CHAPTER 1
INTRODUCTION

Burn injuries involve coagulative necrosis of skin and a variable amount of deeper tissues. They are produced by the dissipation of thermal energy from fires or hot liquids, and less commonly from chemicals, electricity, and various forms of irradiation. Burns represent a largely preventable cause of much morbidity and misery especially in the Low and Medium-Income Countries (LMICs) of the world. While burns are on the increase in these LMICs, research based preventive measures have cut down their incidence in the developed or high-income countries (HICs) such as the United States. In these latter nations, preventive measures based on identified risk factors, and epidemiological characteristics have significantly reduced the incidence of burns (Katcher, 1987; Forjuoh, 2006). Globally, fire-related burns account for up to 230,000 deaths annually. More than 80% of burns involve less than 20% of body surface area (BSA). These are classified as minor burns and can be treated on an out-patient basis. Household incidences account for the vast majority of burn injuries with 73% of all burn-related deaths in the United States resulting from fires in household settings (Pruitt et al., 1997; Othman and Kendrick, 2010).

In burns, as in communicable diseases, the problems of low education and poverty take their toll, with over 90% of global fire-related deaths occurring in the developing countries. South-East Asia alone accounts for over half of these deaths. Successful preventive intervention to reduce the incidence of burns will require clear characterization of its epidemiology and identification of associated risk factors. Most
studies from the LMIC on burn epidemiology focus on burns in childhood. In this regard, toddlers from birth to 4 years of age showed a disproportionately higher number of cases (Forjuoh et al., 1995; De Souza et al., 1997; Nega and Lindtjorn, 2002; Marbrouk et al., 2003).

Although males show a higher incidence in childhood burns, when all age groups are considered, different findings occur (Werneck and Reichemheim, 1997; Hemeda et al., 2003; Daisy et al., 2007). In all age groups, scalding from hot liquids accounts for 33 - 50% of all burns. This is followed by flames in adults and flames and hot objects in children (Davies, 1990; Marsh and Sheila, 1996). In recent years, petrol fires from broken pipes or tankers have caused mass mortality and devastating morbidity in Nigeria (Fadeyibi et al., 2009).

Several factors have been identified to be associated with burns as risk or protective factors. A comprehensive population-based study in Ghana, found the following to affect burn occurrence: pre-existing impairments such as lameness, epilepsy and blindness. Others include a history of burn in a sibling and storage of flammable liquids in the home (Forjuoh et al., 1995). Others have identified overcrowded dwellings slum locations, use of loose clothing, man made fabrics and low socio-economic status as putative risk factors (Werneck and Reichemheim, 1997).

Optimum performance of testicular function requires precision in homeostasis in the physical, endocrine and chemical environment of the testis. Spermatogenesis is for
instance affected by changes in scrotal temperature, blood perfusion rate, and testosterone levels (Sweeney et al., 1991). Normal scrotal temperature in most mammals is lower by 4 – 5 °C than intra-abdominal temperature. Any sustained elevation of the temperature exceeding 20°C impairs testicular function. Normal body temperature disrupts spermatogenesis in man, as seen in cases of cryptorchid testes (Chowdhury and Steinberger, 1970; Rommerts et al., 1980). Congenitally unilaterally cryptorchid rats had 0% fertility rates (Patkowski et al., 1992).

Apoptosis is a normal feature of the seminiferous epithelium. It is probably responsible for controlling the relative quantities of the germ cells, eliminating defective cells and ensuring the production of mostly functional spermatozoa (Quinn et al., 1990). Apoptosis is however induced by several other conditions such as ionizing radiation, systemic trauma, hormone depletion and heat stress (Hikim et al., 1995 & 2003). Even mild heat stress (42-43°C) has been shown to induce both apoptotic and necrotic cell death (Lue et al., 2000 and Matsuki et al., 2003). Testes are also damaged by cytotoxic agents and a number of chemical substances (Artessahin et al., 2006). Lower oxygen tension and blood perfusion pressures are characteristic of the testicular capillary bed (Sweeney et al., 1991). Many of the deleterious effects of heat and varicoceles are related to the fact that there is increased blood flow in these conditions. Cells of the spermatogenic series have been shown however to be particularly sensitive to free oxygen radicals, which generate oxidative stress (Lewis et al., 1995).
Deleterious changes at several levels of the immune system have been characterized in burns. Most of them occur in lymphocyte behaviour and involve changes in cell immunity. For instance there is decreased lymphocyte proliferation and reaction, causing prolonged allograft survival. There is also decreased delayed type hypersensitivity (Ferrara et al., 1988).

It has been established that testicular function is abnormal in a variety of chronic illnesses where both spermatogenesis and androgen production are affected (Semple, 1986).

In both chronic renal failure and liver disease, there is sub-fertility, oligospermia or azoospermia, with low testosterone production and raised gonadotrophin levels. Even the hypothalamic-pituitary feedback systems are affected in these conditions (Semple, 1986).

Niemann and Mechanick in a study of 30 chronically critically ill, (CCI), ventilator-dependent men, found both total and bio-available testosterone (BIOT) levels, to be well bellow average for their age range in 96% of the men. BIOT levels expressed as a percentage of mean for their age range correlated positively with the length of stay in the intensive care unit (ICU). As a result of a concentrating mechanism, intra-testicular testosterone levels are normally far higher than serum levels.

Studies of men who recovered from major burns showed that most of them had hypospermia, with abnormal levels in all key sperm parameters such as total counts, motility and percentage of cells with abnormal morphology (Jewo et al., 2009). When scalding heat was applied to the scrotum for periods ranging from 30 to 90 seconds, a variety of changes occurred in testicular histology. These ranged from increased
apoptotic activity to diffuse degeneration and necrotic cell death, affecting all testicular cells - spermatogenic, Sertoli and Leydig cells (Sakallioglu et al., 2007).

**STATEMENT OF THE PROBLEM**

The mechanisms underlying testicular damage in severely thermally-injured men are poorly understood at the present time. There is a dearth of published studies on the subject in the reviewed literature. For ethical reasons it is difficult in humans to carry out studies that will follow the progress of morphological changes in the testis. This will for instance require serial needle biopsies of the testis to obtain tissue. It is still more difficult in these situations to carry out controlled studies. This requires taking testicular tissue from fertile men. Even if the infection risk in this procedure is avoided studies show that needle biopsy causes histologic changes in the testis. It has been difficult to obtain semen from fertile men to develop reference values for semen parameters in most places (Lewis, 2007). Animal studies with suitable models are therefore required.

The problem is to achieve a satisfactory explanation of how disruptions in structure and function occur in the testes of animals suffering from severe burns, and how this knowledge may be applied to design interventions that can limit or control the damage in humans with burn injury.

**OVERALL AIM OF STUDY**

This study will investigate alterations of testicular structure and function in rats exposed to severe thermal injury that does not involve the perineum and the potential benefits of
ascorbic acid as a protection against such alterations. It should also elucidate the morphological, biochemical and endocrine disruptions that account for them.

**SPECIFIC OBJECTIVES**

The specific objectives of this study are to:

i. Determine histological changes produced in the testes of animals affected by severe burn injury.

ii. Investigate the effects of severe burns on three major sperm parameters – total counts, motility, and percentage of abnormal morphology.

iii. Determine alterations in serum levels of testosterone (T), FSH and LH, and the effects of ascorbic acid on burn-induced testicular damage.

iv. Investigate the role of oxidative stress in the response of the testes to severe burn injury.

**SIGNIFICANCE OF THE STUDY**

Young men recovering from burn injuries may include those who are yet to raise or complete their families. They will expect to be able to do so later in life. Male infertility is then an added challenge in dealing with the many often intractable late sequelae of burns. This carries the risk of potentiating any affective disorder that develops in the individual. The male factor already accounts for up to 40% of infertility in the general population and its treatment is a considerably difficult task (Otubu, 2003). Where finance and facilities are available recourse is now often made to various modalities of
treatment including intra-cytoplasmic sperm injection (ICSI), and in-vitro fertilization (IVF) where sperm counts are too low for standard insemination procedures. The cost of assisted reproduction is however clearly prohibitive for the general population from which burn patients usually come.

Even in the best centers world-wide, conception rates from IVF is 25-30% of treatment cycles (Lewis, 2007). Having children of one’s own is a serious matter among many African people. Laws guiding options for dealing with male infertility, such as donor insemination and adoption are generally poorly developed in most developing nations.

This study will increase our understanding of one key long term consequence of severe burns in males. It also has the potential to address one contributor to male factor infertility in the human population. In a society such as ours where childless couples can do unbelievable things in search of elusive babies, such a contribution is quite important.
OPERATIONAL DEFINITION OF TERMS

Apoptosis: Genetically programmed cell death in which cell debris is not left.

Anti-oxidants: Substances, often naturally occurring, which are capable of reducing oxidizing species and rendering them harmless.

Burns: Tissue damage in skin usually caused by thermal injury.

Cytokines: Chemical substances, often naturally occurring, which act locally on cells and affect their activity in various ways.

Free radicals: Atoms that possess unstable electrons that are readily donated thereby oxidizing various kinds of molecules.

Hypermetabolic state: A condition in which cellular metabolic processes are proceeding at supra-physiologic rates.

Immu-no-suppression: A situation in which the body’s natural defense mechanisms are suppressed.

Necrosis: Cell death associated with nuclear and cellular degeneration.

Oxidative stress: A situation of increased production of free radicals so that pro-oxidants exceed anti-oxidants.

Seminiferous epithelium: The main content of the testis. It consists of various cell types arranged in definite patterns within the testes.

Severe burn: Any burn with burn surface area in excess of 20% in adults.

Spermatogonia: Primitive cells, also known as germ cells. They line the basement of the seminiferous epithelium.

Spermatogenesis: The process whereby mature spermatozoa are produced in the testes from primordial cells known as spermatogonia.
**Spermiogenesis:** The process by which rounded spermatids produced from differentiation of spermatogonia are converted to mature spermatozoa.
CHAPTER 2

LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF BURNS

Burns are a largely preventable cause of much morbidity and disability, especially in the low and medium income countries (LMICs) of the world. Children are commonly affected, especially infants and toddlers. This is believed to be because they are dependent on others and less able to move themselves early enough from points of danger (Forjuoh, 2006). Some studies of age distribution of burns show that prevalence is low in young adults, and begins to rise in the 30-39 years age group. In studies of childhood burns from Brazil, Cote d’Ivoire and India, the 0-4 year age group accounted for nearly half of all childhood burns (Vilasco and Bodurand, 1995). When all age groups were considered, this group still accounted for nearly one-third of all cases (Adamo et al., 1995). The prevalence of burns tapers off after age 4 till late in adolescence when it begins to rise again, probably due to entering work and careless handling of flammable agents (Forjuoh, 2006).

Studies report different findings in gender distribution of burns. There is no agreement in their findings. However studies in children report slightly higher prevalence among males. Some studies suggest a considerable variation in gender ratio for differing age groups within a given country. Studies from Ghana and India reported a higher incidence in males from birth till 4 years of age, with a reversal of the trend beyond this age (Davies, 1990; Forjuoh et al., 1995). Duran has suggested that the fall in male
preponderance after 4 years of age is due to changing activities of both genders at this time: females staying closer to their mothers in the kitchen and therefore becoming exposed to fires and hot liquids, while boys spent more time outdoors (Duran, 1990).

In children, the vast majority of burns occur at home (Zhu et al., 1988; Rossi et al., 1998; Lari et al., 2002; Hemeda et al., 2003). In adults, the setting for burns was roughly equally distributed between home, outdoors and the work place (Vilasco and Bodurand, 1995 and Hemeda et al., 2003). For all age groups, the kitchen is the commonest scene for burns. This is followed by the backyard and veranda, and the living room (Gupta et al., 1992; Kumar, 2003). A study from Egypt reported the bathroom to be a common scene for burns among the elderly, due to the handling of heated water in this location (Marbrouk et al., 2003). For all age groups, hot liquids account for between a third and half of all burns (Shani V, 2000; Lari et al., 2002). This is followed by flame fires in adults and hot objects and flames in children (Adamo et al., 1995; Marsh and Sheila, 1996). Electrical and chemical burns also occur, though these were reported to be rare in epidemiologic surveys from LMICs (Achebe and Akpuaka, 1989; Nursal et al., 2003). Burns also arise from intentional settings, and assault and maltreatment. A study from Eastern Sri Lanka reported several cases of women, who had quarreled with their husbands or other family members, pouring gasoline over their bodies and setting themselves ablaze (Lahoe and Ganesa, 2002). Attempting suicide by burns has also been reported in children from Iran (Lari et al., 2002).
Only a few studies describe burns by the part of the body affected. When all cases are considered, the upper extremity is the most affected, followed by the lower extremities. However when burns from flames alone are considered, the lower extremities are the most affected (Iregbulem and Nnabuko, 1993; Kalayi, 1994; Forjuoh et al., 1995).

Most burns affect less than 10% of the total body surface area (TBSA). Such cases are classified as minor. A few cases involve more than 50% of TBSA, especially in cases from flame fire (Gupta et al., 1993). Overall mortality has been estimated at 9.9% with figures of 21.3% being reported for hospitalized cases (Forjuoh, 2006). Mortality from outpatient cases is estimated at 3.6%.

There is a seasonal variation in the incidence of burns in many parts of the world. Even a diurnal variation has been found, with peak period, being late mornings and in the evening. These times reflect the period when meals are being prepared, and people are in the kitchen. In some temperate areas, more burns occur in winter, being related to home heating procedures (Learmonth, 1980; Adamo et al., 1995). In many tropical regions burn cases are evenly spread across the year. (Forjuoh, 2006).

Certain factors predispose to burns, especially in children. Studies from LMICs, show that pre-existing impairment such as seizure disorders, hearing loss or polio increase burn risk. Socio-economic factors such as low maternal education and overcrowded homes have been shown to also increase burn risk in children (Forjuoh et al., 1995; Forjuoh, 2006). Other significant risk factors identified from studies in Baghdad and Pakistan
include: poor child supervision, location in slum or congested areas, using clothing from synthetic fabrics, and low socio-economic status (Forjuoh, 2006; Daisy et al., 2007).

As a cause of morbidity and long term disability, burns constitute a major public health issue throughout the world. Developing countries are however far more severely affected, accounting for 95% of the over 200,000 annual global death burden of fire-related burns (Heimbach et al., 1999; Peden et al., 2002; Mashreky et al., 2008).

Children are the most vulnerable because they have less ability to discern dangerous situations, and for mounting a prompt and appropriate response. It has been reported that about 173,000 children suffer moderate to severe burns yearly in Bangladesh (Mashreky et al., 2008). Burn prevention strategies based on identified risk factors have reduced the overall incidence as well as the fatality from burns in developed countries such as the UK and the United States. They are nevertheless still a leading cause of unintentional injury, morbidity and mortality in these nations (Rivera, 2000; Krug, 2004).

Society bears a considerable economic burden from burns. A survey of burn injury in children under 18 years in Bangladesh using nationally representative data reported that average annual loss of school days was 21 days, and average direct expenditure by a family was $462. This was a study in which more than 61% of the families earned less than $50 a month (Mashreky et al., 2008). A study based on “cost of burns” model suggests that the economic consequence of childhood burns to the United States is about $3.5 million yearly (McLaughlin and McGuire, 1990).
The petroleum industry is characterized by the presence of several highly flammable substances, and unless appropriate safety regulations are applied, a considerable rate of fire-related injuries and cost is borne (Sharma, 2001). In Nigeria, a developing country, years of neglect by oil industry operators and regulators alike, together with endemic poverty have made mass casualties from petroleum-related fires a recurrent problem in recent decades. Petroleum products spilled from trucks and pipelines set off fires sometimes killing hundreds of people at a time (Fadeyibi et al., 2009).

2.2 PATHOPHYSIOLOGICAL CHANGES

2.2.1 HEMODYNAMIC AND CIRCULATORY CHANGES

Burns cause coagulative necrosis of skin and may include a variable amount of deeper tissues. Burns produce devastating morbidity, with outcomes across the entire spectrum; from circulatory shock and physical disabilities to longer term sequelae such as impaired emotional, mental and reproductive functions (Baker et al., 1992; Bars et al., 1998). Pathologic changes in burns may be grouped into three categories: early, intermediate and late. The earliest of the major consequences of burns are hemodynamic and circulatory compromise. Respiratory impairment may occur especially when inhalation of smoke has attended burns. Subsequent to these early changes seen in the first 48 hours, cell injury may occur in several systems and cause death from multiple organ failure (Godwin, 1990). Late complications include skin contractures, hypertrophic scars and keloids. Fundamental changes occur in substrate metabolism and in the endocrine and immune systems as well. The extent and duration of organ dysfunction is proportional to the extent of burns (Pruitt et al., 1997).
Thermal injury produces immediate effects on blood vessels in the burned area. The direct effect of heat and that of vaso-active substances released from the injured area alter trans-vascular pressure relationships and capillary permeability in such a way as to cause loss of fluid and protein from the intravascular into the extra-vascular tissue compartment. Studies show that within minutes of thermal injury, there is a fall in cardiac output proportional to burn size. Any burn affecting more than 25% of the TBSA is considered extensive (Pruitt et al., 1997). This fall is associated with an initial increase in peripheral vascular resistance (Martyn, 1998). The CVS response is in two distinct phases. An acute phase characterized by hypovolemia and fluid loss, lasts for about 48 hours. This is followed by a hypermetabolic hyperdynamic phase associated with increased blood flow to tissues and organs, and an increase in core temperature (Demling, 1987). During this phase rapid edema develops in tissues in and around the burned area. During the prolonged second phase events, there is increased catecholamine production resulting in higher adrenergic stimulation of the heart and myocardial dysfunction. There is increased end-diastolic volume and reduced right ventricular ejection fractions. These changes together with increased blood viscosity from tissue break down products and impaired fibrinolysis may cause cardiac overload and myocardial infarction (Coumel, 1996; Verrier and Mittelman, 1996; Kelsey et al., 2000).

