchoalveolar lavage (BAL) has been studied by Chastre (Chastre et al. 1990). Patients with trauma in whom the syndrome was evident or suspected underwent lavage, and the results were compared with those in unaffected patients with trauma, patients without trauma but who had ARDS and normals. The lavage specimens were stained with oil red O. In those with the fat embolism syndrome, 63 % of the lavage cells contained fat droplets as compared with less than 2 % of the cells from the other groups. Furthermore, if a cut-off of 5% of cells containing fat droplets was used, there were no false positive or false negative results. It was postulated that this technique might permit early diagnosis and intervention in the asymptomatic, high risk group of patients or those with multiple trauma where clinical signs are difficult to assess. The value of this technique has however been refuted in a more recent study which revealed that most patients in respiratory failure on ventilators have fat present on BAL and that the FES could only be considered in patients with a BAL fat concentration of greater than 30% (Mimoz et al. 1995).

Cytological analysis of blood withdrawn from the lumen of a pulmonary artery catheter has also been suggested as a potentially useful method of identifying patients with the FES but the specificity of this technique has not been evaluated (Masson and Ruggierei, 1985). Furthermore, the finding of fat in the pulmonary circulation does not necessarily mean that the patient will develop the FES.

## 1.9 TREATMENT

It has been suggested that early intervention in patients with FES may improve their outcome (ten Duis, 1997). Various studies have indicated that patients who have the following features may be considered to be high risk:

- fracture of lower limbs
- male sex
- age under 30
- early hypoxia ie. $PaO_2 < 60$ mmHg
- multiple fractures (Levy, 1990.

These patients need to be carefully observed.

Numerous treatment modalities have been used over the past 70 years. Unfortunately, few have proven to be of any benefit when scrutinised

in controlled trials and management in 1998 remains largely supportive. However, some empirical evidence exists indicating possible benefit from some treatment modalities.

### *1.9.1 Supportive*

Hypovolaemia is considered to be fundamental to the development of the FES, therefore fluid resuscitation is of utmost importance (Durst et al. 1976; ten Duis. 1997). This should preferably be initiated on the road side and continued in hospital. Some consider human albumin to be the best form of fluid replacement as it binds circulating FFA (van Besouw and Hinds, 1989).

In hospital, respiratory function should be carefully monitored, either with pulse oximetry or arterial blood gas measurement. Laboratory investigations should include a full blood count in order to monitor haemoglobin and platelet count. Any sign of respiratory deterioration should be managed with oxygen and the patient monitored carefully for other signs such as confusion and petechiae. Early pain relief may limit the sympathomimetic response which results in increased liberation of FFA by accelerated lipolysis. This should be instituted early, bearing in mind the risk of respiratory depression in a

compromised individual. Ventilate early if necessary.

### *1.9.2 Early Surgery*

The role of early surgery has received much recent scrutiny. Many feel that persistent limb movement results in further bleeding and embolization. Serial blood gas measurements reveal intermittent periods of hypoxia which may be related to ongoing shift in the fracture site (Tachakra, 1976).

Several studies have shown that early immobilisation and fixation of long bone fractures may be beneficial (Riska and Myllynen, 1982; Riskan et al. 1976; Behrman et al. 1990) This does not apply to all surgical techniques and it is accepted that intramedullary nailing increases intramedullary pressure and can cause fat embolization (Christie, 1995). This was not supported in a recent study which failed to show an increased incidence of FES with early intramedullary fixation (Bulger et al. 1997).

### *1.9.3 Medical*

Over the years, many drugs have been used to treat FES. Heparin has been advocated for the treatment of fat embolism because of its ability to increase lipase activity, its anti-serotonin effect and its ability to inhibit intravascular coagulation (Bulger et al. 1997). Unfortunately, the clearing of neutral fat from the bloodstream results in increased free fatty acids which are now known to be toxic to the lungs and other tissues, and may actually aggravate the situation. This and the fact that heparin increases the risk of bleeding in a trauma patient has led to it being abandoned as a therapeutic modality in the FES.

Because of its anti-platelet effects, aspirin also has its advocates. Trials have shown an increase in platelet counts and an improvement in arterial blood gases when compared to controls (Shier and Wilson, 1980; Shier et al. 1977). However, whether this has an effect on mortality is not known.

Although alcohol solutions were advocated in the 1960's because they decrease plasma lipase and therefore the concentration of FFA, studies have failed to demonstrate any benefit and its use is not recommended (van Besouw and Hinds, 1989).

Glucose and insulin have been given on the theoretical basis that the combination may lead to a decrease in the arterial levels of unesterified fatty acids. Controlled trials have yielded mixed results and their use is not recommended (Horne and Horne, 1974; Shier and Wilson, 1980).

Low molecular weight dextran complexes with fibrinogen, alters the surface charge of erythrocytes, decreases viscosity in injured blood vessel walls and reduces platelet adhesiveness. There have been a few isolated reports of benefit but most do not advocate its use (van Besouw and Hinds, 1989).

Clofibrate was used on the assumption that it may decrease triglyceride levels post trauma (Shier and Wilson, 1980). It is believed to act by virtue of its affinity for serum albumin, resulting in a reduction of the adrenalin stimulated release of fatty acids from fat stores. Its value has not been established.

Steroids have previously been used on the premise that they decrease capillary leak by stabilising lysosomal and capillary membranes. They

also have the potential to decrease the inflammatory reaction caused by free fatty acids and may inhibit the complement mediated aggregation of neutrophils (Levy, 1990). It has also been noted that patients given steroids prophylactically tended to have higher platelet counts (Shier and Wilson, 1980).

Many of the older studies indicated that steroids were beneficial and its use was very popular during the late 1970's and 1980's (Fischer et al. 1971; Shier et al. 1977; Schonfeld et al. 1983). Some clinical studies concluded that prophylactic administration minimized the fall in arterial oxygen tension seen in FES (Lindeque et al. 1987; Kallenbach et al. 1987). However, benefit is difficult to document as patients tend to fare as well with only supportive treatment (Bulger et al. 1997). The problem is that no large scale studies exist and standardisation of diagnostic criteria between those trials that do, is poor (Guenter and Braun, 1981). Added to this is the fact that the incidence of the syndrome is relatively rare. Steroids may be of some benefit, but their use is still controversial.

Despite ongoing research, the treatment for FES remains largely

supportive with careful fluid management its cornerstone.

## 1.10 HYPOTHESIS

The above review on the current thinking behind the pathogenesis and management of FES illustrates that we are still far from understanding it. It was with this in mind that we set about designing a study which would attempt to shed some light on the contentious issue of the pathogenesis of FES. Our hypothesis was based on the fact that more and more diseases are being explained on the basis of immune mediated mechanisms. We therefore felt that it was likely that the acute lung injury that is associated with long bone fractures could also be explained in a similar way. The fact that these patients embolize fat is not disputed. However, fat particles could be altered in certain individuals in such a way to cause the clinical picture of FES. The development of the acute respiratory distress syndrome is associated with increased oxidant stress. Fat particles could become oxidized and the resultant lipid peroxides may result in acute lung injury. Why only a small number of patients develop FES may involve an imbalance in their oxidant/antioxidant systems related to either the degree of oxidant

stress or to systemic antioxidant status.

The following chapters will summarize issues relating to phagocyte function and systemic inflammation which are central to an understanding of the cellular interactions which may give rise to the FES.

# CHAPTER 2

# THE CELLULAR RESPONSE TO INFLAMMATION

## 2.1 INTRODUCTION TO PHAGOCYTES

Phagocytic cells play a key role in the body's immune system, both for surveillance and protection from potentially harmful bacterial and fungal pathogens. There are two major groups, the polymorphonuclear leukocytes (PMNL) and the mononuclear cells. Once exposed to a chemotactic stimuli, the cells move to become associated with the invader. This is followed by degranulation and the release of preformed lysozymal elements and the formation of reactive oxygen species resulting in the destruction of the offending organism. As we shall see later, there is often considerable tissue destruction associated with the above process.

Although the PMNL's primary function is to defend the host against invading microbes, the neutrophil has little intrinsic ability to differentiate between foreign and host antigens and relies on other arms of the immune system (antibodies, complement and cytokines) to select its targets. If normal host tissues are identified inappropriately as foreign, the cell's destructive potential can lead to extensive tissue damage.

### *2.1.1 Chemotaxis*

The release of substances such as C5a, IL-8 (Fijishima and Aikawa, 1994) and bacterial derived N-formylated polypeptides direct the movement of cells toward the site of tissue invasion (Schleimer et al. 1991; Charo et al. 1986). The microtubule organizing centres of the cell are located on the same side of the nucleus as the chemoattractant (Zigmond et al. 1981). This appears essential to determine the direction of locomotion of the PMNL and the orientation of the pseudopodia. Contact with the endothelial cells and subsequent diapedesis is accomplished (without damage to the PMNL) via the interaction of various adhesion molecules (Froese et al. 1994). The in vivo adherence of PMNL to the endothelium is greatly augmented by tissue damage and other inflammatory stimuli and with more intense inflammation, adhesion between PMNL occurs, greatly enhancing the numbers localised to the site of damage (Brown, 1997).