It has also been suggested that soon after burn injury there is a rise in intra-capillary pressure associated with a fall in interstitial fluid pressure. This creates a gradient that significantly increases capillary filtration for several hours after burns (Lund et al., 1985 and 1986). Although micro-vascular permeability due to the direct effect of heat on
capillaries generally accounts for fluid loss from the vascular space, humoral substances and several cytokines released from activated leukocytes play an important role in this process. Arachidonic acid metabolites such as thromboxane A₂, substance P, fibrin degradation products and platelet aggregation factor (PAF) are all believed to contribute to the process (Pruitt et al., 1997).

Vasoactive substances released from activated immune competent cells are also believed to contribute to micro-vascular permeability increase after burns. Such agents include the products of complement activation, lysosomal enzymes, xanthine oxidase and several lymphokines (Peterson, 1989; Williams and Saviour, 1995; Pruitt et al., 1997). However, and quite fortuitously, pulmonary vascular resistance tends to increase in the immediate post-burn period. This partly explains the rarity with which pulmonary edema occurs, in spite of the large quantities of fluid normally administered to counter fluid loss in burn cases (Harms et al., 1982; Demling et al., 1984, Pruitt et al., 1997). In addition to plasma loss, some of which may ooze out and be lost directly from the burn surface, there is considerable destruction of red blood cells. This cell destruction is proportional to the extent of burns. In severe burns, especially in the third degree type, up to 8-12% of the body’s red cell mass may be lost daily. This represents the sum of cells lost to lysis by heat, micro-vascular thrombosis and necrosis (Pruitt et al., 1997).

Jeschke and others (2008) have shown that in children with severe burns, marked changes occur in several indices of cardiac function. They studied 200 children with mean burn TBSA of 41%. They found that in the immediate post burn period, cardiac output increased, then decreased, and stayed significantly reduced till discharge. Heart rate and
predicted heart rate were significantly increased (up to 160%) and was still high at discharge.

Intriguing changes have been found in cardiac function in burned patients. When more than 40% TBSA is burned, there is an increase in both cardiac output and cardiac index, with an increase of up to 170% in heart rate. Heart rate can remain high for up to two years. This cardiac stress and dysfunction may be a key factor in mortality from extensive burns. It may also be a reason for higher mortality at the extremes of age. The WHO delineates highest mortality from burns at ages below 4 years and above 65 (Hendon et al., 2001). Propranolol decreases cardiac work, and improves oxygen delivery to cardiac muscle. It is therefore a useful agent in therapeutic plans to reduce cardiac morbidity in these cases (Hendon et al., 1999)

2.2.2 METABOLIC RESPONSE TO BURNS

Studies show that severe thermal injury leaves people in a hyper metabolic, disabled and debilitated state for a period now known to be as long as 24 months. Whereas the inflammatory response to burns begins immediately, the hyper-metabolic response begins about the fifth day post-injury (Przkora et al., 2006; Jeschke et al., 2008). The metabolic state is characterized by a hypo-dynamic state with increase in body temperature, oxygen and glucose consumption, CO₂ production and glycogenolysis resulting in futile substrate cycling (Herndon et al., 2004). The hyper-metabolic response is biphasic, being more marked in the first 8-12 weeks (Jeschke et al. 2004). It is believed that the early response
is driven by shock phase hormones such as catecholamine and cortisol, while cytokines may drive the later phase of this response.

The metabolic demands and energy requirements in the thermally injured are often immense. In cases where TSBA is more than 30% the need exceeds what routine calorie-generating carbohydrate substrates can supply. Increased protein degradation and a negative nitrogen balance therefore develop as are characteristic of chronic critical illness whatever the cause may be. In children, it has been demonstrated that severe burns caused a fall of 4%, 3% and 2% in lean body mass (LBM), bone mineral content (BMC) and bone mineral density (BMD) respectively. There was up to 4-8 fold fall in serum level of constitutive hepatic proteins post burn. This affected such proteins as pre-albumin, transferrin and retinol binding protein (Jeschke et al., 2008).

Non-fasted blood glucose is markedly increased in the acute post-burn period and reaches 170-180 mg/dl. This rise occurs in spite of elevation in insulin levels, implying insulin resistance. Free fatty acids and total triglycerides levels are elevated for weeks post-burn (Jeschke et al., 2008).This elevation of total fats at a time when proteins required for fat-transport from the liver are reduced explains at least in part, the fatty infiltration of the liver seen post-burn. This hepatomegally and fatty infiltration has been shown to increase sepsis and burn mortality (Barret et al., 2001). A therapeutic plan to reduce lipolysis and fatty infiltration may therefore improve morbidity and mortality.
Perturbations in glucose kinetics are a general finding in severely burned people. Increased glucose consumption goes through the inefficient anaerobic route with raised lactate production (Wilmore et al., 1974 and 1976; Jeschke et al., 2008). Glucose production from gluconeogenic pathways is increased, and alanine and other amino acids become less available for producing body proteins. Nitrogen is excreted as urea, and a negative balance begins to develop (Rennie, 1985). Hypermetabolism, assessed by resting energy expenditure is elevated up to 130-140% of predicted levels. Prolonged hypermetabolism and the resulting loss in essential body tissues have a serious effect on morbidity. Several agents that can attenuate catabolism, and preserve protein and amino acid stores have been tried therefore in treating these people. They include insulin, growth hormone (GH), insulin-like growth factor-1(IGF-1), oxandrolone and propranolol. The responses to these substances in controlled trials have been varied (Hendon et al., 1999 and 2004; Jeschke et al., 2004 and 2007).

There is a clear correlation between body tissues depletion and mortality. Chang et al, 1998 have shown that a 10% loss of LBM leads to impairment of immune function; a 20% loss of LBM to decreased wound healing and 30% mortality; a 30% loss of LBM leads to pneumonia, pressure sores and 50% mortality and 100% mortality occurs if up to 40% of LBM is lost.

There is therefore a great deal to recommend the use of anti-catabolic agents in these people. Propranolol, a beta blocker has shown some benefits in these cases. It decreases peripheral lipolysis, fatty infiltration of the liver and reverses hepatomegally (Barrow et
al., 2006). Insulin has also been used to attenuate many harmful changes in these cases (Jeschke et al., 2008).

### 2.2.3 OXIDATIVE STRESS

Metabolic activity in living organisms generates unstable atoms with oxidizing power known as free radicals. Free radicals are atoms or small molecules possessing unpaired electrons. These chemical species are therefore pro-oxidants and are inherently unstable. Free radicals include hydroxyl ion (HO\(^-\)), superoxide anion, (O\(_2^\cdot\)), hydrogen peroxide (H\(_2\)O\(_2\)), and nitric oxide (NO). Many of them are oxygen-bearing, and are called reactive oxygen species (ROS). Free radicals are essential components of the aerobic energy pathway. They are vital for cell killing activities in phagocytosis and nitric oxide is particularly useful in molecular signaling roles. The activity of these oxidizing species is normally offset by a balanced production of anti-oxidants in the body. It is difficult to achieve perfection though in this balance. Molecules such as DNA and proteins damaged by free radicals are being continuously repaired or replaced in the body. When pro-oxidants exceed anti-oxidants, a state of oxidative stress occurs (Pryor, 1993).

Oxidative stress may therefore result from presence of substances which produce pro-oxidant metabolites, or a fall in enzymes which generate anti-oxidants such as glutathione reductase (GTH) or superoxide dismutase (SOD). Virtually any cell in the body can become subjected to ROS attack if the conditions are suitable. Oxidative stress causes a range of effects in living tissues. This can vary from an adaptive response based on up-regulation of anti-oxidant enzymes, to cell injury and death, depending on the severity of
the condition. (Nicotera and Orrenius, 1994; Hampton and Orrenius, 1997; Halliwell and Gutteridge, 1999).

Cells and their organelles are walled off by membranes composed mainly of proteins and lipids, the later usually rich in polyunsaturated fatty acid (PUFAs). These lipids which are particularly rich in sperm cell membrane enhance membrane fluidity (Kato et al., 2001). PUFAs are attacked by peroxides. Lipid peroxidation generates malondialdehyde (MDA). This occurs when there is peroxidation of PUFAs with more than two double bonds such as linolenic or arachidonic acids (Halliwell and Gutteridge, 1999). Oxidative stress from excess ROS is believed by many to be a major cause of sperm dysfunction. They are believed to induce a lipid peroxidation cascade that damages cells and tissues (Aitken et al., 1989). Lipid peroxidation weakens membrane integrity, and this may result in rupturing of lysosomes, with spillage of hydrolytic enzymes and cell death. Oxidative stress is believed to be the major pathway by which many toxicants and pathologic processes exert their deleterious effects on body organs.

The superoxide anion (O$_2^-$) is mostly produced in cell mitochondria, but it is very unstable and therefore has a very short half life. It is quickly converted to hydrogen peroxide by superoxide dismutase (Mates and Sanchez-Jimenez, 1999). The HO$^-$ anion is generated from a wide range of reactions and also has very short half-life. H$_2$O$_2$ is metabolized to water and oxygen via catalase or through the glutathione peroxidase (GPX) pathway (Pryor et al., 2006).
An array of anti-oxidant defense systems has been developed in aerobic organisms to cope with oxidative stress. ROS-metabolizing enzymes such as superoxide dismutase, catalase and GPX, all well expressed in the testis, constitute a first line of defense (Mariono et al., 2003; Ischi et al., 2005). The GPX system involves a number of other enzymes such as glutathione reductase and glutathione-S- transferase which are required for recycling glutathione. Naturally occurring free-radical scavenging anti-oxidants constitute a further line of defense. These include various tocopherols (Vit-E), ascorbic acid (Vit-C), flavonoids, carotenoids and melatonin. Many of them have been proved by a large body of studies to be effective in relieving free radical and ROS-mediated testicular damage (Narra et al., 1993; Garazza and Catala 2003; Smerscioz et al., 2003; Artessahin et al., 2006).

Oxidative stress is now believed to mediate the fertility suppressing effects of several pathologic conditions such as varicoceles (Suzuki and Sofikitis, 1999); torsion (Qzturk et al., 2003; Duru et al., 2007; Kaleli et al., 2003); cryptorchidism (Tan et al., 2000; Wakatsuki et al., 2001) and toxicant damage (Santoro et al., 2001; Jewo et al., 2006; Vardi et al., 2009). The effects of ROS are however not uniformly deleterious to sperm function. Nitric oxide (NO) for example is critical to sperm motility. However optimum sperm function requires that the concentration of this agent is kept within a narrow range (Donnelly et al., 1997). NO synthase has been demonstrated in the head and mid-piece areas of spermatozoa, with more intense immuno-flourescence for the enzyme in normozoospermic samples, suggesting a physiological role for NO in fertility (Lewis et al., 2007).
A significant increase in MDA levels has been reported in the liver after burns. This was associated with increased vacuolization in parenchymal cells (Demling and Lalounde, 1990). Similar changes were observed in gastric and hepatic cells by Yegen and others (Guneyesel et al., 2002). In subsequent studies, the degree of liver and gastric damage was shown to be significantly reduced when a NOS inhibitor was applied, and the benefit was reversed when L-arginine, an NO precursor was added. This suggests that NO has a role in remote organ damage post-burn (Gurbuz et al., 1997; Guneyesel et al., 2002). A number of studies have established a correlation between oxidative stress and structural and functional damage in sperm (Twigg et al., 1998; Irvine et al., 2000). As a result tests have been developed to determine the presence of irreversible sperm damage from lipid peroxidation and to assess anti-oxidant protection capacity in sperm and semen (Lewis et al., 1995; Agarwal et al, 2006)). This has been spurred by the fact that these tests have shown strong prognostic values in the outcome of assisted reproductive procedures (Aitken and Baker, 2006).

2.2.4 THE ENDOCRINE RESPONSE

In burns, as in critical illness from whatever cause, abnormal patterns appear in several key axes of the endocrine system. Striking changes occur in the hypothalamo-pituitary-hormone axis in these cases, the severity of which have been shown to be a predictor of morbidity and mortality (Van-Horebeck et al., 2006). This axis is a regulator of several organs and cellular functions. Like the cytokine response, endocrine changes in a severely injured animal are intended to assist homeostasis and improve survival. But they
drive or accentuate the inflammatory and hyper-metabolic states, and exaggerated levels of all these responses often end up destroying tissues and organs and threatening life.

Catecholamines -- Adrenalin, Noradrenalin and stress hormones such as cortisol drive the hyper-metabolic response to burn injury. Serum and urinary cortisol levels rise after major burns, sometimes, up to 5-8 fold. These hormones are believed to be key factors in proteolysis and catabolism in these cases, making a number of researchers to begin studies to control protein breakdown and hyper metabolism by blocking cortisol production (Jeschke et al., 2008). It has been suggested that hypothalamic corticotrophic releasing factor (CRF) may drive pituitary secretion of adrenocortico-trophic hormone (ACTH). ACTH concentration in the severely ill has been shown to be higher than in cases of intravenous injection of CRF.

Aldosterone, an adrenal hormone is an important regulator of fluid and electrolyte balance. Aldosterone levels are raised in burns; due to activation of rennin-angiotensin system. It promotes sodium retention and so attenuates the volume depletion which accompanies burns and severe trauma (Semple, 1986). The adrenal gland also secretes androgens, though their functions are not clearly understood.

Studies in men with burn injury showed that there were changes in adrenal and testicular steroid hormone secretion. There was increased cortisol and urinary 17-hydroxy-corticosteroid secretion, while both adrenal and testicular steroid secretion diminished. There was a fall in levels of androstenedione, dehydro-epiandrosterone, and testosterone
levels. Low levels of dehydro-epiandrosterone sulfate and testosterone was found in some burned men and their levels remained low for many months after their wounds had healed (Lephart et al., 1987).

It therefore appears that the adrenal gland is able by auto-regulatory mechanisms to shift from production of androgenic substances to that of gluco-corticoids, as part of an adjustment aimed at improving the survival chances of the injured individual (Semple, 1986). In the longer term however, the fall in androgens reduce the level of hormones which may have been helpful in combating protein breakdown and tissue wasting. A study of ventilation-dependent chronically-critically-ill (CCI) men found that both total and bio-available (bio-T) testosterone levels were significantly lower than mean for their age range; in 29 out of 30 men, bio-T levels correlated with the number of days spent in intensive care unit (ICU), (Nieman and Mechanick, 1999). Therapeutic approaches to improve androgen function in these cases by administering testosterone have however been unsuccessful. Testosterone caused complications in these cases and was replaced with oxandrolone (Jeschke et al., 2008). Jeschke and others have found in a study of children that there was a fall in estradiol, and testosterone accompanied by a rise in progesterone levels. Such a pattern in the sex hormone milieu can seriously compromise spermatogenesis if it was to persist (Jeschke et al., 2007). This is notwithstanding the fact that estradiol is now known to play a key role in spermatogenesis.

Many of these changes in sex hormones in males have effects on the function and histology of the testis. Previous studies have concentrated on the fact that androgens were
anabolic and tried to manipulate these hormones in therapeutic approaches to encourage building tissue and countering tissue wasting. Several other key endocrine axes are affected by burns. These include the growth hormone (GH) and insulin like growth factor (IGF-1) axis, the free thyroid iodine (FTI) and thyroxine (T4) axis, and the insulin-glucose axis.

The GH - IGF-1 axis is particularly important in major burns. Recombinant human growth hormone (rhGH) has been shown to: improve immune function, (Takagi et al., 1997 and 1998); improve wound healing (Herndon et al., 1999) and to reduce the hyper-metabolic response to burns and severe trauma (Voerman et al., 1992 and Knox et al., 1995). In animal and in in-vitrio studies, it modulates the hepatic acute phase response as well as cytokine expression (Johnson et al., 1991). Unfortunately when rhGH was used in double blind trials in European ICUs in the 1990s, it was found to increase mortality in trauma patients almost three-fold, 42% against 18% for placebo (Takala et al., 1999).

The thyroid hormone axis is also disturbed by severe illness. Van der Bergh and others have showed that in people who died after intensive care, there was a reduced level of bioactive thyroxine. Jeschke and others also showed that both FTI and T4 levels were significantly reduced in the first two weeks post-burn. However the magnitude of changes in the thyroid axis is less than that seen in most other affected hormones (Jeschke et al., 2008).
Severe burns cause increased insulin secretion. Serum glucose is nevertheless significantly increased for 4-5 weeks post burn. Hepatic glucose production is elevated, in spite of the rising insulin levels. This implies hepatic resistance to insulin and a disturbance of the glucostatic function of the liver, because gluconeogenesis from the liver should normally fall when insulin levels rise. Hyperglycemia or high glucose levels are associated with increased mortality in the critically ill (Jeschke et al., 2008).

2.2.5 THE CYTOKINE EXPRESSION PROFILE IN THE POST-BURN ERA

Marked changes occur in the cytokine expression profile of burned individuals and animals. In one study in children there was a drastic elevation in the levels of 16 out of 17 cytokines measured. The most significantly increased were 1L-6, 1L-8, MCP-9, MIP-1β, and G-CSF. Significant elevations also occurred in the levels of IL-2, IL-13, IL-10, IL-17, TNF and INF-γ though the levels were returned to close to pre-burn levels in many of the latter by the end of 60 days post-burn (Jeschke et al., 2008).

Cytokines are humoral substances released from activated cells which affect the action of other cells in the vicinity. Studies show that the development of major complications after burns is affected by a pro-inflammatory cascade induced by several mediators primarily produced from activated macrophages. TNF-α seems to be a major trigger in this cascade involving PGE-2, IL - I and IL - 6. Apparently, impairments in the mechanisms for regulating macrophage function lead to excess cytokine production, and apparently play a key role in the development of post-burn immune dysfunction (Meakins, 1990; MacMicking et al., 1997).
Several of these substances especially IL-1, TNF-α and PGE-2 are capable of increasing the production of inducible nitric oxide synthase (iNOS) in macrophages. This increases the production of this reactive nitrogen species (Renz et al., 1988; Milano et al, 1995 Salvemini et al., 1995).

Cytokine perturbation may affect clinical outcome in patients. Increases of up to 100 to 200-fold were found in serum levels of G-CSF, 1L-6, 1L-8, MCP-1 and MCP-1B in studies of burned children (Jeschke et al., 2008). Only 1L-5 levels was found to fall in that study. 1L-5 is a TH-2 cytokine produced from mast cells. It stimulates B-cell growth and immunoglobulin production. A depletion of this cytokine, a pro-immunity agent may partly explain the general immune exhaustion found in the post burn era.