### *2.1.2 Adhesion*

The mechanisms of adhesion between PMNL and endothelial cells is complex but important in the localisation of these cells at sites of inflammation and their subsequent migration into tissue. The receptors or adhesion molecules as they are known, have been classified into

four main superfamilies (Campbell et al. 1994; Froese et al. 1994; Smith, 1990):

1. ***Immunoglobulin superfamily***

   These are characterised by one or more immunoglobulin like domains. Examples include intercellular adhesion molecules (ICAM-I and II) and vascular cell adhesion molecules (VCAM-I) which are found on endothelial cells and effect leukocyte migration.

2. ***The Integrins***

   They are heterodimeric transmembrane glycoproteins found on the surface of a large variety of cells with target receptors which may include extracellular matrix proteins such as fibrin and fibronectin.

3. ***The Selectins***

   Consisting of p, e, and l selectins, are dynamically expressed on the surface of platelets, endothelial cells and leukocytes depending on their state of activation.

4. ***The Cadherins***

   They establish molecular links between adjacent cells. They form zipper like structures at adheren junctions which are

membrane regions where cells make contact with one another (Frenette and Wagner, 1996).

### *2.1.3 Endothelial Interactions*

Neutrophil/endothelial interactions resulting in cell/cell attachment occurs via three steps ie. reversible adhesion, leukocyte activation and activation dependent binding.(Froese et al. 1994). Neutrophil rolling and slowing takes place as a result of the neutrophil expressing various l selectins which interact with its counter receptors the p and e selectins on the surface of the endothelial cell.( Froese et al. 1994; Stoolman, 1993). All 3 members of the selectin family can mediate rolling. The release of pro-inflammatory cytokines including tumour necrosis factor (TNF) and interleukins 1, 4 and 6 (IL-1, IL-4 and IL-6), (Schleimer et al. 1991) leads to the activation of the PMNL and the expression of activated $\beta_2$ integrins, MAC-I and LFA-I on the neutrophil cell surface (Smith, 1990). Their interaction with ICAM-I leads to the arrest and spreading of rolling neutrophils on the endothelial surface (Froese et al. 1994). This adhesive interaction is also necessary for the migration of adherent neutrophils through the endothelial monolayer (Stoolman, 1993).

### *2.1.4 Degranulation and Phagocytosis*

Chemotactic factors that are responsible for the accumulation of PMNL at sites of inflammation also appear to be involved in the release of storage granules and production of toxic oxygen products by these cells (Fantone et al. 1987). Binding of these chemoattractants to receptors on the surface of the cells leads to the stimulation of a guanine triphosphate (GTP) protein linked second messenger system within the cell. The initiation of the second messenger pathway leads eventually to phospholipase C activation and subsequently in the formation of inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) from the cleavage of phosphatidylinositol 4,5 biphosphate, a membrane bound phospholipid (Linder and Gilman, 1992).

IP3 induces the release of calcium from intracellular stores which results in the activation of phospholipase $A_2$ with the consequent formation and release of arachidonic acid. Arachidonic acid via its many by-products which include leukotriene $B_4$, have potent biological effects on inflammatory cells. The DAG stays in the cell membrane where it activates one of seven subspecies of protein kinase C which activates dihydronicotinamide adenine dinucleotide phosphate

(NADPH) oxidase to form reactive oxidant species (ROS) (Fantone et al. 1987).

Coincident with the production of ROS, the organism is opsonised and engulfed by the phagocyte and sequestered in a vesicle which is lined by what was plasma membrane ( Klebanoff, 1980). Phagocytosis also results in the release of preformed granule contents to the outside of the cell (Fontane et al. 1987). The process is complex and is triggered by an increase in the intracellular ionised calcium level. In the PMNL, the primary (azurophil) granules contain myeloperoxides (MPO), various acid hydrolases, elastase and cationic proteins. The secondary granules contain lactoferrin, vitamin $B_{12}$ binding protein, collagenase, gelatinase, lysozyme and alkaline phosphatase.( Klebanoff, 1980). The enzymes with the greatest destructive potential include elastase, proteinase-3, collagenase and gelatinase (Weiss and Peppin, 1986). Their release leads to the destruction of the surrounding extracellular matrix.

Other proinflammatory substances are also released. The arachidonic cascade is initiated leading to the production of various

proinflammatory prostaglandins and leukotrienes. Platelet activating factor (PAF), which is generated in neutrophils via the activation of phospholipase $A_2$ and PAF acetyl transferase has various biological effects including a direct cytotoxic effect on endothelial cells (Fontane et al. 1987). Neutrophils have also been shown to produce numerous cytokines (Fijishima and Aikawa, 1994). These include:

- TNF, IL-1 and IL-6, which are involved in the initiation and maintenance of inflammation
- IL-8 a potent chemotactic factor
- granulocyte/macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and IL-3 which induce proliferation and differentiation of haematopoietic cells

## 2.2 REACTIVE OXIDANTS

Of possibly greater importance however, is the release of reactive oxygen metabolites by activation of NADPH-oxidase which catalyses the one electron reduction of oxygen to superoxide $O_2^{\bullet -}$ (Clark 1990). The stimulated cell markedly increases oxygen consumption and converts this to oxygen radicals. NADPH is generated via the hexose-monophosphate shunt. NADPH oxidase is a membrane associated oxidase capable of generating superoxide radicals at the exterior of the

cell (Babior, 1984). This process involves the transfer of an electron from NADPH via a b type cytochrome to $O_2$ (Clark, 1990). Activation occurs via the G-protein associated second messenger system discussed earlier.

Oxygen radicals are unstable forms of oxygen which, due to the presence of one or more unpaired electrons are highly reactive (Halliwell, 1994). Although the oxygen molecule is itself a diradical, having two unpaired electrons, its reactivity is limited by the fact that the electrons have parallel spin. This imposes a restriction on electron transfer resulting in a sluggish reaction with non-radicals. Once reduced (having accepted an electron), the oxygen molecule produces a superoxide radical (Klebanoff, 1980). The subsequent reduction of the superoxide radical leads to the formation of $H_2O_2$, which is a potent oxidising agent.

Under normal circumstances, 98% of the oxygen consumed by cells can be accounted for by the catalytic reduction of oxygen to water by cytochrome c, without the release of radicals. The other 2% undergoes sequential one electron reductions to form superoxide radical and

hydrogen peroxide (Tanswel and Freeman, 1995). The reactivity of these compounds is counterbalanced by the presence of various anti-oxidants inside and outside the cell such as superoxide dismutase (SOD), catalase and glutathione (GSH). However, once stimulated, the resting neutrophil is able to markedly increase its production of free radicals, overwhelming the anti-oxidant system and leading to tissue damage.

The stimuli for the production of free radicals are numerous. Some examples are: oxidant enzymes, phagocyte membranes, tobacco smoke, ozone, ionizing radiation and drugs (paracetamol, chemotherapy agents). Low wave electromagnetic radiation can split water in the body to generate the hydroxyl radical $OH^{\bullet}$, which is highly reactive. Nitric oxide is also a free radical produced by endothelial cells which when paired with a superoxide radical, produces peroxynitrite, a highly toxic substance (Halliwell, 1994; Tanswell and Freeman, 1995).

### *2.2.1 Chemistry of Free Radicals*

Free radicals produced by phagocytes are generated in the following way:

1. $2O_2 + NADPH \xrightarrow[\text{NADPH oxidase}]{} 2O_2^{\bullet -} + NADP^+ + H^+$

$H_2O_2$ is subsequently formed in a dismutation reaction, catalysed by the enzyme SOD which is found in the mitochondria and the cytosol.

2. $2O_2^{\bullet -} + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2$

Both $O_2^{\bullet -}$ and $H_2O_2$ can react with a number of important biological substrates (Fridovich, 1986). However, intact neutrophils appear to be somewhat limited in their ability to use either metabolite alone to cause extracellular damage. In fact, the bulk of $H_2O_2$ is consumed by the neutrophils themselves and only a small portion can actually be detected in the extracellular pool (Test and Weiss, 1984). This fact has helped to fuel interest in the possibility that the cell uses these metabolites to generate a more powerful oxidant, $OH^{\bullet}$ (Stephens, 1989; Tanswell and Freeman, 1995).

$OH^{\bullet}$ is thought to be produced in a reaction known as the Haber-Weiss Reaction which requires the presence of a so called transition metal catalyst (Babior, 1984; Klebanoff, 1980).

3. $O_2^{\bullet -} + Fe_3^{+} \text{-------} > O_2 + Fe_2^{+}$

4. $Fe_2^{+} + H_2O_2 \text{-------} > Fe_3^{+} + OH^{-} + OH^{\bullet}$

5. $O_2^{\bullet -} + H_2O_2 \text{----------} > O_2 + OH^{-} + OH^{\bullet}$

This reaction has not been shown to occur in neutrophils due to the production of myeloperoxidase which diverts $H_2O_2$ to other chemical reactions (Halliwell, 1994). Furthermore, an iron binding protein, lactoferrin is released by the neutrophil, resulting in the sequestration of available iron in a form that fails to catalyze $OH^{\bullet}$ formation (Britigan et al. 1988). It should be noted that other metals including copper have also been implicated in the formation of $OH^{\bullet}$ (Halliwell, 1991).