Kowal-Vern et al, (2006) have compared levels of anti-thrombin (AT) and several cytokines in plasma and peritoneal fluid of patients with intra abdominal hypertension from abdominal compartment syndrome in severe burns. They found AT levels were decreased while there was raised levels of INF-γ, IL-10, 1L-6, 1L-4 and 1L-2 in both fluids. The levels of these cytokines were higher in the peritoneal fluid of those who died than in survivors in that study. Therefore some of these substances may serve as predictors of mortality. It seems clear that pro-oxidants and pro-inflammatory mediators produced from cells and tissue metabolism in the burned area account for most of the effects seen in local and distant organs of the body. These processes are intended to help the burn victim survive the major injury, but sometimes they get to such exaggerated levels that they instead cause tissue destruction, multiple organ failure and death.
2.2.6 THE IMMUNOLOGIC RESPONSE TO SEVERE BURNS

Thermal injury exerts a considerable effect on the immune system. The effect is predominantly on cellular immune responsiveness. Immune suppression is the general case, and this is exemplified by prolonged allograft survival, suppression of delayed hypersensitivity and alteration in mixed lymphocyte reactions (Sakai et al., 1974; Mc-Irvine et al., 1982; Calvano et al., 1988). Beyond a certain level immune suppression is determined by burn size.

Although the loss of the mechanical barrier that skin provides, and exposure of vulnerable tissue to microbial invasion contributes to infection in burn patients, impaired immune mechanisms are a major factor in infections in these people. The importance of these processes is underscored by the fact that for a long time now, infection has been the leading cause of death in burns (Pruitt et al., 1997).

Thermal injury causes a reduction in the number and types of lymphocytes in the peripheral blood. There is a reversal for instance of the normal CD8/CD4 count, though these parameters return to normal after a few weeks. Apart from alteration in the total number of circulating lymphocytes, changes of the expression of surface markers on these cells are also important indices of impaired function in them (Pruitt et al., 1997). Cell cytokine markers that indicate lymphocyte activation are expressed in greater quantity after burns. It has been shown that serum IL-2 and soluble IL-2 receptor levels are increased after burns (Deitch et al., 1985; Theodoczyk et al., 1991). These studies
suggest that burn injury initially activates the cell immune system, but finally produces cellular immune suppression.

It appears that burns and infections produce differing changes in the circulating lymphocyte population. Sometimes the fact that infection itself alters lymphocyte levels makes it difficult to tell the effects of burns from those caused by infection. The proportion of CD4+ and CD8+ among circulating lymphocytes is increased in infection (Pruitt et al., 1997). Infection also decreases natural killer cell levels and the percentage of cells expressing the alpha integrin, CDII, an important cytokine in cell function. Altered levels of IL-1, a cytokine that is expressed in reduced levels in vitro from circulating monocytes has been suggested to be related to the development of multiple organ failure in severe burn cases. Serum levels of a naturally occurring IL-1 inhibitor, IL-1Ra, an IL-1 receptor antagonist are raised in burn patients. This may block the action of IL-7, a pro-inflammatory cytokine, and promote immune suppression. (Maldroup-Poulsen et al., 1995). IL-2 receptor antagonist levels are equally elevated after burns in proportion to burn size. Some investigators have shown a correlation between burn size and the reduction of IL-2 from circulating lymphocytes (Wood et al., 1984, Theodocyck et al., 1987).

Studies in which IL-2 was administered to mice, which then had their cecum ligated and punctured, showed that it improved survival. Unfortunately just as in the trials of recombinant human growth hormone, when IL-2 was tried in animal models of burn wound sepsis, conflicting results were obtained (Gough et al., 1988; Pruitt et al., 1997).
Macrophage and monocyte secretion of IL-6 increases in burns. This is under induction of IL-1 and TNF. IL-6 causes signs and symptoms of inflammation such as fever, elevated levels of C-reactive proteins, and immunoglobulin production. Stimulated Kupffer cells may be an additional source of tumour necrosis factor (TNF). TNF has been shown in studies by He and others to be contributors to the pathogenesis of inhalational injury (He et al., 1992, Minei et al., 1994) When TNF transcription by macrophages is blocked by use of pentoxyphylline, lung injury was reduced in animals, just as leukocyte number in broncho-alveolar lavage was reduced in those animals ((Pruitt et al, 1997).

There are also reduced levels of circulating B-lymphocytes and immunoglobulin G (IgG) after burns, though the levels return to normal after about four weeks (Calvano et al., 1998). B-lymphocytes are responsible for producing antibody in response to antigen challenge. Studies show that the ability of these cells to carry out a normal IgG response is impaired after burns. Unfortunately although the exogenous administration of IgG to burn patients restores IgG levels to normal, it does not affect morbidity or mortality. Tabata and Meyer (1993) observed increased numbers of IgM+ cells in mesenteric lymph nodes and spleens of sheep after burns, but their production of IgM was reduced.

Apart from lymphocytes, functional impairment is found in other immune competent cells after burns. Neutrophils for instance ingest microbes and destroy them after “digesting” them. An oxidative burst involving the production of oxygen radicals, such as hydrogen peroxide takes place. This generates the chemicals that destroy bacteria. Several studies show that the ability of neutrophils to mount this oxidative burst is
severely impaired for long periods in cases where burns exceed 40% of BSA (Allen, 1982; Parment et al., 2008). Polymorphs from burn patients also demonstrate a reduction in chemotactic activity in proportion to burn size (Alexander, 1989).

Because of the functional defects in polymorphs and monocytes in burn patients, granulocyte-macrophage colony stimulating factor (GM-CSF) has been tried to enhance survival in “burned animals”. GM-CSF produced a range of pro-immune effects in these cases: it prolonged the lifespan of macrophages and neutrophils, enhanced phagocytosis by neutrophils, enhanced their response to chemotactic agents, and induced cell-surface expression of complement receptors such as CR-1 and CR-3 and CD-118. Mice pre-treated for 5 days with GM-CSF before being challenged with a combination of bacterial gavage and burn injury insult, showed better survival than control, (Gennari et al., 1994).

Soluble immune suppressing agents have been demonstrated from the serum of people with severe burns, and even from sub-eschar fluid (Constantin, 1978; Ferrara et al., 1988). These factors may include complement degradation products that inhibit phagocyte action, immunoglobulin fragments, breakdown products of coagulation and fibrinolysis, prostaglandins and endotoxins (Okzan et al., 1987; Anderson et al., 1989; Warden, 1989).

Burn injuries and other conditions that cause tissue under-perfusion increase the expression of heat-shock proteins. A 68-kd protein closely related to the 70-kd protein (HSP-70) has been identified by antibody studies to be present in serum after burns. Some heat shock proteins including HSP-70 and HSP-90 play a key role in activating
steroid building activity. As a result manipulation of these proteins can be applied to influence the local and systemic response to burn injury (Pruitt et al, 1997). Thermal injury if severe enough affects very many points in the immune defense machinery. The way these effects interact is extremely complex. This complexity makes therapeutic designs much less successful than single experiments often suggest.

It is difficult to arrive at cause-and-effect relationships in the various components of the immune system affected by burns. Moss (1998) has clearly demonstrated a correlation between defects in cell immunity and susceptibility to infection. Many therapeutic approaches have been tried to counteract specific points of suppressed cell function without uniformly positive results. Pruitt and others have observed that the limited or total lack of effectiveness of various vaccines, immuno-modulators and serologic agents, such as IgG, IL-2 and interleukin receptor antagonists in combating burn wound infection and improving survival may represent the inability of any single agent to correct the multiple immune defects induced by a severe burn (Pruitt, 1997).

2.3 EXPERIMENTAL WOUND MODELS

Animal models have been applied to the study of different types of wounds. Several types of animals have been used. Both acute and chronic wounds may be developed in experimental animals. Acute wounds are simple to create. They include incisional, excisional and burn wounds, and have been used by researchers since early in the 20th
Experimental wounds may also be created by using diathermy, scalding, laser beams, and irradiation (Saulis and Mustoe, 2009).

Incisional wounds are made by cutting through skin to a measured depth. They therefore resemble surgical incisions. They are technically easy to perform and reproducible. Healing occurs in them within 1-2 days if the edges are joined, though tensile strength develops over several months. If incisional wounds are left unclosed, they gape. The amount of gape depends on animal species, amount of subcutaneous tissue present, tensional forces acting on the wound and the level to which dermis is adherent to underlying tissues (Saulis and Mustoe, 2009).

In loose-skin animals such as rodents, including rats, guinea pigs and rabbits, such wounds have a large gape. In pigs, as in humans, less gaping occurs because the dermis is more strongly attached to underlying structures. In gaping wounds, healing by secondary intention occurs, producing greater scarring because of prolonged inflammation and the longer time required for re-epithelization to occur (Saulis and Mustoe, 2009).

Excisional wounds involve removing a variable amount of skin tissue. In partial thickness (PT) wounds and burns, all epidermis, and the papillary layer of the dermis are removed. The reticular layer and the bases of most epidermal appendages however remain. Healing is rapid in these wounds, by proliferation and migration of epithelial cells from the surviving structures. In full thickness wounds and burns on the other hand, all epidermis, and dermis are removed as deep as subcutaneous structures, whether fat,
fascia, muscle or cartilage. Healing in these wounds occurs by a combination of wound contraction, granulation tissue formation, collagen deposition and the migration of epithelial cells from the edges (Carrel and Erbeling, 1971; Fluck et al., 1998, Carmelit, 2000).

In pathophysiologic terms, burn wounds differ from incisional and Excisional wounds in a number of ways. In burns, the necrotic tissue continues to spread into deeper tissues for a considerable time after the initial injury. As a result of this extra damage, burn wounds require more time to re-epithelise than the other injury types (Kraz, 1998; Shakespeare, 2001; Greenhalph, 2002).

Healing full thickness wounds begins by the organization of a fibrin clot into granulation tissue which then fills the wound. Capillary tufts grow into the clot, and fibroblasts in the area begin to lay down ground substance and collagen fibers. The developing scar is then re-epithelised by the migration of epithelia cells, usually keratinocytes from the edges of the wound. The process of wound closure is accelerated to a variable extent by wound contraction. In this process, fibroblasts in the wound area assume the characteristics of smooth muscle and induce the contraction of the developing granulation, thereby reducing the dimensions of the wound. In loose-skin animals, up to 90% of wound closure is achieved through contraction.

In humans and pigs, only 25-50% of closure results from contraction (Carmelit, 2000). When wound healing is to be studied human wounds can be simulated in loose-skin
animals by splinting the wound edges. This reduces gaping and wound closure by contraction.

Rapid wound closure by contraction may be avoided even in a loose-skin animal such as the rabbit by creating wounds on areas where the skin adheres more strongly to underlying tissues such as the ear (Folkman, 1985). Such wounds can be used to study cellular and extra-cellular events in healing because reduced contraction allows for more granulation tissue formation and scarring. (Fajado et al., 1988). The wounds described above are also easily reproducible and substances like growth factors may be applied topically on them, making it possible to study a wide range of agents that affect wound healing (Aurebach et al., 1974; Cullington et al., 1989).

Full thickness (FT) excisional wounds may be created by punch biopsy, using scalpel or skin grafting knives. They may result in more bleeding and infection because of injury to underlying tissues. Ischaemic wounds can be created in animals as well. This can be done for example by ligating the main arteries supplying the ear in mice or rabbits. This procedure can be used to simulate hypoxic wounds in humans and to study the effects of therapeutic agents on such wounds (Dugelman et al., 1986).

The effects of age on wound healing have been studied using aged mice and other animal models. In humans, healing propensity decreases with age. Animal studies show age-related impairment in wound healing. Processes affected include: inflammation, macrophage function, fibroblast proliferation, angiogenesis, wound contraction and the
development of tensile strength (Viljanto, 1964; Levenson *et al.*, 1965; Van Winkle, 1969; Goodson *et al.*, 1982; Charles *et al.*, 1997). The causes for this deficiency are not entirely clear, but they may be related to a reduction in the secretion of growth factors. Many of these defects are reversed by the administration of exogenous growth factors (Van Winkle *et al.*, 1969).

### 2.3.1 ASSESSING STRENGTH IN HEALING WOUNDS

The strength of healing wounds may be assessed in a variety of ways. Indirect methods include counting fibroblasts in the wound. Healing wounds can also be studied by histology, immuno-histochemistry and electron microscopy. It is advisable in these studies to limit the biopsy to within 1mm around the wound. Direct methods of studying healing wounds involve measuring the tensile or breaking strength of the wound. Tensile strength is applied load per unit cross-sectional area, and breaking strength is the force required to break a wound or tissue regardless of its dimensions. Tensile strength is better for comparing wounds or tissues. The tissue strip is attached to a tensiometer and pulled. Breaking strength is more important to surgeons however, because higher levels correspond to higher amounts of mature cross-linked collagen in wounds (Deltan *et al.*, 1994).

### 2.3.2 BURN WOUND MODELS

Burn wounds have been developed in a wide range of animals for studying the effect of several agents and treatment variables such as dressings, infection control, and timing of debridement and skin grafting (Fieldlander *et al.*, 1995). Burn wounds have been
generated by the application of heated metal plates (Smahel 1993; Jewo et al., 2009),
scalding and by laser beams (Cuttle et al., 2006). They may be partial thickness or full
thickness depending on whether epithelial tissue and dermis are completely removed as
previously described.

The guinea pig is a docile animal, when compared for instance, to the domestic pig. It
has been used to simulate human burns because it mounts a systemic response to burns
quite similar to that seen in humans. It has been used in nutrition studies (Sakurai et al.,
1997). It has also been used to study wound healing and myocardial contractility
(Murphy et al., 1999; Horton et al., 1999). The rat model was initially used to study the
effects of varying temperatures and humidity on burn wounds. It is now applied to the
study of just about any issue that interests researchers in burns, including mechanisms of
wound healing, kidney and liver changes, inhalational injury, cardiovascular responses
and the neurologic effects of burns (Saulis and Mustoe, 2009).

In most of these studies, the animal’s skin is shaved and exposed under anesthesia to a
source of heat such as steam or hot water, for periods ranging from 3-10 seconds
depending on the wound thickness in view. This method tends to produce uniform burns
that can be histologically ascertained. Animals survive burns on 25% of their total body
surface area (TBSA) without resuscitation. Larger burns may however require fluid
resuscitation. Scalding procedures are technically simple, reproducible and less
demanding in equipment.
Burned rats show a correlation between burn size and a number of physiologic and biochemical parameters that are similar to what occur in humans (Saulis and Mustoe, 2009). Therefore, though mice are easier to handle, rats are now the most frequently used animal in burn research. They have been used in the study of wound healing, infection, metabolism and the effects of various treatments (Walker and Mason, 1986; Herndon et al., 1998). They have also been used to study myocardial function and lung perfusion, as techniques became available for measuring vascular pressures in burned rats (Gulati et al., 1996; Jeschke et al., 1999).

2.4 ANIMALS

The goals of animal experiments in the field of burns have metamorphosed over the last half century or so. In the 1960s and ‘70s, research focused on pathophysiology and the control of burn induced hypovolemic shock. As effective fluid resuscitation regimen became available for preventing death from shock, research focus shifted to identifying and analysing mediators, mostly chemical substances that modulate the systemic response to thermal aggression. This occurred as it became known that they play an important role in the alterations that occur in the immune system, in metabolism and in the vascular system.

Once circulatory shock is avoided, infection is the leading cause of death in burns (Santos et al., 1996; Pruitt et al., 1997). Accordingly another key field of burn research is wound infection. It is second to the study of chemical mediators which affect infection outcomes through their effects on the immune and related systems. The goals of these
studies have been to reproduce in the experimental model, pathophysiological conditions similar to those produced in burned humans. This then enables local and systemic consequences of the burns to be evaluated and ways to modify these effects are also developed in the process.

The Wistar and Sprague-Dawley are both albino rats and are variants of *Rattus norvegicus*. Even though mice are easier to handle, these rats are the most widely used animals in burn research today. The Wistar is very resistant to infection. It is for this reason very suitable for studying any situation in which prolonged survival is required (Santos *et al.*, 1996). The rabbit, *oryctolagus cuniculus*, has as many as 70 varieties in use as experimental animals – from the giant Flanders which can weigh 5kg to the minute Dutch rabbit. They however are far more susceptible to infection than rats, and surgical procedures in them require sterile conditions. They are also costlier than rats, making them less attractive in places where funding is a challenge (Santos *et al.*, 1996).

Pigs are being used more frequently in burns and in other experiments. This is influenced largely by the great anatomical and physiological similarity between pigs and humans. Pigs, as have been pointed out already have tight skin like humans, and wound closure does not receive as much contribution from contraction as happens in loose-skin animals such as rodents.

Though the domestic pig costs far more than smaller animals, one can generate for instance several 2cm burn wounds on only one side of a pig. If the local effects of an
agent were to be studied, control wounds can be generated on the other side of the same pig. This will require 8 rats to do. On a general basis however, pigs are disadvantaged on account of higher costs and the fact that anesthetics are required, and post-operative care requires more elaborate infrastructure than is for example required in rats or mice (Santos et al., 1996).

Scalding is the method in widest use at the moment for generating burns of uniform depth in animals. It is easy, cheap and reproducible. Various techniques can be applied including the use of a perspex surface, or metal cage to immobilize the anesthetized animal (Walker et al., 1968; Cuttle et al., 2008). The animals show the classic signs of burns. Skin on the dorsum a rat is roughly 30% of the body surface area, and this is where most burns are generated.

In an attempt to create deep dermal partial thickness burns (DDPTB), studies have been conducted with exposure to water at different temperatures for different durations. Cuttle and others tested water at temperatures ranging from 76°C to 96°C for 20 seconds and finally 92°C for 10, 15, and 20 seconds. They settled for 92°C for 15 seconds. When examined histologically burns created by water at temperatures lower than 92°C for 10 seconds are partial thickness but do not extend deep enough into the dermis. In making scalds with hot water the minimum duration required for the desired burn should be applied. Maintaining constant temperatures for long periods requires adding hot water along the way and increases the hazards in the procedures (Cuttle et al, 2006; Sakallioglu et al, 2007).
Feifel and others have perfected a technique for generating burns by contact though the method has a drawback. It is difficult to generate burns of uniform depth. It involves applying a copper chip heated to 250°C to the shaved skin for a variable length of time. Ensuring constant pressure over the entire skin under the chip however presents the difficulty (Feifel et al., 1992).