Most of the $H_2O_2$ formed is catabolised in the presence of a halide (usually chloride), and the enzyme myeloperoxidase to form hypochlorous acid.

6. $H_2O_2 + X^{-} + H^{+} \xrightarrow[MPO]{\text{----------}} HOX + H_2O$

$X = Cl^{-}; Br^{-}; I^{-}$

HOCl is highly reactive and has many potential targets including, amines, thiols, thioethers, nucleotides, haemoproteins, and polyenoic acids. However, because of its high reactivity, it does not actually accumulate in biological systems but instead, almost instantly disappears in multiple reactions with available substrates (Test and Weiss, 1986). A portion reacts with low molecular weight amines to yield chloramines (Babior, 1984).

7. $R - NH_2 + HOCl \text{ ---------> } R - NHCl + H_2O$

These, although less reactive are available to chlorinate or oxidise various targets (Test and Weiss, 1986). Chloramines have been shown to exert strong microbicidal activity with little if any damage to surrounding tissues. An interesting property of these amines is that they may have a long half life (up to 16 hours) which allows for their accumulation at inflammatory sites and their diffusion over large distances (Stephens, 1989).

### *2.2.2 Mechanisms of Tissue Damage*

The products resulting from PMNL oxygen metabolism therefore include:

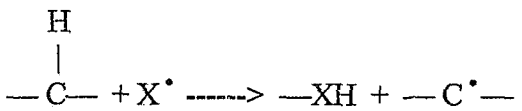
1. $O_2^{\bullet -}$ and $H_2O_2$
2. $OH^{\bullet}$
3. HOCl
4. N-chloramines

Direct tissue injury is most likely mediated by the above substances (Fridovich, 1986). However, with the protective mechanisms in place such as MPO system, the fact that certain substances such as the chloramines do little damage to tissues suggests this may not represent the entire oxidant stress (Weiss and Peppin, 1986). Whatever the final toxic product, injury is mediated either directly, through lipid peroxidation and DNA injury or indirectly by depriving cells of both energy and of the anti-proteolytic screen.

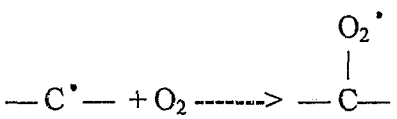
#### *2.2.2.1 Direct oxidant mediated tissue injury*

One of the primary mechanisms proposed for free radical mediated cell damage is the formation of lipid peroxides within cell walls which interfere with the function and integrity of the membrane. A large percentage of most cell membranes consist of a lipid bilayer and are susceptible to damage via this process. The sequence of events involves:

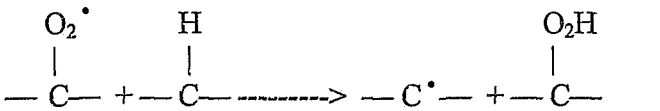
1. The reactive radical eg. $OH^{\bullet}$ or $NO^{-}$ abstracts an atom of hydrogen from the polyunsaturated fatty-acid side chain in the membrane (Dargel, 1992). This leaves an unpaired electron on carbon.



2. Carbon radical reacts with oxygen

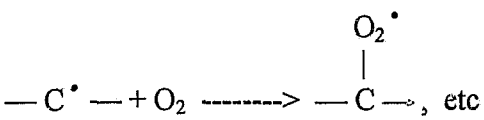


3. Resulting peroxyl radical attacks adjacent fatty acid side chain to generate new carbon radical



Lipid peroxide

4. And chain reaction continues:



, etc

(Halliwell, 1994).

The reaction becomes a self propagating chain reaction terminated by bond rearrangements to form diene conjugates eg. Cyclic peroxides

and lipid hydroperoxides (Dargel, 1992). These undergo degradation to form aldehydes. Aldehydes may react with the amine and sulphydryl groups of membrane proteins, damaging sodium and potassium ATPase pumps and membrane bound enzymes. Cell permeability is altered leading to cell death (Logani and Davies, 1980). There is also evidence that the presence of lipid peroxides leads to increased intracellular calcium and subsequent cell death due to induction of intramitochondrial calcium stasis (Dargel, 1992).

Oxidant mediated DNA damage is a poorly understood process. One mechanism is that $OH^{\bullet}$ in the presence of iron oxidatively damages adenine, guanine, thymine and cytosine and induces DNA strand breaks (Cross et al. 1994). Other effects may be via the inhibition of key enzymes in DNA replication and repair (Jackson et al. 1989).

Oxidant induced endothelial injury can be mediated by depletion of intracellular adenosine triphosphate (ATP). It has been shown that HOCl and $H_2O_2$ can inhibit ATP generation and that this may result in severe cellular damage from cellular energy depletion (Anderson et al. 1990). This may be due to inhibition of glycolytic enzymes and cofactors in the Kreb's cycle.

Reactive oxidants can interfere directly with protein components of membrane receptor systems. Lipid peroxidation affects receptor structure leading to diminished coupling of the receptor to effector proteins (Cross et al. 1994).

### *2.2.2.2 Indirect oxidant mediated injury*

Reactive oxidants are short lived, react only once and injury is non-selective. Enzymes however, remain active for exceptionally long periods, repetitively catalyse a given reaction, and will react only with targets dictated by the range of its substrate spectrum (Weiss, 1989). Neutrophil granules contain a large family of over 20 enzymes, but 3 proteolytic enzymes, the serine proteinase, elastase and the two metalloproteinases, collagenase and gelatinase, seem to have the greatest potential to destroy tissue (Weiss, 1989; Weiss and Peppin, 1986; Fantone, 1987).

Elastase is a serine proteinase which functions to attack the carbonyl carbons of peptide bonds (Lonky and McCarren, 1983). The destructive potential of this enzyme is immense, having the capacity to degrade almost all components of the cell matrix (Buhl et al. 1996).

Other enzymes which act in concert with elastase include cathepsin G and proteinase-3.

Neutrophil collagenase can cleave each of the interstitial collagens (ie. Type I, II and III collagens) while gelatinase has been reported to degrade native type V, type IX and type IV collagens (Weiss and Peppin, 1986). Both are secreted in an inactive form and require the presence of oxidants in order to degrade collagen (Stephens, 1989).

Unopposed, these enzymes would result in considerable damage to surrounding tissue. Therefore, powerful regulatory mechanisms restrict their activity to their major physiological role of bacterial digestion and killing. Alpha-1-antiprotease (AAP), a 52-kd glycoprotein that irreversibly inhibits neutrophil elastase by forming an enzyme-inhibitor complex and alpha-2-macroglobulin, a 725-kd, broad spectrum antiproteinase are major systemic antagonists while anti-leukoproteinase is found only in the interstitial fluids and mucous secretions (Lonky and McCarrel, 1983; Janoff, 1985). Of the three, AAP is the most important. Generally speaking, the quantity of elastase secreted by neutrophils is insignificant compared to that of

AAP, however excessive production of ROS decreases functional AAP. Oxidation of AAP causes a 2000 fold decrease in the rate of association between AAP and elastase (Janoff, 1985). Thus tissue damage depends upon the rate at which the neutrophil derived ROS inactivate AAP and the rate at which AAP inhibits released elastase. Of note is that both $\alpha$1-macroglobulin and anti-leukoproteinase's effectiveness is also reduced by the presence of reactive oxidants.

## 2.3 THE ANTI-OXIDANT SYSTEM

Superoxide dismutase, catalase and glutathione peroxidase (GSH-P) are the primary preventative intracellular anti-oxidants.

### *2.3.1 Superoxide Dismutase and Catalase*

SOD's are metal (primarily zinc and copper) containing enzymes that cause superoxide to react preferentially with another superoxide anion to form hydrogen peroxide (Tanswell and Freeman, 1995). Theoretically, SOD is not an anti-oxidant, in that it could result in the generation of excess $H_2O_2$ which can interact either with MPO to form HOCL or with $Fe_3^+$ to form $OH^\bullet$ (Halliwell, 1994). Which reaction occurs depends on the presence of Fe and PMNL and the quantity of

catalase and GSH peroxidase available. If Fe is unable to react and if MPO is not present, then catalase and peroxidase can reduce the $H_2O_2$ generated to non-toxic products (Halliwell, 1991).

Catalase is found in cellular peroxisomes. Its activity increases linearly with $H_2O_2$ concentration until inactivated at high concentrations. Its primary role is to catalyse the following reaction (Halliwell, 1984):

$$2\ H_2O_2 \xrightarrow[\text{Catalase}]{} 2\ H_2O + O_2$$

### *2.3.2 Glutathione*

Glutathione, the substrate for glutathione peroxidase is a selenium containing compound that is a tripeptide of glutamate, cysteine and glycine and exists in both the thiol reduced and disulphide oxidised forms (Lomaestro and Malone, 1995). High activity is found in liver, moderate activity in heart, lung and brain while low activity is found in muscle (Halliwell, 1984). GSH/GSSG (Oxidised/Reduced glutathione) is maintained in a high ratio intracellularly by a cellular transport system which transports only GSSG out of the cell, and by the reduction of GSSG in the presence of GSH reductase (Dargel, 1992).

Glutathione synthesis is dependent upon the availability of its precursor, L-cysteine which is derived from the diet or protein breakdown. The regeneration of reduced GSH requires the presence of NADPH from the hexose monophosphate shunt (Lomaestro and Malone, 1995). It is the most abundant mammalian anti-oxidant and is found primarily intracellularly but also extracellularly including the alveolar lining fluid of the lung.

Glutathione peroxidase reduces $H_2O_2$ more efficiently than catalase at low $H_2O_2$ concentrations, and has greater ability to reduce intracellular lipid peroxides (Halliwell, 1991).

$$H_2O_2 + 2GSH \xrightarrow[\text{peroxidase}]{\text{Glutathione}} GSSG + H_2O$$

In addition, glutathione is a scavenger of hydroxyl radicals. As the enzyme glucose-6-phosphate dehydrogenase is necessary for the production of NADPH, patients with a deficiency are prone to oxidative stress (Meister, 1988) (as manifest by red cell haemolysis). Organisms undergoing oxidative stress are able to increase levels of GSH production (Cross et al. 1994).

### *2.3.3 Nutrient Anti-oxidants*

Anti-oxidant enzymes are primarily intracellular whereas nutrient anti-oxidants derived from the diet are responsible for inactivation of extracellular free radicals. These are vitamin E (VE), ascorbate (VC) and β-carotene (BC), the major carotenoid precursor of vitamin A (VA).

VE is the major lipid soluble anti-oxidant present in all cellular membranes and as such effectively protects against lipid peroxidation and mutagenesis (Tanswell and Freeman, 1995). VE inhibits the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals (Halliwell, 1994).

$$\alpha TH + -C(O_2)- \longrightarrow -C(O_2H)- + T$$

The tocopheral radical (T) is much less reactive in attacking adjacent fatty acid side chains and can be converted back to α-tocopherol by VC (Halliwell, 1984). The natural oxidant scavenging effect of VE is weak and to be effective, it must interact over prolonged periods with oxidant generating systems. There appear to be other mechanisms by which VE exerts its protective effects on cells which are not well

understood. One possibility however is that VE, on incorporation into membranes stabilises lipid membrane structures, and influences permeability (Burton, 1994). This could limit release of reactive oxidants and generation of lipid peroxides within the cell in a nonspecific fashion unrelated to its anti-oxidant properties (Halliwell, 1984). Its therapeutic use is uncertain as only patients with deficiency states have consistently been shown to respond to therapy.

Despite the fact that BC is the major carotenoid precursor of VA, the later has itself no major anti-oxidant function. BC quenches singlet oxygen and also functions as a chain breaking anti-oxidant in the lipid phase by neutralising peroxyradicals (Burton, 1989; Burton and Ingold, 1984). It also functions as an extra and intracellular scavenger of oxidants generated by the MPO/$H_2O_2$/halide system with effects that are complementary to VE.

VC, present in plasma from healthy individuals at concentrations of 50-200μmol/L has many anti-oxidant properties. These include the ability to regenerate α-tocopherol by the reduction of α-tocopheryl radicals at the surface of lipoproteins and membranes (Halliwell,

1991). It can directly scavenge $O_2^{\bullet -}$ and $OH^{\bullet}$. This results in the formation of the semihydroascorbate free radical whose cytotoxic metabolite, oxalate is further reduced by a GSH dependent dehydroascorbate reductase (Meister, 1994; Halliwell, 1984). Humans are unable to synthesize ascorbic acid and must acquire it from their diet.

Ascorbate has the ability to function as either a pro-oxidant or an anti-oxidant. This depends on the availability of metal ions. Mixtures of ascorbate and $H_2O_2$ with iron have powerful pro-oxidant properties, because they form $OH^{\bullet}$ by the Fenton reaction (Wayner et al. 1986; Halliwell, 1984). Thus, there is the need for careful sequestration of free iron in the body in order for ascorbate to exert an anti-oxidant action (Halliwell, 1991).

# CHAPTER 3

# SYSTEMIC INFLAMMATORY RESPONSE SYNDROME

# INFLAMMATION AND CYTOKINES

## 3.1 INTRODUCTION

For a number of years now, it has been recognised that many patients who die in intensive care units from multi-organ failure, succumb for reasons unrelated to their initial insult (Moore and Moore, 1995). Furthermore, despite the fact that patients are clinically septic, it is often not possible to isolate an organism. This has led to the concept that the response of the immune system to an insult is vital to determining the course of their disease. In 1991, the Society of Critical Care Medicine and the American College of Chest Physicians held a consensus conference to produce a series of universal definitions for the systemic inflammatory response syndrome (SIRS), sepsis and other clinical conditions related to sepsis (Bone et al. 1992)

## 3.2 THE CONCEPTS OF SIRS and SEPSIS

The presence of SIRS is implied by a clinical response arising from a nonspecific insult and includes 2 or more of the following defined variables:

- Temperature ≥ 38°C or ≤ 36°C
- Heart Rate >90 beats per min
- Respiratory rate >20 breaths per min or $PaCO_2$ <4.3 kPa
- WCC >12 000 cells per $mm^3$, <4000 cells per $mm^3$ or 10 percent immature forms.

Sepsis is defined as SIRS with a documented infection. Multiple-organ dysfunction syndrome (MODS) is defined as failure to maintain homeostasis without intervention (Bone et al. 1992). There is a continuum from the development of SIRS to multi-organ dysfunction (Rangel-Frausto et al. 1995) and the mortality increases proportionate to the number of organs that fail (Rangel-Frausto et al. 1995; Moore and Moore, 1995)

Inflammation is the body's initial response to tissue injury. There are four major events in the inflammatory process: vasodilatation, increased microvascular permeability, cellular activation and coagulation (Davies and Hagen, 1997). The major metabolic change that occurs in response to inflammation is an increase in oxygen consumption. The initiation of this process has been previously discussed but involves the interaction of macrophages and neutrophils.

The mechanisms of macrophage activation are generally not well understood except in gram negative sepsis where lipid A (a component of endotoxin) stimulates the release of proinflammatory cytokines from macrophages (Dinarello, 1997)

In the process, various cytokines, (which are communication proteins) are secreted. These enhance neutrophil migration and adhesion and amplify the inflammatory process. Relevant cytokines include tumour necrosis factor α (TNF-α), interleukins 1 and 6 (IL-1 and IL-6), interferons and colony stimulating factors (Foex and Shelly, 1996). It is these substances that are responsible for the maintenance of immune homeostasis.

## 3.3 SIRS AND THE PATHOPHYSIOLOGY OF INFLAMMATION

Bone proposed that there are three stages in the development of SIRS (Bone et al. 1992):

1. In response to injury or infection, the local environment produces cytokines. These help promote wound repair and they recruit cells to combat pathogenic organisms.

2. Small amounts of cytokines are released into the systemic circulation. Macrophages and platelets are recruited and production of growth factors is stimulated. At this stage the response is not pathological but represents a normal defence mechanism. This cytokine response is usually tightly regulated.

3. Occasionally pro-inflammatory stimuli cause regulatory mechanisms to be overwhelmed and homeostasis is lost. This results in a massive systemic reaction in which the effects of the cytokines become destructive. Capillary walls leak and organ dysfunction ensues. Unless this process can be brought back under control, MODS and subsequently death will occur.

The processes by which neutrophils become activated and migrate have been discussed in the previous section. The mediator response may be divided into four phases based on the cytokine/cellular response; induction, triggering of cytokine synthesis, evolution of the cytokine cascade and elaboration of secondary mediators with ensuing cellular injury (Davies and Hagen, 1997). The most influential are TNF, IL-6, and IL-1 (Foex and Shelly, 1996). Persistent levels of TNF and IL-6 are highly predictive of the development of MODS and death (Pinsky et al. 1993)

### *3.3.1 TNF and Interleukins*

TNF is produced primarily by mononuclear and polymorphonuclear leukocytes (PMNL). It elicits the release of PMNL from bone marrow, initiates adhesion and the production of superoxide radicals. It

activates macrophages and stimulates synthesis of acute phase reactants. TNF increases endothelial procoagulant activity and promotes the release of IL-1 (Foex and Shelly, 1996). Administration of TNF to animals evokes the pathophysiological responses associated with SIRS (Tracey et al. 1986). TNF given to human volunteers causes myalgia, chills, headaches, nausea, and tachycardia with an increase in cardiac output and a decrease in peripheral vascular resistance (Suffredini et al. 1989).

IL-1 appears to be released either in parallel or in response to TNF secretion with monocytes or macrophages the primary source. IL-1 is a strong inducer of GM-CSF and hepatic acute phase proteins. Excessive release results in excessive adhesion of activated neutrophils and also stimulates endothelial cell procoagulant activity (Nawroth et al. 1986). Animal studies showing improved survival with IL-1 blockers provide strong evidence supporting an important role of IL-1 in SIRS and sepsis (Pruitt et al. 1995). Unlike TNF, IL-1 is not directly lethal, but it can reproduce many of the effects of TNF and is equipotent in inducing the synthesis of other cytokines (Fong et al. 1990)

IL-6 comprises a family of six differentially modified phosphoglycoproteins. They function as B cell stimulation and T cell differentiation factors (Foex and Shelly, 1996). IL-6 also functions in conjunction with IL-1 and TNF to augment T cell proliferation and promote PMNL activation and adhesion (Dinarello, 1997). IL-6 administration does not cause haemodynamic instability, regardless of dose given (Davies and Hagen, 1997).