Generating chemical burns presents problems in that the agent applied tends to diffuse beyond the desired area. However in 1994, Kim and others perfected a technique that overcame this problem. This involved putting the rat in a Teflon hemi-cylinder into which a hole had been cut in the center. This hole is then placed over the shaved area and the chemical is applied (Kim et al, 1994).

Electric burns have also been studied in animals. Generally this requires the use of larger animals, as smaller ones cannot survive the kind of currents that generate electric burns in humans. In 1990, Chilbert and others applied currents of 1amp, at 60Hz frequency to the legs of dogs. Various effects including physiologic evidence of muscle cell necrosis occurred (Chilbert et al., 1990). However it was Zelt and his team (1998) who carried out a more comprehensive study of the effect of electric burns on animals. They applied currents of between 3000—4000 V and 4.2 amps for 2.5 seconds to animal limbs. This produced patchy cell necrosis in skin and muscle, as well as narrowing of large vascular trunks and reduction in nutrient vessels in the affected areas. Though no evidence of progressive necrosis was found in these animals, they found that there was a loss of conduction in the ulna nerve which failed to recover with time.
In humans who suffer burns in closed spaces or in outdoor situations with a lot of hot smoke or toxic gases, lung injury termed inhalational syndrome occurs. This condition has been produced in experimental burn models by putting the animal in a cylinder connected to a source of smoke immediately after scalding (Santos et al., 1996).

A variety of techniques are available for quantifying the depth of burns. It may be estimated live by ultra-sonography. The depth can also be graded from histology of the wound tissue. The Suzuki scale is a simple method that allows comparison of animals, and samples. In this scale, first degree lesions show minimal epidermal injury with vasodilatation and slight inflammation. In second degree injuries, there is infiltration in the skin, along with peridermal necrosis, vacuolation and dense perivascular infiltrates in superficial dermal burns. There is collagen degeneration limited to the papillary dermis. In third degree, collagen degeneration reaches the deepest layers of the dermis. Scalding cause dermal lesions depending on water temperature and exposure time (Suzuki et al., 1991).

Techniques have also been developed for obtaining samples of body fluids when analysis is required on them on a repeated basis over the course of experiments on animals with burns. One way has been to implant a catheter (commonly 18 G, Venecath) into the common iliac vein at its bifurcation. It is extended from there (it has to have a bevelled tip) into the vena cava. It is then filled with sodium heparin, which is withdrawn and replaced each time blood is withdrawn for analysis. The catheter is secured to the vessel with silk sutures and capped externally. Urine is collected for analysis by a variety of
procedures as well. This includes pressure on the bladder just above the pubic symphysis or by bladder puncture at the same site (Santos et al., 1996).

2.5 HISTOLOGY OF THE TESTIS

The mammalian testes contain germ cells arranged along a tubular structure, the seminiferous tubule which is open at both ends. The tube is tortuous and opens at the rete testes which connect it with efferent tubules and the epididymis. The seminiferous tubule (ST) canalizes at puberty from solid sex cords. In primates the ST are packed into lobules. In the human testes there are 200-300 lobules. Cells of the seminiferous epithelium are arranged from basement membrane up to the lumen, according to their level of differentiation (Stranding, 2005).

The most primitive germ cells – spermatogonia, lie on the basement membrane, and the mature free spermatozoa in the lumen. A succession of cell types – spermatocytes and spermatids lie between these groups of cells. The testis consists mostly of seminiferous tubules, and the space between them is the interstitial tissue. Leydig cells which secret testosterone lie in this tissue. It constitutes about 25-30% of the testis in man, and 15% in rats (Mori and Christenson, 1980).

In most species the tubules are 200 to 250μm in diameter. They are a bit smaller in man. In rats there are approximately 400-600 tubules in per testis and each tubule is 70-80 cm long. Their diameter varies from 120-300 μm and they contain a surface area of roughly 120 cm²/g (Wing and Christenson, 1982). At each point along the tubule, the germ cell
series occur in particular combinations and each such distinct aggregation is a stage of spermatogenesis. The stages repeat themselves along the tubule in distinct time intervals known as cycles. The number of stages and cycles from the entry of particular stem cells into differentiation to the time they are mature and are shed into the lumen is species specific and invariant (Clemont and Antar, 1973; Franca et al., 1998). In this scheme, the length of the tubule or cylinder containing a complete cycle is termed a wave. The waves are linear in many animals but have been shown to be helical in man (Schulze, 1982).

Spermatogenesis may be considered in three phases – stem cell renewal, germ cell proliferation and spermiogenesis. Two types of stem cells are found in primates: dark A (Ad) and pale (Ap) spermatogonia. Intense mitosis among these cells ensures that an undiminishing number of germ cells are continually available for the subsequent step of spermatogenesis. The Ap cells give rise to B spermatogonia which are the first differentiated cells in this epithelium. Division of this cell then produces a primary spermatocyte. Meiosis I and II of this cell produces four haploid spermatids and terminates proliferation. Spermiogenesis consists of the process whereby these round spermatids are transformed into the mature elongated mammalian spermatozoa (Heller and Clemont, 1964).

Intense apoptosis is a normal part of the spermatogenic process and the passage from Ap to B spermatogonia has been shown to be associated with no more than 25-30% yield in rats (Swedloff et al., 1998). Although part of the loss is due to the limitation that arises
from Sertoli cell number, it is also used to weed out developing cells with faulty genetic make up.

### 2.6 BLOOD TESTIS BARRIER

The seminiferous epithelium contains germ cells and large cells, Sertoli cells which stretch from the basement membrane to the tubule lumen. Membrane bound lateral processes from adjacent Sertoli cells form tight junctions which constitute a barrier between blood and the germ cells in the epithelium. The junction is established at a level just above the spermatogonia. This barrier therefore divides the seminiferous epithelium into a basal compartment containing spermatogonia and an adluminal area containing more differentiated cells of the spermatogenic series.

This barrier allows selective passage for substances that reach the lower compartment from the interstitium. Steroids and inhibin for example pass freely while gonadotropins and immunoglobulins are excluded. The barrier is established in humans at puberty and begins to develop in rats at about the 18th day. It is believed that the junction opens periodically for brief periods to allow developing germ cells to advance into the adluminal compartment (Lamb and Forster, 1988; Kumar et al., 2000; Young and Heath, 2002; William and Warick, 2005).

The barrier serves several functions. Germ cell differentiation produces cell bound proteins that may be regarded as foreign if they were not shielded from immune-
competent cells. It also protects spermatogenic cells from many potentially toxic substances that may reach them from the blood stream.

2.7 SPERMATOGENESIS

Spermatogenesis is the main activity of the testes and takes place within the epithelium of the seminiferous tubule. Daughter germ cells derived from each spermatogonium develop as a unit, connected to each other by intercellular cytoplasmic bridges. This arrangement ensures rapid communication between cells and synchronous development (William and Warick, 2005).

Spermatogonia, the first germ cells, begin to differentiate out of primordial cells in the testes at puberty. At puberty, sex cords acquire a lumen and become seminiferous tubules. Mitotic divisions of these B-spermatogonia give rise to spermatocytes. These are recognizable by their having nuclear chromatin in sparse clumps and filaments. Primary spermatocytes undergo the first meiotic division and produce secondary spermatocytes. Each secondary spermatocyte generates two spermatids on completion of meiosis, each of which has haploid number (23) chromosomes. Thus, in humans, four daughter cells, connected by laterally placed cytoplasmic bridges, develop from each primitive germ cell (William and Warick, 2005).

A maturation process known as spermiogenesis transforms the rounded spermatids into the elongated, recognizable mature spermatozoa. The process involves condensation of nuclear material in the head, a drastic reduction of cytoplasm, and the development of an
acrosome, neck, middle piece, and tail. Mature spermatozoa are shed into the lumen of the tubule and then into the rete testis. From here they enter the caput epididymis, where motility is acquired. These movements involve contraction of myoid cells in the tubule and testicular capsule, and the presence of fluid in the tubules. (Sharpe, 1994; Qin and Lung, 2000; Young and Heath, 2002).

Knowledge of the duration of spermatogenesis is crucial to evaluating the effects of substances that are potentially harmful to spermatogenesis, especially when sperm density is a key parameter in such evaluations. In the 1960s, Heller and Clemont incorporated labeled H-thymidine into the nuclei of testicular germ cells. They then examined radio-autographed sections from the tubules at given intervals, and discovered the time it took for labeled cells to first reappear along them. This was roughly 16 days. By injecting H³-thymidine into the testis and following the progress of the label in pre-leptotene spermatocytes, the most advanced of the germ cells to take up this label, Heller and Clemont discovered that it took 16 days to appear in round spermatids, and another 16 days to appear in mature elongated forms about to be shed into the lumen. They established that there are 4 cycles of spermatogenesis in humans, lasting 16 days each, making 64 days. It takes about 48 days in rats. This is the time duration for B spermatogonia to transform into free mature spermatozoa.

In mammals, it was discovered that if two adjacent sections are cut from any given seminiferous tubule, the relative proportions of the different germ cells will be different in them. Particular cell associations, appear in a cross section, in the entire circumference
of the tubule, disappear, and reappear, a certain distance further in the tubule. Particular cell associations were therefore appearing in waves, which is the length of tubule or cylinder between these appearances. Each particular cell association represents a stage. The time interval between these recurring stages is a cycle. The number of stages within a cycle, cycle duration, and the number of cycles it takes a given primitive cell to reach the epididymis are species-specific (Heller and Clemont, 1964; Hess, 1999). There are 14 stages in rats, 12 in guinea pigs and monkeys and 6 in man (Hess, 1999).

In subhuman primates, a wave of spermatogenesis occupies a longer segment of the tubule, up to 10mm. The cylinder in humans is of much shorter length and particular cell associations or stages do not occupy the entire circumference of the tubule. Waves are therefore not as distinct as they are in lower primates, or sub-primate mammals.

2.7.1 SERTOLI CELLS AND CONTROL OF SPERMATOGENESIS

Sertoli cells are large, tall cells scattered in the epithelium of the seminiferous tubule. They usually span the entire depth of the epithelium from basement membrane to the ad-luminal area. They bear and nourish successive generations of the germ cell series in membrane invaginations present all over their surface. Their function is therefore critical in spermatogenesis, the process of converting spermatogonia to recognizable sperm cells capable of fertilizing ova. Sertoli cells are required to maintain the normal cyto-architecture of the germinal epithelium and provide energy substrates and general nutritional support for the developing germ cells. They are the only cellular component of the blood-testes barrier in primates (Dym, 1973; Cavichia and Dym, 1978). It has been
shown however that each Sertoli cell can only support a limited number of germ cells. Therefore the complement of Sertoli cells in the testes determines to a large extent sperm production and fertility (Orth et al., 1988; Sharpe 1994; Silber, 2000). Fecundity is known to begin to decline if sperm concentration falls below certain levels WHO, 1992).

Sertoli cells first appear in the human fetal testis at about 8 weeks (Pelliniemi et al, 1993). At birth, approximately 10% of the adult cell number of 4 billion cells is present. Proliferation of Sertoli cells occur through the pre-pubertal and pubertal periods until this figure is achieved. Gonadotropins, especially FSH drive this process at puberty (Marshall and Plant 1996). Sertoli cell number was found to nearly double when juvenile rhesus monkeys were administered pulsatile doses of either recombinant human FSH (rh-FSH) for 11 days or rh-LH (Ramaswamy et al., 2000). It is now felt that FSH and LH levels rise about the same time in boys at puberty, (Plant, 1994; Mitamura et al., 1999). LH action on Sertoli cells proliferation is probably mediated by a local action of testosterone which is secreted by Leydig cells in response to this gonadotropin. This position is supported by the finding that juvenile monkeys given T alone underwent Sertoli cell divisions.

There are significant differences between species in the time at which the full complement of these cells is attained in the seminiferous epithelium. Studies have shown that Sertoli cell proliferation in rats is complete by 14-21 days, ahead of puberty which is only achieved at 25-30 days of age (Sharpe 1994; Ojeda and Urbanski, 1994). In the marmoset (Callithrix jacculus), a new-world monkey, adult numbers of the cells are
attained at between 18-22 weeks of age (Sharpe et al., 2000). The fact that the adult number takes many years to be attained in humans must be considered when extrapolating findings from animals onto the human situation.

2.7.2 HORMONAL CONTROL OF SPERMATOGENESIS

Androgen Receptors (AR) are already expressed in the fetal gonad to a large extent by the 8th week, even though it lacks sex differentiation at this time. It has therefore been shown that the expression of this receptor is not responsible for sex differentiation. All androgens act through this single intracellular androgen receptor (Sajjad et al., 2004). FSH and LH receptors are present in Sertoli and Leydig cells respectively. The signals from FSH reach the germ cells through Sertoli cells. FSH signals cause elevation of intracellular c-AMP, producing protein kinase A, (PKA). This latter act on cAMP-responsive element (CRE), which is present on the promoter sites of many genes, up-regulating the activity of such genes (Santen, 1987; Sassone et al., 1995; Mundy, 2005).

The first generation of testicular spermatozoa in primates is produced at puberty. This process known as spermatogenesis is driven by pituitary gonadotropins, FSH and LH. At this time a transition from the relatively hypo-gonadotropic state of the pre-pubertal period to the eugonatropic state of adulthood occurs (Plant, 1994). If FSH and LH levels are prematurely elevated either experimentally, or pathologically, spermatogenesis will begin in primates, confirming the role of these gonadotropins in initiating spermatogenesis (Plant et al., 1989; Grumbach and Styne, 1992).
Several studies have been mounted to clarify the differential contribution of FSH and LH to the initiation and maintenance of spermatogenesis. The direction of the evidence from these studies suggests that relative to LH, FSH plays a subordinate role in the process. Pre-pubertal monkeys administered LH alone for 12 weeks, did have stimulation of the seminiferous epithelium, but no cells matured beyond B spermatogonia were found in them (Arslan et al., 1993; Schlatt et al., 1995; Ramaswamy et al., 2000). In men with hypogonadotropic hypogonadism, combined treatment with FSH and T failed to generate any spermatozoa after 2 years (Schaison et al., 1993).

On the contrary, spermatogenesis was initiated in boys in whom a mutant gene for the LH-receptor (LH-R) caused premature LH activity. The same thing occurred in cases of Leydig cell hyperplasia (Muller et al., 1998; Kim et al., 1985). Also examination of men where FSH production was attenuated by a mutant FSH-R gene, spermatogenesis was found to begin (Tapanainem et al., 1997). Interpretation of these findings present problems though, because in two men where FSH deficiency occurred due to deletion of the gene coding for the B-subunit of the hormone, azoospermia and infertility was the uniform finding (Maroulis et al., 1977; Linstadt et al., 1997).

The differing clinical situation in the cases of FSH-R and FSH-B subunit deficiencies suggest that a wholesale conclusion on the role of FSH in initiating spermatogenesis is not yet achievable. Further difficulty with interpretation of these findings, arises from the fact that the absolute levels to which the FSH-R mutant gene was expressed in the testes.
of these men was not determined, and reduced FSH signaling up-regulates its own receptor (Themem et al., 1991, Mc-Guire et al., 1997).

It has been suggested from the kind of findings described above that LH alone is enough to initiate spermatogenesis. FSH has been felt not to be obligatory to the initiation of the process. The role of these gonadotropins in the maintenance – the sustained production of spermatozoa in the adult testis is another subject of considerable research (Moudgal and Sairam, 1998; Nieschlag et al., 1999). Experimental evidence also suggests that FSH is not obligatory to the maintenance of spermatogenesis in primates. Spermatogenesis has been found to be maintained in men rendered FSH-deficient by the administration of human chorionic gonadotropin (Martsumoto et al., 1986). Immuno-neutralizing circulating FSH has not produced consistent impairment in the maintenance of spermatogenesis – in the rhesus macaque (Murty et al., 1979) or in men, (Moudgal et al., 1997).

Is LH obligatory for the maintenance of spermatogenesis?. Indeed immuno-neutralizing circulating LH has been shown to result in severe depletion of maturing germ cells (Suresh et al., 1995). A similar finding occurred when LH attenuation was induced in cynomolgus monkeys by simultaneous administration of GnRH-R antagonist and FSH replacement (Weinbauer et al., 1991). On the balance, the real physiologic situation may be that combined action of both LH and FSH, as postulated by classic endocrinology is required to both initiate and maintain spermatogenesis at optimum levels (Steinberger, 1971; Nieschlag et al., 1999). The exact pattern of the interaction between these two
hormones to synergistically affect spermatogenesis is however yet to be clearly elucidated.

It is known that even though chronic application of testosterone to the juvenile primate testis can induce spermatogenesis, combined LH/FSH stimulation reduces the time for undifferentiated Sertoli cells to reach maturation in this situation. It is believed that FSH facilitates the LH-dependent differentiation of Sertoli cells, and reduces in this way, the time these cells will require to begin to support developing germ cells. However the progression of spermatogenesis – its kinetics, is similar, whether it is initiated by FSH or LH (Moudgal et al., 1998; Nieschalg, 1999).