Other cytokine which have been shown to interact in the inflammatory cascade include IL-2, IL-4, IL-8, TNF-β and GM-CSF.

### *3.3.2 Arachidonic Acid*

The leukocytes and the endothelial cells produce pro-inflammatory second messengers which include prostaglandins, leukotrienes, thromboxanes, platelet activating factor, nitric oxide and proteases which are responsible for most of the manifestations of the SIRS. Arachidonic acid metabolites, particularly those of lipoxygenase and cyclo-oxygenase are significant mediators of SIRS and their release is stimulated by TNF and IL-1 (Schlag and Redl, 1996; Davies and Hagen, 1997). The major endothelial derived prostoglandin is prostacyclin ($PGI_2$). $PGI_2$ is a potent vasodilator, prevents platelet

aggregation and inhibits thrombosis. Thromboxane ($TXA_2$) induces platelet aggregation and neutrophil accumulation, and increases vascular permeability leading to capillary leak.

### *3.3.3 Nitric Oxide*

Nitric oxide is synthesised from the conversion of l-arginine to citrulline by the enzyme nitric oxide (NO) synthetase. This exists in both a cytosolic and a membrane bound form and acts via a membrane cyclic guanine mono-phosphate (cGMP) dependent kinase. It functions by relaxing smooth muscle and inhibiting platelet aggregation and is therefore responsible for many of the clinical manifestations of septic shock. Other adverse effects are due to the conversion of NO to peroxynitrite which is a potentially toxic free radical (Nussler and Billiar, 1993; Mizutani and Layton, 1996). It is also a myocardial suppressant and may lead to sepsis related cardiomyopathy (Finkel et al. 1992).

### *3.3.4 Reactive Oxidant Species*

These play a major role in the initiation and propagation of SIRS, sepsis and MODS. They have been discussed in the previous section.

### *3.3.5 Platelet Activating Factor*

This is produced by macrophages, PMNL, platelets and endothelial

cells. It has numerous negative effects which include, negative inotropism, activation of free radical production, platelet aggregation, and the promotion of PMNL activation and adhesion. Receptor blockers have been shown to be beneficial in preventing septic shock in animal studies (Moore et al. 1991; Schlag and Redl, 1996)

### *3.3.6 CARS and MARS*

Recent changes in the concept of inflammation have centred on the theory that not only is there a pro-inflammatory response but there appears to be an anti-inflammatory response that occurs in critically ill patients in an attempt to preserve homeostasis. This is termed the compensatory anti-inflammatory response syndrome or CARS (Bone, 1996a). The function of TNF, IL-1 and IL-6 have been well documented. However, there is also an anti-inflammatory cytokine network which inhibits immune function by decreasing monocyte, B cell and T cell function. Furthermore, these cytokines inhibit TNF and IL-1 production and function, blocking the SIRS component of inflammation. These cytokines include IL-4, IL-10, IL-13 and transforming growth factor β (Dinarello, 1997). Over-expression of these cytokines may actually lead to a situation in which a patient is immunosuppressed (Moore, 1998). It is therefore necessary for a

balance to be established between these opposing effects in order for patients to survive.

### *3.3.7 Disseminated Intravascular Coagulation*

Disseminated intravascular coagulation (DIC) has been noted in patients with SIRS irrespective of whether the initial insult is traumatic or infectious.

Tissue factor, primarily mediated by TNF, initiates the coagulation pathway, and is a key regulator of disseminated intravascular coagulation. Tissue factor is expressed in perivascular cells and is exposed to endothelial cells by tissue trauma. This results in the conversion of factor VII to VIIa and factor X to Xa leading to the generation of thrombin. Thrombin augments this pathway by activation of factor XI. As a consequence, diffuse thrombosis occurs on the damaged endothelium in a process resulting in the consumption of clotting factors and platelets (Gawaz et al. 1997; Gando et al. 1997). This results in tissue hypoperfusion and exacerbates end organ dysfunction.

Recently, attention has been focused on the role of antithrombin (AT) in the pathogenesis of DIC in SIRS. Antithrombin is a single-chain

glycoprotein in plasma which belongs to a family of molecules called the serpins and is synthesized in liver parenchymal cells. AT is a unique inhibitor of the clotting system and neutralizes most of the enzymes generated during activation of the clotting cascade, especially thrombin and factors Xa and IXa. During acute DIC, clotting factors and inhibitors are consumed faster than they can be reproduced (Mammen, 1998a). This consumption of AT is of great significance in DIC and sepsis, and plasma AT levels have been shown to predict outcome (Penner, 1998). AT levels drop early in sepsis and laboratory signs of DIC can already be found in patients with SIRS and early sepsis (Mammen, 1998a). In the future antithrombin concentrates may be useful as an additional therapeutic modality (Mammen, 1998b)

## 3.4 MULTIPLE ORGAN DYSFUNCTION SYNDROME

Many factors contribute to the pathogenesis of MODS. Hypotension and decreased cardiac output leads to tissue hypoxia. Subsequent cell death results in the release of cytokines and proinflammatory mediators which aggravate the SIRS (Moore and Moore, 1995). Furthermore, if organ perfusion is re-established, there is the danger of xanthine

oxidase mediated reperfusion injury (Zimmerman and Granger, 1992). Organ injury can also occur at sites distant to the initial insult (Moore and Moore, 1995). For instance, gut ischaemia has been shown to aggravate pulmonary capillary leakage and subsequent ARDS (Koike et al. 1992). Microvascular obstruction secondary to thrombin/platelet adhesion further compromises organ perfusion and PMNL adhesion and migration is associated with the release of reactive oxidants and proteolytic enzymes in the tissues (Moore and Moore, 1995).

In addition to the development of disseminated intravascular coagulation, massive thrombin generation and activation contributes to the pathogenesis of multiple organ dysfunction through a newly found thrombin receptor (Brass et al. 1994; Rinaldo and Rogers, 1982). Endothelial injury up-regulates thrombin receptors (Gando et al. 1997). Furthermore, thrombin production:

- increases cytokine production and adhesion molecule expression
- increases endothelial permeability
- acts as a chemoattractant for monocytes and neutrophils.

It is therefore one of the main factors in the pathogenesis of MODS (Gando et al. 1997)

## 3.5 FACTORS THAT PREDISPOSE TO SIRS

It is not known why some patients should develop SIRS and MODS while others with similar injuries do not. What does seem likely, is that the initial stages of inflammation which involve local tissue reaction and a well controlled systemic reaction may become overwhelmed leading to a massive systemic reaction. Cytokines become destructive rather than protective. The flood of inflammatory mediators trigger numerous humoral cascades and result in sustained activation of the reticular endothelial system with loss of microcirculatory integrity and insults to distant organs.

Recently, there has been a great deal of work on the pathogenesis of SIRS and numerous theories have been advanced. The following is a review of the current concepts as to the cause of individual predisposition to SIRS.

- Oxidant stress may be excessive and overwhelm antioxidant defences. One postulate as to why some patients develop FES and others do not may be related to the degree of oxidative stress and the quality of anti-oxidant defences. This is supported by evidence that the greater the degree of trauma, the more likely FES is to develop (Levy,1990; Nixon and Brock-Utne, 1978).

- Some studies have found that monocytes can respond differently to the same stimuli, depending on their previous state of activation (Szabo et al. 1991). For example, IL-6 normally prompts release of TNF and IL-1. However, in some settings IL-6 can inhibit release of these other cytokines (Bone, 1996b). Furthermore, the type of monocyte may be important. An increased proportion of FcγRI monocytes is produced by the marrow in patients with SIRS (Szabo et al. 1991). These produce relatively more IL-6 and TNF may contribute to the increased susceptibility to SIRS.

- Patients with ARDS that have the highest plasma and BAL levels of TNF, IL-1β, IL-6 and IL-8 also have the highest mortality.

Increased activation of the transcription regulatory nuclear factor NFκB, a proximal activation mechanism for the simultaneous expression of multiple cytokines, has been demonstrated in the alveolar macrophages of patients with established ARDS (Meduri, 1997).

- Genetics may also play a role in predisposing certain individuals to SIRS. TNF is recognised as a central mediator of MODS in patients with SIRS after trauma, haemorrhage, surgical intervention, and infection. High levels of TNF-α are found to correlate with the outcome of patients in fulminant septic states (Waage et al. 1989). Genes encoding TNF-α and TNF-β are positioned next to each other within the cluster of human leukocyte antigen class III genes on chromosome 6. The two alleles are named TNFβ1 and TNFβ2. In severe sepsis, the TNFβ2 allele is associated with poor prognosis and high TNF-α plasma concentrations. As TNF is a major mediator in the pathogenesis of sepsis and its sequelae, individuals with a genetic determination for high TNF responses may be at high risk for development of organ failure and death when challenged with severe infection, trauma, and other noxious stimuli that can

evoke a generalized SIRS (Stuber et al. 1996)

- ICAM I is a glycoprotein belonging to the immunoglobulin superfamily. It is located on the endothelial surface and is subject to up regulation by TNF-α, IL-1 and IL-8. Following the activation of circulating neutrophils, ICAM I facilitates the migration of neutrophils out of the vascular spaces. Recent studies have shown that the levels of ICAM I are markedly increased in patients with SIRS and are predictive of mortality (Sessler et al. 1995). Whether this is a consequence of the degree of sepsis or related to specific pathogenicity of ICAM's not known.