The role of these hormones in the restoration of spermatogenesis after temporary suppression or cessation is another issue that has been studied. Sometimes, the adult testis in which spermatogenesis was temporarily suppressed has been used to study the initiation of the process. Inferences drawn from such studies however will apply more properly to restoration rather than initiation of spermatogenesis. Significant differences exist, at the cellular level in the characteristics of the testis in both situations. During initiation, the germ cell maturational process is closely related to Sertoli cell proliferation and differentiation. Secondly, Sertoli cell function is partly dependent on the composition of the basement membrane which will still be undergoing developmental changes at puberty. These situations do not apply when spermatogenesis is to be restored in the formerly active testis (Dym, 1994; Loveland et al., 1998).
Both FSH and LH have been used to restore spermatogenesis in men after suppression. FSH alone restored sperm production in men rendered hypogonatropic by chronic injection of T until the serum level of this hormone reached 11-14 ng/ml (Matsumoto et al., 1983). There is abundant evidence that LH alone will restore sperm production, though it was below normal levels in men (Brenner et al., 1981). In hypophysectomized monkeys where intra-testicular T was fully restored by exogenous sources, testicular volume recovered up to 90% of former levels and spermatid count was 15-20 per cross section compared to 40 per cross section in control animals (Marshall et al., 1995). LH directly stimulates T secretion from Leydig cells. However though FSH administration to normal primates enhances spermatogenesis and leads to germ cell proliferation such increases has unfortunately not been translated into consistent results in attempts to treat oligospermic men with FSH (Bartoov et al., 1994; Foresta et al., 2000).

A selective elevation of serum FSH by unilateral orchietomy stimulated spermatogenesis in the intact testis. It also caused 70% increase in testicular volume, and increased the diameter of ST and number of all germ cells matured beyond pale spermatogonia (Ap), in spite of an unchanged Sertoli cell number. Thus circulating levels of FSH are the main determinant in normal situation of the level to which spermatogenesis is driven in primates. Though the details of how these gonadotropins interact with testicular T to drive this process are unsettled, it is believed that FSH amplifies the effect of LH on T secretion. This makes it possible, at least in primates to achieve intra-testicular concentration of T, required for optimal sperm production (Majumdar et al., 1997; Young and Heath, 2000). This is important, because there is
evidence that intra-testicular T in rats is usually maintained at levels well in excess of what is required for optimal sperm production (Zirkin et al., 1989).

Attempts to delineate the particular cells that respond to FSH in the testes are on-going. In the T-treated, hypophysectomized rhesus monkey, FSH administration caused a selective amplification of all differentiated spermatogonia without altering pale spermatogonia cell number (Marshall, 1995). Others have however carried out experiments in which FSH was reported to cause increase in number of both types of spermatogonia in both rhesus and cynomolgus monkeys (Van Alphen et al., 1988). It has been pointed out however that the increase in the population of both B and pale germ cells in their time relation run against the settled conclusions on the kinetics of spermatogenesis in this species. (Clemont and Antar, 1973).

A proliferation of B spermatogonia, resulting from sustained increase in the Ap stem cells, requires that the latter must have proliferated in the preceding cycle of the seminiferous epithelium. This should be at least 18 days before the level of B cell proliferation. Examination of the seminiferous epithelium of these macaques for such a time-dependent proliferation showed it did not occur (Van Alphen et al., 1988). Some have suggested that the proportion of Ap cells dividing is reduced in the absence of FSH. It is however well established that in most monkey species, the entire population of Ap cells divide in stage IX of the cycle of the seminiferous epithelium (Clemont, 1969; Clemont and Antar, 1973). They have also shown that these stem cells are tough; their numbers do not reduce as they age, and pass along from stage IX to stage VIII of the
subsequent cycle, when they next divide. It is therefore more likely that the action of FSH to increase B spermatogonia population might result from its ability to promote the survival of the first generation of these cells to differentiate, possibly by stimulating secretion of a Sertoli cell factor that suppresses apoptosis of this cell type.

In primates FSH secretion is regulated by pulsatile release of GnRH from a network of GnRH expressing neurons in the hypothalamus referred to by some as the GnRH pulse generator (Karch, 1980). The same neural impulses drive LH secretion. LH secretion is under direct negative feedback control of testicular T on the hypothalamus. However FSH levels appear to be independent of direct feedback control from GnRH (Spratt et al., 1987) of T in the same manner even though castration of primates causes a marked elevation of FSH levels which is not seen if both estradiol and T are immuno-neutralized (Medharmarthy et al., 1990). A non-steroidal agent of testicular Sertoli cell origin, inhibin B has been established as being responsible for negative feedback control of FSH secretion probably by regulating FSH-β gene expression in the hypophysis (Plant et al., 1997, Ramaswamy et al., 1998). However, FSH levels are not completely insensitive to testicular steroids. When circulating levels of T or E$_2$ are raised beyond physiologic margins FSH levels are suppressed (Resko et al., 1977; Plant, 1989). The contribution of steroid negative feedback control is clearly less than that of inhibin, at least at physiologic levels.

The current evidence suggests that the FSH-inhibin B feedback loop as well as FSH feed-forward signals play important roles in the maintenance of spermatogenesis. FSH might
provide signals that set the level of spermatogenesis above that induced by testicular T. The action of FSH is mediated by Sertoli cells through paracrine actions that serve as a survival factor for the first differentiated spermatogonia, thereby amplifying the basal level of spermatogenesis generated by testosterone.

2.8 SEMEN PARAMETERS AND FERTILITY

Semen is a complex fluid consisting of spermatozoa suspended in a liquid derived from several sources: the testis, epididymis, ductus deferens, seminal vesicles and the prostrate gland. These last two are accessory organs that contribute important substances such as fructose, prostaglandins and acid phosphatase to seminal fluid. Its volume in humans is 3-5ml, of which spermatozoa constitute only 10%, usually 200-300 millions per ejaculate (Lamb et al., 1988; Mundy et al., 2005).

Studies show that sperm density has declined over the last several decades, especially in the developed nations. It has been reported that sperm counts of men in Paris dropped by 24%, from 86 m/ml in 1973 to 60 m/ml in 1992 (Auger et al., 1995). More worrisome is the report of a Danish study which showed that 30% of 19 year-olds drawn from the general population had sperm counts in the oligospermic or subfertile range (Jenson et al., 2002).

In 1992 Carlsen and his team startled the world by announcing that sperm concentration in men had fallen by half during the preceding five decades of that century. Many studies
and voices came out disputing this conclusion, at that time. Though the causes are yet to be clearly established, few doubt today that male fertility has declined over the last several decades – not just in the western world, but worldwide (Carlsen et al., 1992; Ginsburg et al., 1994; Auger et al., 1995; Andolz et al., 1999).

A recent study showed that 20% of the young men in the general population of Norway and Denmark, had sperm counts below the WHO reference levels of 20 million per ml (m/ml) and about 40% had counts below 40 m/ml (Haugen et al., 2006). The figure of 40 m/ml has been showed to be a threshold below which fecundity begins to decline (Jorgensen et al., 2006). Minimum requirements for semen analysis and semen parameters were first established in 1951 by the American Fertility Association (Abarnabel, 1951), and later by Freund and Ellianson (Freund, 1966; Ellianson, 1971). The World Health Organization (WHO) published manuals setting reference values for normal parameters in 1980, 1987, 1992 and 1999.

It had been established however as far back as 1951, that values of semen parameters varied between males from fertile and infertile marriages (McCleod, 1950; McCleod and Gold, 1951). The WHO values were derived from studies of a fertile population and represent cut offs from those results. They do not therefore consider infertile marriages and do not represent values below which conception will be unlikely in such marriages. The minimum values that still give a reasonable chance of conception will clearly be lower than those establishing normality within a population with proven fertility such as that used by the WHO. In response to these criticisms and the fact that regional
variations occur in semen parameters, the 1999 WHO manual recommended that each laboratory should determine its own reference range for each parameter.

The clinical value of the traditional semen analysis in predicting fertility is therefore seriously questioned at the present time, and some have suggested it should be completely abandoned (McDonough, 1997; Menkveld et al., 2001; Lewis 2007). Some have however suggested that while semen analysis may still be useful for identifying infertile cases, serious limitations must be placed on its prognostic value (Jecquier, 2005). She pointed out that the analyses were confined to visual observations of a continually variable biologic product at a single point in time. Besides, these analyses give no information on the causes for any deficiency.

Even when they are used, there is little consensus as to which of these parameters – sperm concentration, motility and morphology – usually measured during standard semen analyses is the best in predicting fertility. While Guzick and his group reported morphology to have the greatest discriminatory power Nallela and others identified sperm concentration and motility as being more effective ((Glazener et al., 1987; Polansky et al., 1988; Guzick et al., 2001; Nallela et al., 2006). Interestingly, in a study supporting morphology, in 243 men whose wives were pregnant at the time of study, mean sperm morphology was below the WHO cut off for normality (Chia et al., 1998).

WHO cut offs are 31% morphologically normal spermatozoa, and 45% motility, 8% acrosome index. When morphology score is done by strict criteria, 4% becomes the cut
off for normal cell count. In a study comparing these values in fertile and sub-fertile populations, Menkveld and others (2001) have recalculated new cut off figures from receiver operating characteristic curve analysis. They arrived at 21% and 3% normal cells by WHO and strict criteria respectively, 20% for motility, 3% acrosome index and 2.09 teratozoospermia index (TZ1), They found morphology to be the best discriminator between the two populations.

Many factors account for the lack of agreement in the result of studies of these parameters from different parts of the world. First, there are considerable differences in the way spermatozoa are evaluated morphologically in different laboratories. Secondly, large variations occur in sperm concentration between countries and regions. Seasonal variations even occur between ejaculates in any single individual (Fitch et al, 1996). A dramatic example of intra-personal semen heterogeneity is the case reported in the WHO manual of 1987 of 60 consecutive samples from one man over 120 weeks, in which the count ranged from a peak of 170 m/ml to well below 20 m/ml on several occasions. Studies by Mallidis and others (1991) have confirmed this report.

None of the parameters measured in the usual semen analysis addresses sperm function – the power of the sperm being examined to fertilize an egg. A variety of assays to evaluate this power have been developed over the last few decades. These include DNA structure assays, sperm motion analysis, hyper-activation and zona penetration assays, (Yoger, 2000; Lin et al, 2004; Lewis and Aitken, 2005; Agarwal and Said, 2005; Lewis, 2007).
The development of intra-cytoplasmic sperm injection techniques (ICSI), has led some to suggest that improving methods for performing and interpreting semen analysis are unnecessary. In ICSI, only a single viable spermatozoon is needed to achieve conception. However, ICSI is a relatively recent technique. Though preliminary evaluation of the DNA make up of babies born through this technique has not revealed any effect on their genome, it can be said to be too early in the day to pronounce confidently on its safety. For as others are pointing out by by-passing all the natural barriers that helped select spermatozoa on their way to the egg, ICSI might weaken the gene pool of the general population over time (Lewis, 2007).

One useful value of working out appropriate thresholds for semen parameters in sub fertile men is that it will reduce cases of over diagnosis and referrals to assisted reproduction units. It will even stop some cases being referred directly to ICSI, by-passing IVF. These points are important when one considers that Health Insurance in most European Union countries do not cover IVF costs though negative population growth has forced a number of European countries to allow use of state funds for couples in assisted reproduction situations (Ombelet et al., 2005). The jury is still out on the potential hazards of procedures like ICSI on the human gene pool. Better prognostic tests for fertile sperm are still needed therefore if the least invasive procedures are to be used. Of all the new tests of sperm quality, DNA assays present the least variability and are therefore very powerful measures of reproductive competence. Unfortunately no agreement exists yet on the application of these newer measures, and none of them are in general clinical use at the moment (Lewis, 2007).
CHAPTER 3
MATERIALS AND METHODS

3.1 ANIMALS AND EXPERIMENTAL PROTOCOLS

200 mature Sprague-Dawley rats were obtained in batches from the laboratory animal center of the College of Medicine, University of Lagos, and Ladoke Akintola University, Ogbomosho for the studies. The animals were authenticated at the department of zoology of the University of Lagos. They were kept in a well aerated room with hygienic surroundings and with natural, 12:12 hour light: darkness rhythm. They were fed with food from Livestock Feeds Nigeria Limited and clean water was provided ad-libitum. They were divided into 6 groups of 7 animals each. The experiments were repeated 3 times over a 24- month period.

3.1.1 INDUCTION OF BURN INJURY

Each animal was anesthetized with an intra-peritoneal injection of ketamine hydrochloride (20mg/ kg b.w.) and diazepam (0.1mg/ kg b.w.). The skin over the entire dorsum of the rat was dabbed with a liquid containing methyl alcohol and chlohexidine, and shaved.

Burn injury was induced by modification of a procedure developed by Cuttle et al, (2006). The animal was placed in the supine position in a shallow 3 mm thick perspex bowl with a rectangular hole cut in its floor, with dimensions giving an area equal to 35% and 45% of total body surface area of the animal. The dorsum of the rat was pressed into this opening and the bowl was lowered into water at a temperature of 99°C for 10
seconds. The animal was then removed and the skin dried immediately with a clean soft cloth (Cuttle et al., 2006). In animals intended to develop more debilitating injuries, drying the scalded skin was delayed for several minutes allowing more prolonged contact with the hot water.

Sham-burned animals were anesthetized and shaved. They were then put belly up in water at 20 °C for 10 seconds and removed. The animals were then kept and observed in separate cages for 8-12 weeks. The percentage of total body surface area (TBSA) to be burned was determined in the animals by applying the Meeh-Rubner equation. According to this rule, the TBSA of an animal is given in the formula:

\[ \text{TBSA} = W^{2/3} \times k. \]

Where \( W \) is the weight of the animal in gram, and \( k \) is a constant. In rodents \( k \) is 10 (Dilpin, 1996). The hole in the perspex plate was made so as to give its dimensions an area corresponding to the required percentage.

### 3.1.2 ANIMAL CARE

All animals tolerated the induction procedure well, recovered and were active and feeding 6-8 hours later. The animals were kept in separate cages, in a room that was periodically disinfected. The animals were carefully observed for evidence of infection, especially fever and reduced appetite. Though chronic slow-healing wounds, with sub-eschar sepsis occurred in some animals, systemic evidence of infection was not found in any animal. Burn depth was assessed clinically on the second and third days post exposure, by checking for pain reaction when the burned surface is pricked with a sterile
hypodermic needle. Third degree burns are painless over the burned areas. The scars were also examined later by histological methods when the animals were sacrificed. This was done by examining sections from the burned skin. Animals which developed repeated hemorrhagic wounds were administered topical silver sulfadiazine (Dermazine) ointment. When healing scars were being injured from scratching, mepyramine maleate (Piriton) cream was applied. Some animals were lost from anesthesia, handling errors and inadvertent injuries. Animals that developed tears in the scrotal skin were excluded from the study.

3.1.3 TREATMENT GROUPS

A---Control, and sham burn.
B---35% TBSA burn, no treatment.
C---45% TBSA burn, no treatment.
D--- 45% TBSA burn, i.m. Testosterone (T) injection, 4 mg/kg b.w. weekly.
E--- 45% TBSA burn, i.m. FSH/LH 1 IU/kg b.w. daily + weekly T injections.
F--- 45% TBSA burn, i.m. Ascorbic acid (AA) injection 8 mg/kg b.w. daily.

All treatments were started on the second day post-burn. The animals were weighed at the onset of the study, every fortnight and again just before they were sacrificed.

One set of animals was sacrificed after 8 weeks and the second after 12 weeks to allow for the 8 week duration of the spermatogenic cycle in rats. They were given ketamine hydrochloride intra-peritoneally, 20 mg/kg b.w. and diazepam 0.1 mg/kg b.w. Blood was withdrawn with a 23G cannula by cardiac puncture into plain haematocrit bottles. After it
settled, serum was separated by centrifuging blood at 3000 G for 10 minutes. The testes and accessory sex organs were dissected out and weighed. The volume of the testes was also measured. The caput epididymis was dissected off the testis, trimmed of fat and used for estimation of semen parameters. One testis was fixed in Bouin’s fluid and used for histologic analysis. The other was crushed in a mortar, homogenized in 10ml of distilled water and used for anti-oxidant assays. The scar was also excised for histologic examination.

3.2 TESTICULAR WEIGHT (TW) AND VOLUME (TV)

Testicular weight was measured by an electronic balance (ADAM, Denmark), while the TV was estimated by water displacement using a 5 ml syringe with the plunger removed. The two testes of each rat were measured and the average value obtained for each of the parameters was regarded as one observation. Values were expressed in g and ml for TW and TV respectively.

3.3 SEMEN ANALYSIS

Semen analysis was carried out by modifying a method previously described (Yokoi et al., 2003). Briefly, the cauda epididymis was dissected out, several incisions were made in it and it was suspended in 1ml of physiological saline. The preparation was allowed to stand for 10 minutes in an incubator at 37 °C to allow sperm swim up. One drop from this preparation was placed on a heated slide for motility estimation. The slides were examined using a CETI (UK), phase contrast light microscope at magnifications of 100 and 400. The microscopic field was
scanned systematically and spermatozoa encountered were assessed as motile or non-motile. An estimate of the percentage of motile sperm was made (Sonmez et al., 2005). Sperm movement was then classified into whether it was linear and rapid, sluggish or non-linear based on the predominant quality of motion observed among motile sperm cells (Saalu et al., 2010).

Sperm count was determined using the improved Neubauer haemocytometer. A dilution ratio of 1:20 from each well-mixed sample was prepared by diluting 50µl of epididymal spermatozoa suspended in physiological saline with 950µl of diluents. The counting chamber was charged from this suspension carefully to avoid sperm accumulating in its trough. All cells within the defined squares were counted taking care to avoid counting cells which crossed the forbidden lines (Gundersen et al., 1998). Both chambers of the haemocytometer were scored and the average count calculated on both sides of the chamber. If the two counts differed by more than 10%, the sample was discarded and the process repeated. To minimize error the count was conducted three times on each of the samples obtained from each epididymis. The average of all the six counts, from each side, from a single rat constituted an observation.

3.4 HISTOLOGICAL ANALYSIS

The testes were carefully dissected out, blotted dry to remove any blood and then fixed in Bouin’s fluid for 72 hours. The fixed tissues were transferred to graded series of ethanol. On day 1, they were placed in 70% alcohol for 7 hours, then
transferred to 90% alcohol and left in the latter overnight. On day 2, the tissues were passed through three changes of absolute alcohol for an hour each and then cleared in Xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58 °C. Three changes of molten paraffin wax at one-hour intervals were made after which the tissues were embedded in wax and blocked out. Prior to embedding, it was ensured that the mounted sections to be cut by the microtome were oriented perpendicular to the long axes of the testes so as to generate cross sections. Serial sections 5µm thick were obtained from each block of tissues using a SHANDON (UK), rotary microtome. They were then fixed on clean slides, stained with hematoxylin and eosin, after which they were passed through a mixture of equal concentrations of Xylene and alcohol. The sections were dried overnight between 35 °C and 40 °C (Sheehan and Hrapchak, 1987).

3.5 SEMI-QUANTITATIVE EVALUATION

Histological changes in the testes were further evaluated by examining 100 tubules in each testis and classifying them as normal, atrophic, sloughing or degenerative depending on the predominant histological feature seen in each tubule. Tubules with disrupted cell association were classed as sloughing. Tubules with noticeable germ cell loss were classed as atrophic, and those with giant cells, (multi-nucleated) were classed as degenerative (Crissman et al., 2004; Sayyim, 2007).
3.6 TESTICULAR (ENZYME) ACTIVITIES

3.6.1 TESTICULAR CATALASE ACTIVITY

Catalase was assayed colorimetrically at 620 nm and expressed as µmoles of H₂O₂ consumed/min. The reaction mixture (1.5 ml) contained 1.0 ml of 0.01M pH 7.0 phosphate buffer, 0.1 ml of tissue homogenase and 0.4 ml of 2M H₂O₂. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent, 5% potassium dichromate and glacial acetic acid mixed in a ratio of 1:3 (Rukmini et al., 2004). The protein content of the samples was determined by the method described by Lowry and others (1995).

3.6.2 TESTICULAR MALONDIALDEHYDE

Testicular malondialdehyde (MDA) level was determined using the modified thiobarbituric acid (TBA) method of Buerge and Aust (1978). This is possible because MDA reacts with thiobarbituric acid to give a red compound absorbing at 532 nm. The stock reagent contains 2 ml 15% w/v trichloro-acetic acid, 0.375% w/v thiobarbituric acid and 0.25 mol/L hydrochloric acid. An aliquot of 2 ml of the stock reagent was added to 1 ml of testicular homogenate, mixed thoroughly and placed in an Equitron water bath (80-90°C) for 15 minutes. It was then cooled and the flocculent precipitate removed by centrifugation at 1000g for 10 minutes. The absorbance of the supernatant was determined with a spectrophotometer at 532 nm against blanks containing all the reagents. The concentration of MDA was calculated using the molar absorptivity coefficient of MDA which is 1.56x10⁵ M⁻¹ cm⁻¹.
3.6.3 TESTICULAR ASCORBIC ACID (AA)

Ascorbic acid levels were determined using its reaction with oxalic acid. The tissue homogenate was added to 0.5M oxalic acid. This oxidized the ascorbic acid to dehydro-5-ascorbic acid, which was then coupled with 2, 4-Dinitro-phenyl hydrazine (DPNH). Thiourea was added to the DPNH to prevent its oxidation by extraneous substances. The resulting osazone was reacted with 85% sulphuric acid which re-arranged the complex to give a colour complex measured at 520nm.

3.6.4 TESTICULAR SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The enzyme was assayed using the method by Sun (1988). The reaction was carried out in 0.5M sodium carbonate, buffer pH 10.2. The reaction was initiated by the addition of $3 \times 10^{-4}$ epinephrine in 0.05N HCL. 0.02 ml of the tissue homogenate was added to 3.0ml of sodium carbonate buffer and 0.03 ml of epinephrine and the absorbance at 480nm was measured for 3-5 minutes (Sun et al., 1988).
3.7 SERUM HORMONE ASSAY

3.7.1 SERUM TESTOSTERONE ASSAY

Serum testosterone concentration was determined by the enzyme-linked immunosorbent assay (ELISA) technique based on the principle of competitive binding between testosterone in the test specimen and testosterone-horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-testosterone, as previously described by Tietz (1995). Goat anti-rabbit IgG-coated wells were incubated with testosterone standards, controls, samples (blood sera), testosterone-horseradish peroxidase conjugate reagent and rabbit anti-testosterone reagent at 37 °C for 90 minutes. During the incubation, a fixed amount of HRP-labeled testosterone competes with the endogenous testosterone antibody. Thus, the amount of testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of testosterone in the specimen increases. Unbound testosterone peroxidase conjugate was removed and the well was washed.

Tetramethylbenzidine (TMB) was added and incubated at room temperature, resulting in the development of a blue colour. The colour development was stopped with the addition of 1N hydrochloric acid, and the absorbance measured at 450nm. The intensity of the colour formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled testosterone in the sample. A standard curve was obtained by plotting the concentration of the
standard versus the absorbance and testosterone concentrations calculated from the standard curve (Tietz, 1995).

3.7.2 SERUM FOLLICLE STIMULATING HORMONE ASSAY

This was determined using the ELISA kit (catalogue number FSH-96 supplied by Teco Diagnostics, Anaheim, CA). In the TC FSH ELISA, 50μl of anti-α FSH monoclonal antibody is coated on micro-titer wells. 100μl of sample and a constant amount of a second anti-β FSH antibody conjugated with horseradish peroxidase (HRP) are added to the wells. Upon mixing, FSH antigen from the sample becomes sandwiched between the solid phase and the enzyme-labeled antibody conjugate. After 60 minute incubation at room temperature, the wells are washed 5 times with de-ionised water to remove the unbound FSH conjugate. A solution of tetramethylbenzidine (TMB) and hydrogen peroxide is then added and incubated for 20minutes in the dark, resulting in the development of a blue colour. The colour development is terminated with the addition of a stop solution, and the absorbance is measured at 450nm. The intensity of colour is directly proportional to the concentration of FSH in the sample.

3.7.3 SERUM LUTEINISING HORMONE ASSAY

This was done using an ELISA kit (Teco Diagnostics, Anaheim, CA; catalogue number: LH-96). Briefly, in the TC LH ELISA, 50μl of anti- α LH monoclonal antibody is immobilized on micro-titer wells.
50µl of sample and 100µl of a second anti-β LH antibody conjugated with horseradish peroxidase (HRP) are added to the wells. Upon mixing, LH antigen from the sample becomes sandwiched between the solid phase and enzyme-labeled antibody conjugate. After 60-minutes incubation at room temperature, the wells are washed 5 times by using de-ionised water to remove unbound LH conjugate. A solution of tetramethylbenzidine (TMB) and hydrogen peroxide is then added and incubated for 20 minutes in the dark, resulting in the development of a blue colour. The colour development is terminated with the addition of a stop solution, and the absorbance is measured at 450nm. The intensity of the colour is directly proportional to the concentration of LH in the sample.

3.8 STATISTICAL ANALYSIS

The results are expressed as means ± standard deviation and subjected to analysis of variance (ANOVA) followed by Turkey’s Post-Hoc test. The significance level was set at p < 0.05. Analysis of data was done with the aid of electronic calculator and the SPSS computer software, version 11.
CHAPTER FOUR

RESULTS

4.1 GENERAL RESPONSE TO BURNS

Scalded skin was blanched immediately after exposure to heat. The burned area turned brown over the next few days as a thick brown eschar developed. In some animals repeated hemorrhagic granulation later appeared on the wound. Wound healing was delayed in such animals. It was observed that this tended to happen when contact with the scalding water lasted a longer time even after the animal had been removed from the water. In over 80% of the animals healing was rapid, and led to a thin scar. In many cases, healing was clinically complete in 8-12 weeks, though the development of tensile strength in the resulting scar would still be in progress.

4.2 MORTALITY

The procedure was well tolerated by the animals, and systemic infection was not encountered as a direct result of the procedure. Animals died in different groups, and at different stages, mostly from injury, chest infections and handling errors. Lost animals were replaced along the way. Animals were lost from respiratory failure, probably aspiration, lung collapse and infections. Others died when there was rapidly falling weight loss, probably due to multiple organ failure, gross injuries and anesthetic accidents. After deaths from handling errors and accidental injuries were discounted, overall mortality was 16.6%.
4.3 EFFECTS OF BURNS ON BODY WEIGHT

Generally the animals lost weight in the first 2 weeks following burn, apparently due to reduced food intake. By 4 weeks, most animals had recovered much or all lost weight. This happened even in cases where the eschar did not lift till the end of the experiment, and clinical evidence of sub-eschar sepsis such as necrotic tissue and sometimes purulent fluid was found. Findings from these animals were retained in the groups because sepsis was localized and the animals retained their weight. Animals in group F which received AA had significant weight gain over the study period (Tables 1 and 2).

Most of the animals tolerated the procedure well and many maintained their weight. Though some gained weight during the course of the experiments, statistically significant changes in body weight were rarely recorded. Animals in which significant reduction in weight occurred were lost and had to be replaced.
### Table 1: Effects of burn injury on body weight of treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>Initial bw (g)</th>
<th>Final bw (g)</th>
<th>% wt gain/loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>292.5 ± 5.42</td>
<td>298.6 ± 4.86</td>
<td>2.05</td>
</tr>
<tr>
<td>B</td>
<td>245.6 ± 5.04</td>
<td>350.8 ± 6.28</td>
<td>2.04</td>
</tr>
<tr>
<td>C</td>
<td>250.8 ± 5.14</td>
<td>256.8 ± 6.12</td>
<td>2.4</td>
</tr>
<tr>
<td>D</td>
<td>320.4 ± 6.24</td>
<td>324.4 ± 5.77</td>
<td>1.25</td>
</tr>
<tr>
<td>E</td>
<td>335.5 ± 6.15</td>
<td>340.7 ± 6.17</td>
<td>1.49</td>
</tr>
<tr>
<td>F</td>
<td>285.8 ± 5.42</td>
<td>331.5 ± 6.12*</td>
<td>15.79*</td>
</tr>
</tbody>
</table>

**BW** - Body weight.

*-- Significant increase in weight occurred, p<0.05.

**Key to table1.**

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 2: Effects of burn injury on body weight of treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial bw (g)</th>
<th>Final bw (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>350.6 ± 6.72</td>
<td>348.4 ± 6.24</td>
</tr>
<tr>
<td>B</td>
<td>296.8 ± 5.81</td>
<td>298.8 ± 5.24</td>
</tr>
<tr>
<td>C</td>
<td>246.4 ± 5.14</td>
<td>256.4 ± 5.88</td>
</tr>
<tr>
<td>D</td>
<td>248.8 ± 6.17</td>
<td>258.2 ± 6.15</td>
</tr>
<tr>
<td>E</td>
<td>312.6 ± 5.84</td>
<td>320.4 ± 6.14</td>
</tr>
<tr>
<td>F</td>
<td>315.8 ± 5.12</td>
<td>321.5 ± 5.23</td>
</tr>
</tbody>
</table>

BW - Body weight.

**Key to table 2.**

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
4.4 EFFECTS OF BURNS ON WEIGHT OF TESTES

A statistically significant reduction in testes weight occurred in hormone-treated groups D and E, \( p<0.001 \). The lowest figures for testes weight occurred in animals administered hormones, when compared to control, or with the other groups. These changes occurred at both 8 and 12 weeks (Table 3 and 4).

4.5 EFFECTS OF BURNS ON VOLUME OF TESTES

Changes in the volume of testis (TV) paralleled those in weight. Statistically significant reduction in volume occurred in group D and E when compared to control groups B and C, (Tables 3 and 4).

4.6 EFFECTS OF BURNS ON WEIGHT OF ACCESSORY SEX ORGANS

In the prostate and seminal vesicle, no statistically significant changes in weight occurred, except for an elevation in group B at 8 weeks. However significant reduction occurred in the weight of epididymis at both durations, in groups D and E, again the hormone-treated groups. These changes parallel those in weight and volume of testes (Tables 5 and 6).
Table 3: Effects of burn injury on weight and volume of testes in treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.22 ± 0.31</td>
<td>1.11 ± 0.21</td>
</tr>
<tr>
<td>B</td>
<td>1.21 ± 0.22</td>
<td>0.92 ± 0.15</td>
</tr>
<tr>
<td>C</td>
<td>1.37 ± 0.28</td>
<td>0.92 ± 0.16</td>
</tr>
<tr>
<td>D</td>
<td>0.73 ± 0.12</td>
<td>0.63 ± 0.12*</td>
</tr>
<tr>
<td>E</td>
<td>1.22 ± 0.21</td>
<td>1.04 ± 0.22</td>
</tr>
<tr>
<td>F</td>
<td>1.32 ± 0.22</td>
<td>1.11 ± 0.18</td>
</tr>
</tbody>
</table>

*--Significant difference exists when compared to control, p<0.05.

Key to table 3.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 4: Effects of burn injury on weight and volume of testes in treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>Weight (g)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.24 ± 0.24</td>
<td>1.10 ± 0.22</td>
</tr>
<tr>
<td>B</td>
<td>1.20 ± 0.12</td>
<td>0.94 ± 0.18</td>
</tr>
<tr>
<td>C</td>
<td>1.27 ± 0.24</td>
<td>0.98 ± 0.14</td>
</tr>
<tr>
<td>D</td>
<td>0.57 ± 0.22</td>
<td>0.41 ± 0.08**</td>
</tr>
<tr>
<td>E</td>
<td>0.71 ± 0.14</td>
<td>0.61 ± 0.12*</td>
</tr>
<tr>
<td>F</td>
<td>1.27 ± 0.18</td>
<td>1.10 ± 0.24</td>
</tr>
</tbody>
</table>

* -- Significant difference occurred when compared to control, p<0.05.

**-- Significant difference occurred when compared to control, p<0.01.

Key to table 4.

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 5: Effects of burn injury on weight of accessory sex organs of treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>SV (g)</th>
<th>Prostate (g)</th>
<th>Epidydimis (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.65 ± 0.24</td>
<td>1.52 ± .11</td>
<td>0.30 ± .04</td>
</tr>
<tr>
<td>B</td>
<td>2.30 ± .25</td>
<td>0.45 ± 0.8</td>
<td>0.31 ± .06</td>
</tr>
<tr>
<td>C</td>
<td>1.18 ± .24</td>
<td>0.38 ± .08</td>
<td>0.32 ± .07</td>
</tr>
<tr>
<td>D</td>
<td>1.89 ± .28</td>
<td>0.66 ± .14</td>
<td>0.22 ± .05 *</td>
</tr>
<tr>
<td>E</td>
<td>1.52 ± .24</td>
<td>0.56 ± .11</td>
<td>0.23 ± 07 *</td>
</tr>
<tr>
<td>F</td>
<td>1.57 ± 0.21</td>
<td>0.55 ± 0.11</td>
<td>0.28 ± 0.04</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

SV: Seminal vesicle.

Key to table 5.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 6: Effects of burn injury on weight of accessory sex organs of treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>GROUP (n=7)</th>
<th>S.V. (g)</th>
<th>Prostate (g)</th>
<th>Epidydimis(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.54 ± .18</td>
<td>0.56 ± .08</td>
<td>0.34 ± .06</td>
</tr>
<tr>
<td>B</td>
<td>1.04 ± .07</td>
<td>0.53 ± .07</td>
<td>0.32 ± .08</td>
</tr>
<tr>
<td>C</td>
<td>1.14 ± .22</td>
<td>0.44 ± .09</td>
<td>0.29 ± .07</td>
</tr>
<tr>
<td>D</td>
<td>1.66 ± .28</td>
<td>0.58 ± .09</td>
<td>0.18 ± .07 *</td>
</tr>
<tr>
<td>E</td>
<td>1.62 ± .14</td>
<td>0.54 ± .08</td>
<td>0.19 ± .06 *</td>
</tr>
<tr>
<td>F</td>
<td>1.56 ± .08</td>
<td>0.57 ± .07</td>
<td>0.33 ± .05</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

SV-Seminal vesicles.

Key to table 6.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
4.7 EFFECTS OF BURNS ON SEMEN PARAMETERS

Sperm density was significantly lower in all experimental groups than in the control group at both durations. Counts were lowest in group B (untreated) and E, (T-treated group) at 8 weeks (Table 7). They were lowest in groups D and E at 12 weeks (Table 8). In the later group severe oligospermia occurred ($p<0.001$).

Sperm motility was reduced compared to control roughly in the same pattern as sperm density. The most significant reduction occurred in groups D and E ($p<0.001$) (Tables 7 and 8).

Sperm progressivity was sluggish in most experimental groups except in group B. Sperm motion was very sluggish in groups D and mostly non-linear in group E (Tables 9 and 10). The changes were similar at both 8 and 12 weeks.

The proportion of morphologically abnormal sperm cells was significantly different from the control group in most groups. It was however, much higher in groups D and E ($p<0.001$) (Tables 9 and 10). Again the differences were similar at both durations. Abnormal cell proportion was significantly higher in group B than in the control but the difference was less than that observed in groups D and E ($p<0.01$). Abnormal cells in group B were observed to possess predominantly shriveled tails.

All key sperm parameters were significantly enhanced in AA group (F) than all other burned groups ($p<0.001$). Total viable count was in fact higher in group F than in the control.
Table 7: Effects of burn injury on sperm count and motility in treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>TSP count (m/ml)</th>
<th>Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>112.2 ± 8.4</td>
<td>&gt;80.5 ± 4.8</td>
</tr>
<tr>
<td>B</td>
<td>34.4 ± 4.72**</td>
<td>&gt;70.8 ± 4.5</td>
</tr>
<tr>
<td>C</td>
<td>48.4 ± 5.48**</td>
<td>&gt;75.6 ± 5.2</td>
</tr>
<tr>
<td>D</td>
<td>8.4 ± 1.14**</td>
<td>&gt;10.8 ± 1.4**</td>
</tr>
<tr>
<td>E</td>
<td>64.6 ± 5.42**</td>
<td>&gt;25.4 ± 4.2**</td>
</tr>
<tr>
<td>F</td>
<td>120.5 ± 6.22</td>
<td>&gt;60.5±4.5*</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

**--Significant difference occurred when compared to control, p<0.01.

TSP: Total sperm count

**Key to table 7.**

A  - Control, sham burn.
B  - 35% TBSA burn, no treatment.
C  - 45% TBSA burn, no treatment.
D  - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E  - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F  - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
### Table 8: Effects of burn injury on sperm count and motility in treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>TSP count (m/ml)</th>
<th>Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128.6 ± 4.22</td>
<td>&gt;80.6 ± 4.22</td>
</tr>
<tr>
<td>B</td>
<td>72.4 ± 5.82**</td>
<td>&gt;65.4 ± 4.15*</td>
</tr>
<tr>
<td>C</td>
<td>66.4 ± 4.84**</td>
<td>&gt;60.4 ± 4.27*</td>
</tr>
<tr>
<td>D</td>
<td>27.2 ± 0.14**</td>
<td>&gt;15.2 ± 1.52**</td>
</tr>
<tr>
<td>E</td>
<td>21.2 ± 0.24**</td>
<td>&gt;5.5 ± 1.13**</td>
</tr>
<tr>
<td>F</td>
<td>145.5 ± 7.84</td>
<td>&gt;70.5 ± 4.5</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

**--Significant difference occurred when compared to control, p<0.01.

TSP count- Total sperm count.