- Adhesion molecules are not only found on cell membranes, but occur in a soluble form as well. Not only are they markers of inflammation, but they may modulate the inflammatory process by acting as a chemotaxin, blocking neutrophil activation, or by competing with the membrane-bound form of cell-cell adhesion molecules (Lobb et al. 1991). It has been shown that advanced age is associated with increased levels of soluble adhesion molecules and an increased likelihood of developing SIRS and MODS (Boldt

et al. 1997)

- A report by Connelly et al suggests that the serum ferritin concentration identifies patients at risk for the development of ARDS. Because superoxide radicals and hydrogen peroxide are involved in the pathogenesis of ARDS and because free iron catalyses the formation of the highly toxic hydroxyl radical $OH^{\bullet}$ from $O_2^{\bullet -}$ and $H_2O_2$ through the Haber-Weiss reaction, it is not surprising that increasing free iron worsens injury. Proinflammatory cytokines such as TNF, and IL6 are involved in the production of ferritin. Other factors such as tissue damage may also increase ferritin levels (Connelly et al. 1997)

- Cholesterol is a basic element of cell membranes and is likely to be essential in large tissue repair processes, for instance in sepsis or after trauma. Therefore, hypocholesterolaemia may be an indicator of the metabolic response in stress. Critically ill patients who are hypocholesterolaemic have a poor prognosis. This may both reflect the degree to which the immune system is stimulated as well as the ability of the body to repair damaged cell membranes (Gui et al.

1996)

- Low levels of nutrient antioxidants would leave patients more prone to oxidant stress. There is some evidence that certain diseases can be prevented by adequate intake of fruits, grains and vegetables which are high in nutritional antioxidants (Halliwell, 1994).

Despite the current intense research into the factors involved in the development of SIRS, we are still far from understanding and being able to control what for many patients leads to premature death.

# CHAPTER 4

# SUBJECTS

# AND

# METHODS

## 4.1 SUBJECTS

The investigation was conducted over a 6 month period starting November 1997. The study group comprised 19 patients with long bone fractures of the lower limbs ie. midshaft tibia or femur fractures, admitted to the Johannesburg and Oliver Tambo Memorial Hospitals. The patients were older than 18 years of age and were required to give verbal consent to be included. Patients with major trauma involving the thorax or abdomen were excluded as were those with severe underlying pulmonary disorders. The study was approved by the Ethics Committee of the University of the Witwatersrand.

## 4.2 METHODS

Blood tests were taken on admission and at 12, 24 and 48 hours post admission. These were sent for full blood count, arterial blood gas (ABG) and C reactive protein (CRP) measurement. Both plasma and serum specimens were separated and frozen at minus 72°C. Lipid peroxides (LPO), serum glutathione (GSH) and vitamin C levels were measured at a later stage (see below).

### *4.2.1 Calculation of Alveolar-arterial Difference*

The alveolar-arterial difference (A-a$DO_2$) was calculated from the ABG for each patient. In order to do this, the alveolar $pO_2$ ($PAO_2$) was first calculated using the alveolar gas equation (Isselbacher et al. 1994):

$$PAO_2 = FiO_2 \times (Patm - PH_2O) - PaCO_2/R$$

where:

$FiO_2$ = fractional concentration of inspired $O_2$

Patm = barometric pressure (635mmHg at Johannesburg)

$PH_2O$ = partial pressure of water vapour 47mmHg when air is fully saturated at 37°C)

R = respiratory quotient (the ratio of $CO_2$ production to $O_2$ consumption, usually 0.8)

### *4.2.2 Criteria for Diagnosis of FES*

Patients who developed signs and symptoms of FES were noted. These were based on Gurd's Criteria in which 2 of the following 3 criteria constitute the diagnosis (Gurd, 1974).

- respiratory distress with tachypnoea and dyspnoea and decreased $PaO_2$.
- cerebral manifestations including drowsiness, lethargy and convulsions

- petechial rash on the mucous membranes and the skin of the anterior part of the thorax and neck

### *4.2.3 Measurement of Vitamin C.*

This technique was modified from a previously described method (Attwood et al. 1974).

0.5ml of 2.5-20µg/ml vitamin C, 0.5ml of tricarboxylic acid (TCA) and 0.3ml of 2,4 dinitophenylhydrazine (DNPH) were mixed together in glass tubes and then incubated for 4 hours in a 37°C water bath. Plasma specimens were thawed. 1.8ml of plasma was added to 1.8ml of TCA. This was then spun for 15 min at 3000 rpm. The supernatant fluid was removed and 1ml was placed in each of 2 glass tubes. Into one tube, 0.3ml of DNPH was added and into the other, 0.3ml of distilled water was added. The later was used as a control for colour. These were then incubated for 4 hours at 37°C. It was necessary to include a background control for DNPH colour (1ml $H_2O$ and 0.3ml DNPH).

After incubation, all tubes were put on ice and 1.5ml of 65% sulphuric acid was added. These tubes were then placed in a spectrophotometer and counted at wavelength 520nm. A standard curve was then plotted

using premixed concentrations of vitamin C and values were read from there. Normal levels ± 10 μg/ml.

### *4.2.4 Measurement of LPO Levels*

These were quantitated in serum by a colorimetric method (Taeishi et al. 1987) based on the haemoglobin-catalyzed reaction of hydroperoxides with 10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine (MCDP), using a commercial system (K-Assay LPO-CC, Kamiya Biochemical Co., Seattle, CA, USA), which, according to the manufacturer, specifically quantitates lipid peroxides and has a coefficient of variation for 10 repeated assays of <3%. Briefly, serum samples (100μl) were treated with ascorbic acid oxidase (14 U/ml) and lipoprotein lipase (1.3 U/ml) and incubated for 10 min at 30°C. Lipid peroxides in the ascorbate-depleted samples were then assayed spectrophotometrically at 675nm 10 min after the addition of MCDP and haemoglobin at final concentrations of 0.03mM and 45μg/ml, respectively. Cumene hydroperoxides (50nmol/ml), supplied by the manufacturer, were included as positive control systems. Normal values are considered to be < 2nmol/ml.

### *4.2.5 Measurement of Glutathione Levels*

This was measured as previously described (Anderson and Meister, 1980). Whole blood (200µl) was added to 200µl of 10mM DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)] in 100mM potassium phosphate buffer, pH 7.5 containing 17.5mM EDTA as anti-coagulant. The tubes were then centrifuged at 200 x g for 6 minutes and 20µl of supernatant added to 800µl of phosphate buffer, followed by the addition of DTNB (250uM) and 50µl of a solution of glutathione reductase (0.5 units). After 45 seconds 50µl of NADPH (5mM) was added to each cuvette and the reactions monitored spectrophotometrically at 412nm for 5 minutes by comparison with an appropriate blank. Total plasma glutathione, most of which is in the reduced form was then calculated as previously described (Anderson and Meister, 1980).

### *4.2.6 Statistical Analysis*

The sum of the results for each investigation at a given time period were determined and the mean and standard deviation were calculated. These were then compared with each other to determine whether there was a statistically significant change over time. eg. Results from time 0 hours were compared to time 12, 24, and 48 hours. This was done using the Wilcoxon sign rank test and a p value of less than or equal to

0.05 was considered significant at a significance level of 95%. Further comparisons were done looking at the maximum change from time 0 hours for each variable to take into account the variability between the time at which the accident took place and the time of their entry into the study.

# CHAPTER 5

# RESULTS

## 5.1 RESULTS

Tables 5.1 and 5.2 show the changes over the first 24 and 48 hour periods respectively.

The white cell count (WCC) was initially raised in 11 out of 18 patients. This subsequently decreased as the patients improved. The following results are expressed as that for times 0, 24 (Table 5.1) and 48 hours (Table 5.2) respectively. The difference in the 0 hours for tables 5.1-5.3 is due to the fact that the N value is not always the same. The mean WCC was initially 13.4 x $10^9$/l (±6.1) (Table 5.1) and 13.8 x $10^9$/l (±6.0) (Table 5.2). This decreased significantly to a mean of 8.9 x $10^9$/l at 24 and 48 hours respectively. The platelet count also showed a steady decline from a mean of 266.6 x $10^9$/l (±89.9) (Table 5.1) and 256.1 x $10^9$/l (± 80.4) (Table 5.2) to 218.4 x $10^9$/l (±92.6) at 24 hours and 193.5 x $10^9$/l (±61.9) at 48 hours.

Serum CRP and LPO concentrations increased over time. The CRP which was only slightly raised initially, 9.3µg/l (±11.5) (Table 5.1) and 9.7µg/l (±11.7) (Table 5.2) showed a significant increase to 82.8µg/l

(±57.8) at 24 hours and 127.3μg/l (±99.4) at 48 hours. LPO increased from 3.79nmol/ml (±3.45) (Table 5.1) and 3.52nmol/ml (±3.34) (Table 5.2) to 5.81nmol/ml (±4.93) at 24 hours which was statistically significant (p=0.03) and 6.42nmol/ml (±6.63) which was not statistically significant (This was due to the high standard deviation).