**Key to table 8.**

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 9: Effects of burn injury on sperm progressivity and morphology in treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>Progressivity</th>
<th>% Abnormal</th>
<th>% Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24.8 ± 2.12</td>
<td>72.4 ± 6.17</td>
</tr>
<tr>
<td>B</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>20.4 ± 2.74</td>
<td>73.2 ± 5.27</td>
</tr>
<tr>
<td>C</td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25.2 ± 2.85</td>
<td>70.4 ± 4.21</td>
</tr>
<tr>
<td>D</td>
<td>c</td>
<td>60.21 ± 5.21**</td>
<td>35.7 ± 5.44**</td>
</tr>
<tr>
<td>E</td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>64.4 ± 4.18* *</td>
<td>32.4 ± 3.71**</td>
</tr>
<tr>
<td>F</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25.5±2.84</td>
<td>74.6±4.54</td>
</tr>
</tbody>
</table>

**--Significant difference occurred when compared to control, p<0.01.

a<sup>1</sup> - Rapid; b<sup>1</sup> - slow; c - non-progressive.

Key to table 9.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 10: Effects of burn injury on sperm progressivity and morphology in treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>Progressivity</th>
<th>% Abnormal</th>
<th>% Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>22.3 ± 2.84</td>
<td>74.2 ± 5.82</td>
</tr>
<tr>
<td>B</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>32.4 ± 4.14*</td>
<td>62.7 ± 5.71</td>
</tr>
<tr>
<td>C</td>
<td>b&lt;sup&gt;1&lt;/sup&gt;</td>
<td>28.4 ± 4.12*</td>
<td>68.6 ± 5.21</td>
</tr>
<tr>
<td>D</td>
<td>c</td>
<td>65.7 ± 6.2 **</td>
<td>30.6 ± 3.14**</td>
</tr>
<tr>
<td>E</td>
<td>c</td>
<td>68.6 ± 5.41**</td>
<td>32.4 ± 3.71**</td>
</tr>
<tr>
<td>F</td>
<td>a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>28.2 ± 3.8</td>
<td>71.8 ± 5.4</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

**--Significant difference occurred when compared to control, p<0.01.

a<sup>1</sup> - Rapid; b<sup>1</sup> - slow; c - non-progressive.

Key to table 10.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
4.8 EFFECTS OF BURNS ON SERUM HORMONE LEVELS

4.8.1 EFFECTS OF BURNS ON SERUM TESTOSTERONE LEVELS

By 8 weeks serum testosterone (T) levels fell only minimally in the burned animals except in the group that received FSH/LH treatment. T level was however significantly raised in group E even beyond the level in the control group \((p<0.001)\). This group was treated with testosterone (Table 11). Considerable reduction of serum testosterone levels also occurred in animals where chronic wounds caused prolonged debility.

4.8.2 EFFECTS OF BURNS ON SERUM FSH LEVELS

By 8 weeks a moderate fall in serum FSH levels occurred in burned untreated animals, groups B and C when compared to control group. The change was not statistically significant though. FSH levels were significantly raised in group E which was treated with the hormone, \(p<0.001\), (Tables 11 and 12).

4.8.3 EFFECTS OF BURNS ON SERUM LH LEVELS

LH levels were within normal range in most groups, though as in the case with FSH, they were almost doubled in group E when compared to control. LH level was significantly lower in group D which received long term testosterone treatment, \(p<0.05\).
Table 11: Effects of burn injury on FSH, LH, and T levels in treated and control rats at 8 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>FSH (miU/ml)</th>
<th>LH (miU/ml)</th>
<th>T (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.51 ± 0.31</td>
<td>5.54 ± 0.35</td>
<td>3.75 ± 0.26</td>
</tr>
<tr>
<td>B</td>
<td>4.62 ± 0.34</td>
<td>5.81 ± 0.41</td>
<td>3.01 ± .24</td>
</tr>
<tr>
<td>C</td>
<td>4.42 ± 0.42</td>
<td>5.62 ± 0.34</td>
<td>2.88 ± 0.31</td>
</tr>
<tr>
<td>D</td>
<td>4.14 ± 0.41</td>
<td>3.12 ± 0.42*</td>
<td>6.12 ± 0.74*</td>
</tr>
<tr>
<td>E</td>
<td>7.51 ± 0.64*</td>
<td>8.24 ± 0.66*</td>
<td>1.94 ± 0.14*</td>
</tr>
<tr>
<td>F</td>
<td>4.52 ± 0.30</td>
<td>5.48 ± 0.38</td>
<td>3.44 ± 0.24</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p <0.05.

Key to table 11.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 12: Effects of burn injury on FSH, LH, and T levels in treated and control rats at 12 weeks

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>FSH (miU/ml)</th>
<th>LH (miU/ml)</th>
<th>T (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.54 ± 0.32</td>
<td>5.68 ± 0.24</td>
<td>3.46 ± 0.22</td>
</tr>
<tr>
<td>B</td>
<td>4.22 ± 0.41</td>
<td>5.44 ± 0.22</td>
<td>2.58 ± 0.24</td>
</tr>
<tr>
<td>C</td>
<td>4.14 ± 0.28</td>
<td>5.12 ± 0.21</td>
<td>2.56 ± 0.21</td>
</tr>
<tr>
<td>D</td>
<td>4.01 ± 0.26</td>
<td>3.04 ± 0.52*</td>
<td>7.25 ± 0.84*</td>
</tr>
<tr>
<td>E</td>
<td>7.64 ± 0.48*</td>
<td>8.42 ± 0.82*</td>
<td>1.64 ± 0.18*</td>
</tr>
<tr>
<td>F</td>
<td>4.48 ± 0.34</td>
<td>5.42 ± 0.23</td>
<td>3.45 ± 0.1</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p <0.05.

Key to table 12.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
4.9 EFFECTS OF BURNS ON TESTICULAR ENZYME ACTIVITIES

4.9.1 EFFECTS OF BURNS ON TESTICULAR ASCORBIC ACID LEVELS

At both durations, ascorbic acid levels were significantly lower in all experimental groups, compared to the control, \(p<0.002\). However the reduction was least in group E, which was a T-treated group. In all experimental groups, ascorbic levels were about half the level in the control group (Fig. 2).

4.9.2 EFFECTS OF BURNS ON TESTICULAR MDA LEVELS

At both durations of the experiments, MDA levels were significantly higher in all experimental groups, including group F which received AA than in control group. Lipid peroxidation in the testes as measured by the level of this metabolite was 3-fold higher in untreated groups, (B and C) than in the control group \(p<0.001\). It was, however, only one and a half fold higher in groups D and E, \(p<0.001\), (Fig. 1).

4.9.3 EFFECTS OF BURNS ON TESTICULAR SUPEROXIDE DISMUTASE LEVELS

Superoxide dismutase levels were significantly raised in all burned groups than in control group, B and C \(p<0.05\). They were raised far more in hormone-treated groups than in the others. They were raised three-fold in group D and two-fold in group E \(p<0.02\). However, the levels of the enzyme in groups B and C were only moderately raised compared to that in control group (Fig. 3).
4.9.4 EFFECTS OF BURNS ON TESTICULAR CATALASE LEVELS

At both durations catalase levels were significantly higher in groups B and C than in control group. Unlike the situation with superoxide dismutase however, catalase levels were significantly lower in the hormone and vitamin-C treated groups than in all other groups including control group, $p<0.02$, (Fig. 4).
Figure 1: Effects of burn injury on testicular malondialdehyde levels (u/mg prot.) after 8 weeks.

β: Significant at p<0.05.

γ: Significant at p<0.001

Key to figure 1.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Figure 2: Effects of burn injury on testicular ascorbic acid levels (mg/100ml) after 8 weeks.

β: Significant at p<0.05.

γ: Significant at p<0.001

Key to figure 2.
A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/kg b.w. daily.
Figure 3: Effects of burn injury on testicular superoxide dismutase activity (u/mg protein) after 8 weeks.

β: Significant at $p<0.05$.

γ: Significant at $p<0.001$

**Key to figure 3.**

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/kg b.w. daily.
**Figure 4: Effects of burn injury on testicular catalase activity (u/mg prot.) after 8 weeks.**

β: Significant at p<0.05.

γ: Significant at p<0.001

**Key to figure 4.**

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
4.10 EFFECTS OF BURNS ON HISTOLOGY OF THE TESTES

Sections from the testes in control rats showed normal features and histology. All germ cells were arranged in the normal order, with spermatogonia on the basement, spermatocytes and round spermatids in the middle, and elongated spermatids and mature spermatozoa in the adluminal area. The seminiferous epithelium also showed the germ cells arranged according to the various stages of the spermatogenic cycle in rats (Plate 1).

In groups B and C, experimental groups without treatment, marked changes were observed in tubular epithelium at both 8 and 12 weeks durations. Cell atrophy, especially of mature luminal spermatozoa was the characteristic finding. Many tubules showed severe cell atrophy. There was considerable loss of cellularity in the adluminal areas, with free mature cells being completely absent in many sections. In some of these tubules, no cells that matured beyond round spermatids were found in the seminiferous epithelium. There was also vacuolation in the basal areas, suggesting exfoliation of cells (Plates 2 and 3).

By 12 weeks, many sections showed the same marked cell depletion affecting all cell types with considerable disorganization of the tubular architecture (Plates 8 and 9). In some sections basally placed cells were considerably detached from the basal lamina. In other sections, spermatocytes clustered in the lumen, suggesting exfoliation into this space (Plates 5 and 13). The Interstitium and Leydig cells appeared normal in these sections.
Hormone treatment generally failed to alter the histologic changes. At both durations, similar changes occurred in the sections from groups D and E which were treated with gonadotropins and T respectively (Plates 3, 4, 10 and 11). In the histopathological score, cell atrophy was the key finding, occurring in about 50% of tubules in experimental groups. When chronic debility occurred due to unhealing wounds, sloughing was the predominant finding (Plates 13 and 14). It was found in 60% of tubules (Table 15). This was followed by degenerative changes. Mean seminiferous tubular diameters (MSTD) were reduced in all experimental groups (Table 14). As was the case with sperm parameters, the histological changes described above were largely reversed by AA administration. The percentages of histologically intact tubules were similar in the AA (60.4 ± 4.2 %) and control groups (61.5 ± 6.5 %).
Table 13: Effects of burn injury on mean seminiferous tubular diameter (mstd) of rats after 8 weeks

<table>
<thead>
<tr>
<th>GROUP (n=7)</th>
<th>MSTD (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24.2 ± 0.1</td>
</tr>
<tr>
<td>B</td>
<td>20.1 ± 0.2*</td>
</tr>
<tr>
<td>C</td>
<td>20.2 ± 0.3*</td>
</tr>
<tr>
<td>D</td>
<td>20.5 ± 0.1*</td>
</tr>
<tr>
<td>E</td>
<td>20.2 ± 0.2*</td>
</tr>
<tr>
<td>F</td>
<td>24.1 ± 0.34</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, p<0.05.

Key to table 13.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 14: Effects of burn injury on histopathological score of seminiferous tubules of rats after 12 weeks

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>INTACT</th>
<th>SLOUGHING</th>
<th>ATROPHIC</th>
<th>DEGENERATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>61.5 ±6.5</td>
<td>5.5 ±2.5</td>
<td>22.2 ±7.5</td>
<td>6.5 ±2.5</td>
</tr>
<tr>
<td>B</td>
<td>37.6 ±9.0*</td>
<td>9.1±1.0*</td>
<td>50.6 ±10.0**</td>
<td>5.0 ±2.5</td>
</tr>
<tr>
<td>C</td>
<td>39.4 ±8.5*</td>
<td>11.4 ±4.5*</td>
<td>53.5 ±8.0**</td>
<td>6.8 ±2.2</td>
</tr>
<tr>
<td>D</td>
<td>13.4 ± 2.4**</td>
<td>12.8 ±4.2*</td>
<td>56.4 ±8.0**</td>
<td>5.5 ± 2.7</td>
</tr>
<tr>
<td>E</td>
<td>39.5 ±9.0*</td>
<td>10.2±2.0*</td>
<td>38.5± 4.5*</td>
<td>9.8± 3.0*</td>
</tr>
<tr>
<td>F</td>
<td>60.4 ± 5.2</td>
<td>5.21 ±0.9</td>
<td>34.4 ± 2.4</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, $p <0.05$.

**--Significant difference occurred when compared to control, $p <0.01$.

Key to figure 14.

A - Control, sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Table 15: Effects of burn injury on histopathological score of seminiferous tubules of rats exposed to chronic debilitating wounds at 16 weeks

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>INTACT</th>
<th>SLOUGHING</th>
<th>ATROPHIC</th>
<th>DEGENERATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>61.5 ±6.5</td>
<td>5.5 ±2.5</td>
<td>22.2 ±7.5</td>
<td>6.5 ±2.5</td>
</tr>
<tr>
<td>G</td>
<td>17.4 ±3.5*</td>
<td>43.2 ±1.5*</td>
<td>19.5 ±4.5</td>
<td>20.2 ±5.0*</td>
</tr>
</tbody>
</table>

*--Significant difference occurred when compared to control, \( p <0.05 \).

Key to table 15.

A - Control, and sham burn.

G - 45% TBSA burn, no treatment.
Plate 1: Micrograph of testis from control rat (group A) after 8 weeks. Note normal arrangement of germ series, with free mature sperm cells in the lumen. The pointer tip is surrounded by spermatogonia and basement membrane lies just above it. (H&E × 400).
Plate 2: Micrograph of testis from a rat with 35% burns and no treatment (group B) after 8 weeks. There is severe loss of cellularity in the adluminal area. Elongated spermatids (ES) are arrested in basal area. Multinucleated cells are visible in the basal area as well. At pointer tip is spermatogonium. (H&E × 400).

Key: SG: Spermatogonia; ES: Elongated spermatids; TF: Tail fragment.
Plate 3: Micrograph of testis from a rat with 45% burns and no treatment (group C) after 8 weeks. The tubule shows marked germ cell atrophy in the adluminal area which also contains tail fragments (TF) and sloughed cell nuclei. Spermatogonia (SG) lie close to the basement membrane. Pointer lies at the border between surviving germ cells and an enlarged lumen. (H & E, × 400).
Plate 4: Micrograph of testis from a rat with 45% burns and T treatment (group D) after 8 weeks. There is severe cell atrophy in the lumen (L). Elongated spermatids are the common cells near the lumen. Secondary spermatocytes with clumped nuclei are seen beyond the spermatogonia. A blood vessel (BV) is visible in the interstitium (I), which is normal. At the pointer tip lies a spermatocyte with heads of mature spermatozoa just beyond it (H&E, × 400).
Plate 5. Micrograph of testis from a rat with 45% burns and FSH/LH treatment (group E) after 8 weeks. There is cell atrophy in the ad-luminal area and sloughing into the lumen (L). Tail fragments and cell debris are visible in the lumen. (H&E x 400).
Plate 6: Micrograph of testis from a rat with 45% burns and ascorbic acid treatment (group F) after 8 weeks. There is normal cell arrangement from basement to the lumen and an abundance of free mature spermatozoa (SZ). (H&E, × 640).

Key: SG: Spermatogonia; I: Interstitium.
Figure 7: Micrograph of testis from a control rat (group A) after 12 weeks. Note normal arrangement of germ series, with tails of free mature sperm cells crowding the lumen, lying at pointer tip. (H&E × 400).

Key: SC: Spermatocyte.
Plate 8: Micrograph of testis from a rat with 35% burns and no treatment (group B) after 12 weeks. Cellularity is normal only in the basal area. The lumen contains only tail fragments and giant cells (arrowhead) are visible in basal areas. Layers of spermatocytes (SC) are also visible. (H&E × 400).
Plate 9: Micrograph of testis from a rat with 45% burns and no treatment (group C) after 12 weeks. There is vacoulation and massive ad-luminal cell atrophy. Tail fragments (TF) are visible in the lumen. (H&E, × 400).
Plate 10: Micrograph of testis from a rat with 45% burns and T treatment (group D) after 12 weeks. Tubules show massive ad-luminal cell loss around the lumen (L). (H&E × 400).

Key: ST: Spermatocyte.
Plate 11: Micrograph of testis from a rat with 45% burns and FSH/LH treatment (group E) after 12 weeks. The tubule has empty lumen, few mature spermatozoa, cell debris and tail fragments (TF) in it. (H&E, × 400).
Plate 12: Micrograph of testis from a rat with 45% burns and ascorbic acid treatment (group F) after 12 weeks. It shows normal cell arrangement from basement to the lumen and abundance of free mature
Plate 13: Micrograph of testis from a control rat (group A) after 16 weeks. There is normal cellularity and abundance of free mature spermatozoa (SZ) in the lumen and at pointer tip. Interstitium is intact with a Leydig cell (L) visible in it. (H&E, × 400).
Plate 14: Micrograph of testis from a rat with 45% burns and chronic wounds (group G). Disorganized cell arrangement is visible. Severe cell loss and sloughing is also present, with a mass of desquamated cells just under the pointer tip. Interstitium (I) is intact. (H&E, × 400).
Plate 15: Micrograph of testis from rat with 45% burns and chronic wounds (group G) after 16 weeks. Cell loss is seen around the lumen. Severe loss of basal cells is also present. Only Sertoli cell nuclei (SN) and a single layer of spermatogonia are seen in some areas. Pointer tip lies between an intact interstitium and the thin basement membrane. Cell debris are visible in the lumen, (L).(H&E, × 400).
Figure 5. Effects of burn injury on percentage of histologically intact seminiferous tubules among the groups after 8 weeks.