The anti-oxidants vitamin C and glutathione both showed a decrease in mean value. Initial vitamin C was 8.07μg/ml (±3.85) (Table 5.1) and 8.26μg/ml (±3.74) (Table 5.2). This decreased to 7.06μg/ml (±3.40) and 7.45μg/ml (±3.76) at 24 and 48 hours respectively. Both were significant (p = 0.05). The serum glutathione levels were assessed in only 8 patients. There was a decrease noted from baseline of 6.04μmol/ml (±2.06) (Table 5.1) and 5.96μmol/ml (±2.21) (Table 5.2) to 5.38μmol/ml (±2.68) at 24 hours and 6.42μmol/ml (±6.63) at 48 hours. Neither change was statistically significant however, possibly as a result of the low numbers.

The A-a$DO_2$ was 13.6mmHg (±10.8) at time 0 hours and increased to 17.0mmHg (±12.9) at 24 hours and 23.2mmHg (±15.7) at time 48 hours. Neither of these was statistically significant despite the large

change.

Table 5.3 records values measured at time 0 hours and compares them with the maximum change from baseline. The reason for this comparison is explained in a subsequent section. All comparisons reached statistical significance. The mean WCC decreased from 13.4 x $10^9$/l (±6.0) to 7.7 x $10^9$/l (±2.5), p=0.0003. The platelet count decreased from 266.6 x $10^9$/l (±89.9) to 199.4 x $10^9$/l (±88.1) with p=0.0001. The CRP increased from 9.2μg/l (±11.6) to 127.7μg/l (±96.1) p< 0.0001. The vitamin C and glutathione both decreased from 8.07μg/ml (±3.85) and 6.04μmol/ml (±2.06) to 6.27μg/ml (±3.08) and 2.95μmol/ml (±1.51) respectively (p= 0.0004 and 0.03 respectively). The LPO level more then doubled from a mean of 3.79nmol/ml (±3.45) to 8.19nmol/ml (±6.99) with p value equal to 0.007. Lastly, the A-a$DO_2$ level increased from 13.8mmHg (±10.8) to 23.3mmHg (±13.9) with p=0.002.

Only one of the patients developed clinical signs and symptoms of the FES including, hypoxia, confusion and a petechial rash over the chest and axillae. His results are shown in Table 5.4. It is interesting to note

that as the CRP and LPO levels increased, so the vitamin C and glutathione levels decreased. At the same time the A-a$DO_2$ showed a marked increase from 19.1mmHg to 64.7mmHg. This patient eventually required mechanical ventilation. He did however, survive.

**Table 5.1**

Change in WCC, Platelets, CRP, Vitamin C, Glutathione, LPO, and A-$aDO_2$ between 0 and 24 hours.

| | N* | 0 hours | 24 hours | p value |
|---|---|---|---|---|
| WCC (x $10^9$/l) | 18 | 13.4 (±6.1) | 8.9 (±3.2) | 0.006 |
| Platelet (x $10^9$/l) | 18 | 266.6 (±89.9) | 218.4 (±92.6) | 0.002 |
| CRP (μg/l) | 19 | 9.3 (±11.5) | 82.8(±57.8) | <0.0001 |
| Vitamin C (μg/ml) | 19 | 8.07 (±3.85) | 7.06 (±3.4) | 0.05 |
| Glutathione (μmol/ml) | 8 | 6.04 (±2.06) | 5.38 (±2.68) | 0.58 |
| LPO (nmol/ml) | 19 | 3.79 (±3.45) | 5.81 (±4.93) | 0.03 |
| A-$aDO_2$ (mmHg) | 16 | 13.6 (±10.8) | 17.0 (±12.9) | 0.34 |

* The difference in the 0 hours for Tables 5.1-5.3 is due to the fact that the N value is not always the same

**Table 5.2**

Change in WCC, Platelets, CRP, Vitamin C, Glutathione, LPO, and A-$aDO_2$ between 0 and 48 hours.

| | N* | 0 hours | 48 hours | p value |
|---|---|---|---|---|
| WCC (x $10^9$/l) | 17 | 13.8 (±6.0) | 8.9 (±2.9) | 0.003 |
| Platelet (x $10^9$/l) | 17 | 256.1 (±80.4) | 193.5 (±61.9) | 0.0005 |
| CRP (μg/l) | 18 | 9.7 (±11.7) | 127.3 (±99.4) | <0.0001 |
| Vitamin C (μg/ml) | 18 | 8.26 (±3.74) | 7.45 (±3.76) | 0.05 |
| Glutathione (μmol/ml) | 7 | 5.96 (±2.21) | 3.84 (±1.77) | 0.22 |
| LPO (nmol/ml) | 18 | 3.52 (±3.34) | 6.42 (±6.63) | 0.20 |
| A-$aDO_2$ (mmHg) | 12 | 13.6 (±10.8) | 23.2(±15.7) | 0.34 |

**Table 5.3**

Maximum Change from baseline value of WCC, Platelets, CRP, Vitamin C, Glutathione, LPO and A-a$DO_2$.

| | N* | 0 hours | Max. Change | p value |
|---|---|---|---|---|
| WCC (x $10^9$/l) | 18 | 13.4 (±6.0) | 7.7 (±2.5) | 0.0003 |
| Platelets (x $10^9$/l) | 18 | 266.6 (±89.9) | 199.4 (± 88.1) | 0.0001 |
| CRP (µg/l) | 19 | 9.2 (± 11.6) | 127.7 (± 96.1) | <0.0001 |
| Vitamin C (µg/ml) | 19 | 8.07 (± 3.85) | 6.27 (± 3.08) | 0.004 |
| Glutathione (µmol/ml) | 8 | 6.04 (± 2.06) | 2.95 (± 1.51) | 0.03 |
| LPO (nmol/ml) | 19 | 3.79 (± 3.45) | 8.19 (± 6.99) | 0.007 |
| A-aD02 (mmHg) | 16 | 13.8 (± 10.8) | 23.3 (± 13.9) | 0.002 |

**Table 5.4**

WCC, Platelets, CRP, Vitamin C, Glutathione, LPO, and A-a$DO_2$ values at 0, 12, 24 and 48 hours for patient MK who develop the FES.

| | 0 hours | 12 hours | 24 hours | 48 hours |
|---|---|---|---|---|
| WCC (x $10^9$/l) | 30.7 | 12.9 | 16.4 | 12.1 |
| Platelets (x $10^9$/l) | 358 | 237 | 127 | 90 |
| CRP (µg/l) | 19 | 59 | 193 | 251 |
| Vit C (µg/ml) | 8.9 | 10.96 | 8.2 | 6.2 |
| Glutathione (µmol/ml) | 5.5 | 3.8 | 1.6 | 2.3 |
| LPO (nmol/ml) | 0.62 | 3.1 | 6.8 | 16.7 |
| A-a$DO_2$ (mmHg) | 19.1 | 12.7 | 59.2 | 64.7 |

# CHAPTER 6

# DISCUSSION

## 6.1 DISCUSSION

Cedric Prys-Roberts in his editorial in 1974 noted that " because of its varied presentation, the investigator with an interest in the FES syndrome faces a number of difficulties, not the least of which is that the syndrome is uncommon and tends to present suddenly, at times and in places which do not readily conform to the ordered process of a research protocol" (Prys-Roberts, 1974). With this in mind we set out to design a study in an attempt to explain the entity of FES in terms of specific host response. Our hypothesis was that the trauma related to long bone fractures would initiate the systemic inflammatory response syndrome (SIRS) and the subsequent generation of lipid peroxides. In most cases, counter-regulatory mechanisms (which include the anti-oxidant system) would be sufficient to restore homeostasis. Occasionally however, lipid peroxides would be generated in excess of antioxidants. These would embolize to the microvasculature and induce capillary leak with the subsequent development of acute lung injury.

Analyzing the data was difficult. Ideally, one would want to take the baseline bloods before the event occurred. Obviously this was not possible and we had to use bloods taken on admission as our baseline. This was problematic because the time between the initial trauma and admission to hospital could be several hours. At that stage, most of the patients already had abnormal blood results. The difference in the time of the initial blood specimen made comparison of patients using a fixed time frame difficult. In an attempt to circumvent these difficulties, the data was analyzed in two ways. Firstly, the variables were compared over time to determine if a significant change took place. Secondly, the variable at time 0 hours was compared with the maximum change from baseline. This was done in an attempt to compensate for the variation in the time taken for patients to get to hospital.

Only one of the 19 patients admitted to the study developed the fat embolism syndrome (FES). It is therefore impossible to extrapolate our results to FES. However, there is sufficient evidence from the other patients to make an oxidant-mediated mechanism for FES very likely. Furthermore, although most of the signs of FES were absent, there was significant biochemical evidence suggestive of a subclinical process

taking place. For instance, there was a progressive lowering of serum platelets over the 48 hours, an increase in CRP and a decrease in antioxidants. There was also a significant increase in the A-aDO2 suggesting a worsening ventilation/perfusion (V/Q) mismatch in the lungs, which is a subclinical indication of acute lung injury.

Most patients showed evidence of a leukocytosis and as a consequence, increased oxidant stress. 13 patients had raised WCC which dropped significantly over the next 48 hours from a mean of 13.8 x $10^9$/l to 8.9 x $10^9$/l ($p= 0.003$). One reason for the initial increase in the WCC is that during injury IL-1 and TNF are produced (Davies and Hagen, 1997; Dinarello, 1997). TNF elicits the release of neutrophils from the bone marrow leading to an increase in the circulating neutrophil count (Davies and Hagen, 1997). The subsequent drop in WCC may be the result of two factors. The first is that there is release of endogenous antagonists following an insult which keep the inflammatory response in check both by down regulating cytokine production and by counteracting cytokines already released (Bone, 1996b). The second possibility is that TNF initiates neutrophil margination by inducing the expression of adhesion molecules and promoting their transendothelial

passage (Brown, 1997; Dinarello, 1997). This results in less neutrophils in the circulation and a decrease in the WCC.

There was evidence of a progressive thrombocytopenia. The initial mean platelet count was 256.1 x $10^9$/l which decreased over 48 hours to 193.5 x $10^9$/l ($p= 0.0005$). This is not a newly described feature of long bone fractures and has been shown in other studies (Riseborough and Herndon, 1976). The initiating factor may be the release of tissue factor from the injury site which activates factor VII and the rest of the clotting cascade. This eventually leads to endothelial damage and diffuse thrombosis with subsequent consumption of clotting factors and platelets (Gawaz et al. 1997). Severe trauma also causes a massive release of tissue thromboplastin and platelet activators, which in turn result in platelet adhesion to any abnormal surface, particularly exposed collagen and fat droplets. This leads to platelet adherence and clumping and deposition in the injured capillaries of the lung (Shier and Wilson, 1980). Platelets are also consumed in the process of haematophagocytosis which is related to levels of macrophage colony stimulating factor and results in a reduction of platelets which is out of keeping with the DIC (Francois et al. 1997). Subsequent obstruction of

microcapillaries leads to worsening tissue perfusion and organ dysfunction including the lung (Shier and Wilson, 1980; Gawaz et al. 1997). This in itself would lead to V/Q mismatching in the lung and hypoxia, a significant feature of FES (Prys-Roberts, 1971).

Most of our patients exhibited a significant increase in CRP, from 9.7 μg/l to 127.3 μg/l ($p < 0.0001$). For many years, the CRP was thought to be only a marker of disease and not pathogenic in its own right. More recently, CRP has been implicated in the pathogenesis of several disease processes including myocardial infarction, thromboembolic stroke (Ridker et al. 1997) and pneumonia (Kragsbjerg et al. 1995).

Interestingly, Hulman (Hulman, 1995) has shown that in vitro, CRP can cause fat particles to coalesce. This may be responsible for the entity of creaming seen in patients receiving intravenous lipid emulsions. Experiments performed on intravenous lipid emulsions suggest that liposomes undergo calcium dependent agglutination by CRP. Although this has not been shown to occur in vivo, there are isolated reports of children who have died from agglutination and

embolisation of Intralipid (Mughal et al. 1984). In a recent autopsy study, 93.3% of patients with non-traumatic fat embolism had elevated levels of CRP (Hulman, 1995).

Like the liposomes of intravenous fat emulsions, chylomicrons and very low density lipoprotein (VLDL) undergo calcium dependent agglutination in the presence of sera containing high levels of CRP (Hulman, 1988b). Both chylomicrons and VLDL may therefore agglutinated into macroglobules by high levels of CRP in the bloodstream, resulting in fat embolism.

The presence of lipid peroxides was central to our hypothesis. Lipid oxidation has been implicated in the pathogenesis of numerous diseases (Logani and Davies, 1980). These include liver cell injury caused by chemicals such as carbon tetrachloride, ethanol and bromide, lung damage secondary to ozone exposure, photocarcinogenesis as a cause of skin cancer, Wilson's disease, iron overload (Halliwell, 1984) and probably most important, atherogenesis (Frei et al. 1988). In the first 24 hours post fracture, there was a significant increase in lipid

peroxides (3.79nmol/ml to 5.81nmol/ml, p= 0.03). At 48 hours, the LPO level had virtually doubled, from 3.52nmol/ml to 6.42nmol/ml (although this was not statistically significant). Furthermore, if one compares the mean of the first LPO level with the highest, (3.79nmol/ml to 8.19nmol/ml) this change was found to be highly significant.

Vitamin C and glutathione are both major antioxidants. Vitamin C decreased significantly at 24 and 48 hours. This coincided with the rising lipid peroxide level. Furthermore, when comparing the mean of the first vitamin C level (8.07μg/ml) and the lowest (6.27μg/ml), this decrease was also highly significant. Previous studies have shown that vitamin C plays a pivotal role in protecting plasma lipids from peroxidative damage initiated by aqueous peroxyl radicals or by activated polymorphonuclear leukocytes (Frei et al. 1988). Clearly a decrease in vitamin C would render lipids more prone to oxidation in the face of increasing levels of LPO. This homeostatic mechanism appears to have been overwhelmed in the patient with FES (Table 5.4). The levels of glutathione, the major intracellular anti-oxidant, (Lomaestro and Malone, 1995) were also noted to decrease over this

period, although due to small numbers this was not statistically significant.

It appears that large amounts of marrow fat are released into the peripheral blood stream in patients with long bone fractures. The presence of pro-inflammatory cytokines released in response to trauma increases oxidant production by increasing the activation and number of neutrophils. This increase in oxidant stress results in lipid peroxidation and accelerated oxidation of membrane phospholipids in those with impaired antioxidant systems or excessive stress.

This is particularly evident in the lung. Hypoxia as a feature of long bone fractures without FES has been well documented (Tachakra and Sevitt, 1975; Nixon and Brock-Utne, 1978). Some of the reasons previously put forward for this phenomenon include the deposition of fibrin and platelets in the airways and an increase in free fatty acid levels in the blood leading to damage to pulmonary capillary endothelium (Nixon and Brock-Utne, 1978; McCarthy et al. 1973).

We measured the alveolar-arterial difference on our patients to assess the degree of V/Q mismatch. 13 patients had an A-$aDO_2$ of greater than 10mmHg at some stage in the first 48 hours. We compared the mean of the initial A-$aDO_2$ of 13.8mmHg with the highest A-$aDO_2$ of 23.3mmHg, which represents a significant increase ($p=0.002$). This may occur because of oxidant-mediated damage to the pulmonary capillary membrane and V/Q mismatch subsequent to extravasation of fluid into the alveoli (Kollef and Schuster, 1995). Lipid peroxides are themselves pro-inflammatory and can cause cell damage (Logani and Davies, 1980). It is possible that the degree of acute lung injury in FES is proportional to the concentration of LPO and that this is due to prior anti-oxidant status. Non-oxidized fat in the pulmonary capillaries is probably harmless, but if oxidized, pulmonary injury may occur.

Previous authors have suggested other reasons for post trauma hypoxia including atelectasis secondary to immobility or the use of sedative drugs such as morphine (Prys-Roberts, 1974; Kallenbach et al. 1987). There is however evidence from previous trials which would indicate that the observed hypoxia is in fact directly related to the long bone fractures themselves. Tachakra looked at 50 patients with long bone

fractures. He noted that patients admitted with only soft tissue injuries did not become hypoxic. Furthermore, half of the patients who were hypoxic on admission had several episodes of hypoxia subsequently, these related to manipulation of the fractures and surgical repair. (Tackakra and Sevitt, 1975). In another study by Nixon, it was noted that the incidence of hypoxia was related to the number of bones fractured ie. the greater the number of long bones fractured, the more severe the hypoxia (Nixon and Brock-Utne, 1978). These studies indicate that in patients with long bone fractures, trauma alone was not the cause of the hypoxia but that it was directly related to the fractures themselves.

As previously indicated, only one of the patients developed the FES. Despite the fact that it is impossible to draw conclusions from a single patient, these results exhibited the large increase in LPO and decrease in vitamin C and glutathione predicted by our hypothesis. The WCC was very high initially and decreased rapidly. This coincided with a decrease in platelets. The LPO increased dramatically, the vitamin C and glutathione levels dropped and the A-a$DO_2$ deteriorated to the point where the patient required mechanical ventilation. All of these

features suggest that in this patient there was evidence of severe oxidant stress, coinciding with the consumption of anti-oxidants.

## 6.2 CONCLUSION

It seems likely then, that it is the balance between the oxidants (as represented by LPO) and the anti-oxidants (glutathione and vitamin C) that explains why most people with fractured long bones do not develop the FES. In the few patients who do develop FES, there is probably an imbalance between these two opposing systems (Bone, 1996a). LPO and other oxidants are highly reactive substances which are capable of causing severe tissue damage leading to the well known features of FES, specifically ARDS and central nervous system dysfunction. The mechanisms have been discussed elsewhere but include damage to both cell membranes and DNA. This leads to sodium/potassium pump dysfunction, leaky capillaries and cell death. Further studies are necessary to fully elucidate the complex immune interactions in these patients.

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