β: Significant at $p<0.05$.

γ: Significant at $p<0.001$

Key to figure 5.

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Figure 6. Effects of burn injury on percentage of seminiferous tubules showing histologic features of sloughing among the groups after 8 weeks.

β: Significant at $p<0.05$.

γ: Significant at $p<0.001$.

Key to figure 6.

A - Control, and sham burn.
B - 35% TBSA burn, no treatment.
C - 45% TBSA burn, no treatment.
D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.
E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.
F - 45% TBSA burn, i.m. Ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
Figure 7. Effects of burn injury on percentage of seminiferous tubules showing histologic features of atrophy among the groups after 8 weeks.

β: Significant at $p<0.05$.

γ: Significant at $p<0.001$

**Key to figure 7.**

A - Control, sham burn.

B - 35% TBSA burn, no treatment.

C - 45% TBSA burn, no treatment.

D - 45% TBSA burn, i.m. T injection, 4 mg/ kg b.w. weekly.

E - 45% TBSA burn, i.m. FSH/LH 1IU/ kg b.w. daily + weekly T injections.

F - 45% TBSA burn, i.m. ascorbic acid (AA) injection 8 mg/ kg b.w. daily.
CHAPTER FIVE
DISCUSSION

5.1 BODY WEIGHT AND WOUND HISTORY

Generally the animals lost weight in the first 2 weeks following burn, apparently due to reduced food intake. By the 4th week however, almost all animals had recovered lost weight though healing was not progressing at the same speed in all animals. The difference in wound healing speed was mostly due to the fact that some wounds got bruised by the animals scratching them. In other animals, the wounds developed hemorrhagic areas and this slowed healing.

Healing was rapid and led to a thin scar, in over 80% of the animals. This is consistent with the natural history of wound healing in animals (Santos et al., 1999). In loose–skin animals the distribution of the paniculus carnosus, fibroblasts which assume contractile capabilities in time of injury, ensures that up to 80% of wound closure is achieved through contraction. Systemic infection was however not a significant problem in this study. Excluding the cases where debility occurred, AA treatment produced only an apparent difference in speed of healing. It has been noted however that weight gain was better in that group.

5.2 SPERM PARAMETERS

A number of studies indicate that severe burns deplete germs cells in the seminiferous epithelium (Da et al, 1999; Fadeyibi et al, 2009). In humans especially, evidence for
reduced sperm density and a pattern of abnormal morphology characterized by sperm head edema has been reported (Jewo et al., 2009). Marked reduction of sperm density and motility was found in this study.

Most previous studies of the effects of severe burns on the reproductive system did not involve semen analysis. This may be because they were done on people still being hospitalized at the time, when it would be unthinkable to ask them for semen samples. This problem has been partly overcome though by asking men attending post-burn follow-up clinics to undergo semen analysis (Jewo et al., 2009). In that study reduced sperm density was associated with increase in percentage of abnormal spermatozoa. Head abnormalities were the preponderant type in that study, contrasting with the finding here where tail anomalies were the commoner type seen.

Normally tail abnormalities predominate in rat sperm, accounting for up to 75% of all abnormalities in control animals in studies (Artessahin et al, 2006). Head abnormality in human sperm from burn survivors were mainly rounded and swollen or edematous heads. This suggests unraveling of DNA, and implies that sperm DNA may be more resistant to burn-induced damage in rats than in humans. There is no agreement on which of the three parameters measured in traditional semen analysis is a better predictor of future fertility (Chia et al, 1998; Guzick et al, 2001; Nallela et al, 2006). The preponderance of studies however favors morphology, whether this is done by the general or strict WHO criteria (Lewis, 2007).
In this study, severe reduction of sperm parameters was found in all experimental groups and this was irrespective of hormone and treatment status. This could imply persisting defects in sperm production even after correction of any significant falls that may have occurred in FSH, LH and T levels, or in spite of the absence of such falls. The fact that considerable damage occurred in testicular histology and function in hormone-treated groups suggests that non-endocrine mechanisms may play a significant role in fertility suppression in thermally injured animals. Even among atrophic tubules those showing necrotic cells and debris were few, plate 11 being the typical example. Apoptosis rather than necrosis may therefore account for more of the massive cell loss found in this study.

Low sperm density paralleled histologic case of tubular cell atrophy. Apoptosis is increased in testis in a number of conditions. Necrotic and apoptotic cell death were all increased when scrotal skin was directly exposed to steam for various durations (Sakallioğlu et al, 2006). Methotrexate, a cytotoxic anti-cancer agent has also been shown by caspace-3 staining to increase apoptosis in seminiferous tubules (Artessahin et al, 2006).

All key sperm parameters were enhanced by AA administration, normalizing the viable sperm count even though MDA levels were not lowered in this group. This suggests that AA has a powerful fertility supporting effect which may involve mechanisms other than its anti-oxidant activity.
5.3 HISTOLOGICAL CHANGES IN THE TESTIS

Marked germ cell depletion in tubular histology was also found in this study. In tubules from all experimental groups, areas of atrophy, exfoliation and sometimes severe disorganization of the architecture were common. By 12 weeks, in many groups, cells that matured beyond round spermatids were absent in many tubules. This is consistent with the findings of previous investigation (Da et al., 1999; Fadeyibi et al., 2009). One significant difference in the result of this study and that of Fadeyibi is that in that study, interstitial edema occurred in all testes samples. In this study, the interstitium was generally intact.

Marked luminal cell loss reflects enhanced susceptibility of the most differentiated germ cells to damage in this condition and underlies the severe fall in sperm density seen in the semen analysis. Cell atrophy was the main finding in experimental groups. Sloughing was the key finding in groups with chronic debility from unhealing wounds (Figs 20-22). Both changes reflect severe damage. They are common findings in testicular damage from other toxic agents, especially with anti-cancer agents, such as adriamycin, doxorubicin and methotrexate (Artessahin et al., 2006; Vardi et al., 2009; Saalu et al., 2010). Severe disruption of cell architecture and giant cell degeneration found in some groups in this study was also found in methotrexate-induced testicular damage (Vardi et al, 2009).

The reduction in mean seminiferous tubular diameters (MSTD) implies shrinking volumes, since in cylinders, length being constant, volume relates to the third power of
the radius. It underlies the reduction in testicular volume in experimental animals. Again, as is the case with sperm parameters, histologic features of testicular damage are largely normalised in the AA-treated group showing that seminiferous tubular damage accounts for the reduced counts. The percentage of histologically intact tubules in this group is similar to that of control animals.

5.4 ENDOCRINE PERTURBATIONS

The facts that gross interstitial injury was not a finding in this study might explain why serum testosterone (T) levels fell only minimally in the burned animals except in the group that received FSH/LH treatment though severe fall occurred in the group with chronic debility. In this group feed forward suppression of T apparently occurred. In humans a marked fall of serum T and bio-available testosterone, (Bio-T) occurred in chronically critically ill men (Semple, 1986).

A moderate fall in serum FSH, LH and T occurred in burned untreated animals. In children who suffered burns, Jeschke and his colleagues (2008) reported a marked fall in T level beginning 4 weeks post burn Dolecek (1983) however reported a fall in T levels in adults beginning much earlier. Perhaps a drastic fall in testosterone level did not occur in many of the animals, because severe debility did not occur in many of them. In animals in which severe debility occurred, a significant fall occurred in serum T levels. In
the study by Jeschke and his team mean burn surface area was 56% of TBSA. In this study burn surface area (BSA) was 35-45% of TBSA.

Rising LH and falling FSH levels, have been reported in burn patients (Dolecek et al., 1983). Da and others (1999) however reported a significant fall in both hormones from day 14-30 post burn. The levels were returned to normal in many of the patients by day 30. This may explain why gonadotropin levels are within normal range in burned animals in this study, as measurements were made from day 64. It is instructive that in Da’s study gonadotropin levels, were still subnormal at 30 days, mainly in people with mean burn in excess of 30% of TSBA, suggesting that 30% of TBSA might be a threshold of burn extent at which altered endocrine priorities occurs in the hypothalamo-pituitary axis (Da et al., 1999).

Previous investigators have reported a marked elevation of estradiol (E$_2$) post burn, altering the normal ratio of E$_2$ to T. Such an alteration may contribute to germ cell destruction and reduced sperm counts (Da et al., 1999; Jeschke et al., 2008). This is irrespective of the fact that some amount of E$_2$ is required for normal spermatogenesis to occur. FSH and LH levels were raised in group E and T level was significantly raised in group F even beyond the level in the control group. These groups were treated with the respective hormones. Hormone treatment resulted in higher levels than in all other groups. However as already pointed out, this elevated levels of gonadotropins and T failed to cause any corresponding prevention of germ cell damage in testicular histology or the severe fall in spermatogenesis, evidenced in reduced counts.
Unsuccessful attempts have been made in humans to use endocrine manipulations to reverse the wasting and negative nitrogen balance induced by major trauma. When testosterone proved to have adverse effects, oxandrolone, a synthetic androgen was tried. Growth hormone promotes protein retention. Yet when recombinant growth hormone (rh-GH) was applied to control tissue loss in intensive care units across Europe the results proved disastrous. Mortality was higher in the treated group than in control (Takala et al., 1999).

In this study attempts to stem the fall in sperm production by attacking the moderate hypogonadotrophic state was not successful. This supports Da’s conclusion many years ago, that the complexity of the systemic effects of burns, especially associated immune events may account for the failure of corrective endocrine manipulations (Da et al., 1999). They also suggest that non-endocrine mechanisms may be involved in the observed damage.

5.5 OXIDATIVE STRESS AND ANTI-OXIDANTS

Most previous studies on the systemic effects of burns, and indeed chronic illness generally on male reproductive function did not investigate the possible contribution of oxidative stress to the changes observed in testicular histology and function. This is probably for the same reason of difficulty of obtaining testicular tissue from people for study purposes. A considerable assessment of oxidation and the anti-oxidant defense systems in the testes was carried out in this study. In spite of the administered treatment
lipid peroxidation as assessed by MDA levels was significantly elevated in all burned groups (B-F).

The effects of burns and treatment on anti-oxidant defense systems were variable. Compared to control groups, AA levels were significantly lower in all experimental groups including group F, suggesting that it was being used up in the injured animals. However the reduction was least in group D, the T-treated group (Fig.1). Malondialdehyde levels were significantly higher in all experimental groups than in control. Lipid peroxidation was 3-fold higher in untreated groups, (B and C) than in control group. It was only one and a half fold higher in groups D and E suggesting that hormone therapy reduced this activity and its contribution to oxidative stress. Catalase levels were significantly higher in untreated groups (B and C) than in control, suggesting an up-regulation of this enzyme in the injured animals. The levels were however significantly lower in the hormone and AA- treated groups, suggesting that these hormones somehow reduced up-regulation of the enzyme (Fig. 4).

On the other hand, superoxide dismutase, levels were higher in hormone treated groups than in both control and groups B and C (Fig.3). However only a moderate elevation of the enzyme level occurred in groups B and C compared to control. Hormone treatment caused enhanced up-regulation of the enzyme in these animals. It appears then that there is an up-regulation of anti-oxidant enzymes, catalase and SOD in the severely burned animal, as a mechanism for dealing with the resulting oxidative stress. Anti-oxidant
enzymes often up-regulate themselves in response to rising levels of pro-oxidant substrates (Lewis, 2007).

Seminal fluid and the testes are furnished with an extensive array of anti-oxidants, notably ascorbic acid and glutathione reductase (GSH), catalase and superoxide dismutase. The latter enzymes are important in inactivating specific free radicals. Normal cellular metabolic activity results in the generation of several oxidizing agents or pro-oxidants. However oxidation of key cell components such as proteins, alkyl side chains, and DNA are potent pathways leading to cell injury and death. A proper balance between the generation of pro-oxidants and anti-oxidants is required to maintain optimum cell function (Aitken et al., 1992 and 2003; Lewis et al., 1995 and Lewis, 2007). Several natural anti-oxidants such as lycopene, melatonin and ascorbic acid have been used to attenuate the deleterious effects of spermatoxic substances on the testis (Artessahin et al., 2006; Duru et al., 2007; Jewo et al., 2009;).

Catalase and superoxide dismutase are the main anti-oxidant enzymes in the testes (Kawakami et al., 2007). As already pointed out, both testicular dismutase and catalase activities rose with rising MDA levels in all groups when compared to control. This contrasts with the case of testicular oxidative stress from methotrexate, a cytotoxic agent, where rising lipid peroxidation was associated with lower anti-oxidant enzyme levels, implying a disruption of self-regulatory up-regulation of these enzymes (Vardi et al., 2009). A higher level of up-regulation however occurred with catalase in groups B and C which had no treatment. This is probably because these treatments reduced the generation
of pro-oxidants, since MDA levels were lower in hormone-treated groups D and E than in B and C.

Although endocrine parameters did not differ significantly in AA-treated group in this study, all sperm parameters were normalized. Testicular AA levels are 8-times serum levels. Several studies have established the fertility boosting power of AA as well as its protective role in several testiculo-toxic conditions (Fraga et al., 1991; Cheng et al., 2008). AA was found to reduce endogenous DNA damage in human sperm. A dietary reduction in AA from 250mg to 5mg daily caused a 91% increase in semen levels of oxo-8DG, a marker of DNA damage (Fraga et al., 1991). AA promotes cross-linkage of hydroxyl-proline residues required for the maturation of collagen and thus enhances wound healing and well being. This effect may have contributed to the benefits observed in this study.

Though oxidizing species are particularly effective in damaging spermatozoa, a certain level of nitric oxide, a pro-oxidant, is critical in male reproduction. It is important in libido and several aspects of reproduction. Optimal sperm function requires that its concentration be maintained within a narrow physiologic range (Donnelly et al., 1997). It is in fact known that semen from sub-fertile samples often contain higher level of pro-oxidant including nitric oxide. The habit in many laboratories of spinning samples from men with low counts together with good quality semen in one centrifuge has been queried (Lewis, 2007).
As was previously pointed out, it is well established that many spermatotoxic agents exert their effects on germ cells through the generation of free radicals. Mature spermatozoa consist mostly of DNA and membrane. Depletion of cytoplasm is a part of the sperm maturation process. Sperm cell membrane is particularly rich in poly-unsaturated fatty acid (PUFAs), (Aitken and Fisher, 1994; Sofitikis et al., 1992). PUFAs assist membrane structure and fluidity in sperm motion. Oxidation of these acids contained in membrane side chains, often in a peroxidation cascade, is a common pathway to motility loss and cell death (Aitken and Fisher, 1994).

It must be stressed that in this study, lipid peroxidation was significantly elevated in all burned groups (B-F), in spite of the administered treatment. Oxidative stress is therefore quite probably a key pathway for burn-induced suppression of testicular function as observed in this study. Other mechanisms, including endocrine, cytokine and altered immune activity are probably involved in burn-induced testicular damage. Whatever the actual mechanisms involved, the results of this study makes a strong case for including high dose AA as an adjunct in the therapy of any young male who suffers severe burns. This is particularly important as the permanence or otherwise of burn-induced testicular damage is yet to be determined.
CHAPTER SIX

6.1 SUMMARY OF FINDINGS

Severe burns caused extensive testicular germ cell destruction.

Severe burns caused significant reduction in sperm density and motility, and increased the percentage of abnormal sperm cells.

Severe burns caused only apparent changes in FSH, LH and T levels though considerable reduction of T levels occurred when chronic wounds caused prolonged debility.

Treatment with FSH, LH or T did not mitigate the anti-fertility effects of severe burns, whereas ascorbic acid treatment significantly improved sperm parameters and reduced histological evidence of testicular damage.

Increased oxidative stress was a key component of burn-induced testicular damage.

6.2 CONTRIBUTIONS TO KNOWLEDGE

This study has demonstrated germ cell atrophy as the characteristic histological change associated with burn-induced testicular damage.

This study has also established a role for ascorbic acid as an adjunct therapy for testicular damage in burns, thus providing experimental evidence to support clinical trials of the agent in young males under treatment for severe burns.
6.3 CONCLUSION AND RECOMMENDATIONS

This study has shown that severe burn injury has deleterious consequences on fertility in the male rat. This manifested as reduction in spermatogenesis and histological damage in the testis. Chronic debility in wounds worsened testicular damage. Consequently, physicians caring for burn patients must make every effort to avoid or reduce the occurrence of wound chronicity. Finally, ascorbic acid treatment showed far better potential to correct the observed testicular damage than hormones did.
REFERENCES


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LIST OF ABBREVIATIONS

AA: Ascorbic acid
ANOVA: Analysis of variance
AR: Androgen receptor
BMC: Bone mineral component
BMP: Bone mineral protein
BSA: Body surface area
CAT: Catalase
CR-1: Complement receptor-1
CCU: Critical care unit
CVS: Cardiovascular accident
DDPTB: Deep dermal partial thickness burn
ELISA: Enzyme-linked immunosorbent assay
FTI: Free thyroid iodine
FSH: Follicle stimulating hormone
LH: Luteinizing hormone
GH: Growth hormone
GM-CSF: Granulocyte-macrophage colony stimulating factor
GTH: Glutathione
HRP: Horseradish peroxidase
HSP: Heat shock protein
ICSI: Intra-cytoplasmic sperm injection
ICU: Intensive care unit
IGF-1: Insulin-like growth factor 1
IL: Interleukin
IgG: Immunoglobulin-G
INF: Interferon
IVF: In-vitro fertilization
LBM: Lean body mass
LMIC: Low and medium-income country
MDA: Malondialdehyde
MCP: Macrophage chemotactic protein
MIP: Macrophage inhibitory protein
MSTD: Mean seminiferous tubular diameter
PGE: Prostaglandin-E
PKA: Protein kinase-A
PT: Partial thickness
PUFA: Polyunsaturated fatty acid
rhGH: Recombinant human growth hormone
ROS: Reactive oxygen species
SOD: Superoxide dismutase
ST: Seminiferous tubule
SV: Seminal vesicle
T: Testosterone
TBSA: Total burn surface area
TV: Testis volume